ADVANCES IN CLINICAL CHEMISTRY

Volume 10

Oscar Bodansky &
C. P. Stewart

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VOLUME 10

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Advances in CLINICAL CHEMISTRY

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FOREWORD

A historian of science in years to come may well be astonished at the explosive burst of scientific activity round about the middle of the twentieth century of our era. He will be puzzled by the interrelationship between the growth of population and the rise of the standard of living; he will be interested in the increased percentage of scientists among the population, their greater specialization, and the resulting fragmentation of science; he will analyze the economic and the psychological motivation of scientists; he will compare the progress of knowledge with the broadness of the current of scientific publication.

Living as we do in the midst of these events, we are hardly aware of their relatively rapid rate. What we notice is a doubling of the scientific output every ten years, regardless of contemporary political events. It is this climate which has engendered the appearance of series of reviews in dozens of disciplines. It may be with yearning or with a feeling of superiority that we look back at such annual compendia as "Maly's Jahresberichte der Thierchemie" of one hundred years ago, which encompassed the annual progress in the zoological half of biochemistry within 300 to 400 pages.

Nowadays, that number of pages would not suffice to record the complete annual increment of knowledge in a single specialized division of a subject such as clinical chemistry. Media already existing furnish a comprehensive list of publications and an encyclopedic summarization of their contents; the present serial publication *Advances in Clinical Chemistry*—like other *Advances*—attempts something different. Its aim is to provide a readable account of selected important developments, of their roots in the allied fundamental disciplines, and of their impact upon the progress of medical science. The articles will be written by experts who are actually working in the field which they describe; they will be objectively critical discussions and not mere annotated bibliographies; and the presentation of the subjects will be unbiased as the utterances of scientists are expected to be—*sine ira et studio*.

The bibliography appended to each chapter will not only serve to document the author's statements, it will lead the reader to those original publications in which techniques are described in full detail or in which viewpoints and opinions are expressed at greater length than is possible in the text.

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C. P. STEWART

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PREFACE

With this current volume the serial publication *Advances in Clinical Chemistry* is completing its first decade. During this period the field of clinical chemistry has expanded greatly in scope and in complexity, and it has been increasingly recognized that the conduct of a clinical chemistry laboratory in a hospital demands its own professional guidance. The clinician has sought increasing reliance on the clinical chemistry laboratory to aid him in the diagnosis and management of his patient. The great expansion in medical investigation has also utilized the technology of the clinical chemist. In countries all over the world these factors have been reflected in the increasing number and variety of determinations that the biochemical laboratory or department is asked to perform.

The preface to the first volume of this publication (1958) stated its aims to be dual in nature: the description of reliable, accurate diagnostic and prognostic procedures as well as the elucidation of the fundamental biochemical abnormalities which underlie disease. The acceptance of this work has led us to believe that these aims have been realized in the volumes that followed.

The editors have attempted to make the current volume responsive to these aims, as modified by the needs of the day. Methodological advances are represented chiefly by the chapters on Automated Techniques in Lipid Chemistry and on Hydroxyproline Measurement in Man. Several of the other articles emphasize the biochemical aspects of disease. It is hoped that such contributions will also stimulate the development of feasible laboratory techniques that will be performed by the clinical laboratory of tomorrow. In their article on Quality Control in Routine Clinical Chemistry, Professor Whitby and his associates have treated in a detailed and definitive manner a subject that is of much concern to many laboratories.

As in the past, it is a great pleasure to thank our contributors and publisher for their excellent cooperation in making this volume possible.

OSCAR BODANSKY
C. P. STEWART

November, 1967

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CALCITONIN AND THYROCALCITONIN

David Webster and Samuel C. Frazer

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1. Introduction

1.1. The Stability of Plasma Calcium Levels

The regulation of the concentration of calcium in body fluids in health has been thought until quite recently to be almost entirely a function of the parathyroid glands, and so well did the parathyroids appear to perform this task that McLean and Hastings (M7) described the plasma calcium level as "one of Nature's physiological constants." Certainly there is convincing evidence to support the view that changes in the concentration of calcium in the blood supplying the parathyroids determine the output of parathormone. In 1942, Patt and Luckhardt (P3) perfused the isolated parathyroid (and thyroid) glands of a dog with decalcified blood and collected the venous effluent from the glands. Subsequent injection of the plasma from this blood into a second animal produced a sustained rise in its plasma calcium concentration, similar to that found after administration of parathyroid extracts. Perfusion of the glands with blood of normal calcium content, however, yielded a venous effluent that produced no significant alterations in the plasma calcium levels in recipient animals. McLean (M6) has proposed a negative feedback mechanism for the control of blood calcium, whereby a fall in the calcium content of the blood reaching the parathyroids stimulates increased secretion of parathormone. The resulting rise in calcium concentration then acts to suppress further parathormone secretion. Further evidence for this humoral control of parathormone secretion as the prime factor in the maintenance of calcium homeostasis came from the experiments of Copp *et al.* (C17), who demonstrated that perfusion of the thyroid–parathyroid system in dogs with blood of low-calcium content led to rises in the systemic plasma calcium concentration, while perfusion with high-calcium blood had the opposite effect.

In 1961, however, Rasmussen (R1) had pointed out that, since parathormone has a somewhat prolonged action on bone, a simple feedback system, such as that suggested by McLean, might be expected to lead to wide fluctuations in plasma calcium concentration rather than to the physiological constancy referred to above. He postulated a second regulating system, more rapid in action but with a more limited range of control, and indicated that this might be found in the action of parathormone on the kidney. Although this secondary renal regulation may well be important, an impressive mass of evidence has accumulated in the past 5 years that points to the existence of a dual mechanism of a different sort, namely, that the action of the parathyroid hormone in raising the plasma calcium level is opposed by one or more faster-acting hormones, secreted in response to hypercalcemia, and acting to lower the plasma calcium concentration.

This review is concerned with the nature of such hypocalcemic agents, factors controlling their secretion, mode of action, and possible clinical significance.

1.2. Evidence Suggesting a Dual Regulating Mechanism for Plasma Calcium Concentrations

Although the classical view of the plasma calcium level being maintained by adjustment of the level of parathormone secretion provided a satisfactory explanation for most of the known facts of calcium homeostasis, some uncertainty persisted whether this was the whole story. In 1960, however, there came a clear indication that there must be a dual mechanism for the control of plasma calcium concentration. In an experimental study of calcium homeostasis in dogs, Sanderson *et al.* (S1) noted that hypercalcemia resulting from calcium infusions persisted longer in thyroparathyroidectomized animals than in intact ones. It was also noted (C1, G2) that hypercalcemia resulting from administration of parathyroid extracts persisted longer in parathyroidectomized rats than in normal animals. Copp (C14) observed that during calcium infusion in the intact dog the plasma calcium level ceased to rise after a few hours, but that removal of the parathyroids at this point, leaving the thyroid intact, resulted in an immediate further rise in the systemic plasma calcium level. Direct perfusion experiments (C17) had earlier indicated that the thyroid–parathyroid system releases a hypocalcemic factor in response to hypercalcemia. Since this agent did not appear to be released when the thyroid gland alone was perfused with hypercalcemic blood, Copp *et al.* (C17) deduced that there must be a second parathyroid hormone, which was named "calcitonin." However, the parathyroid origin of calcitonin was subsequently challenged by Foster *et al.* (F4) on the basis of separate perfusions of goat thyroid and parathyroid glands, the results of which clearly indicated a thyroid origin for the hypocalcemic agent. The question whether or not two distinct hypocalcemic hormones exist will be considered in more detail below, but we propose to follow Hirsch *et al.* (H3) in describing the principle obtained from thyroid as "thyrocalcitonin," while reserving the term "calcitonin" for use in the context of a possible second parathyroid hormone.

2. Sites of Production of Calcium-Lowering Hormones

2.1. The Parathyroid Glands

The concept that the parathyroid glands are involved in the control of hypercalcemia through the production of calcitonin is supported by three main lines of experimental evidence. The first involves high-calcium

perfusion of the isolated parathyroid or thyroparathyroid glands in experimental animals, and the measurement of the effects of the venous effluent on the calcium level in the same animal or in recipient animals. The second is the demonstration of hypocalcemic activity in parathyroid extracts, both crude and relatively pure. In the third type of experiment, parathyroidectomized animals have been shown to control hypercalcemia, produced by calcium loading and parathormone administration, less effectively than intact animals.

2.1.1. *Evidence from Perfusion Experiments*

The earliest perfusion experiments pointing to a hypocalcemic agent of parathyroid origin were performed by Copp *et al.* (C17) in the dog. Because of the close anatomical relationship between the parathyroids and the thyroid in the dog, it is virtually impossible to perfuse parathyroid tissue without simultaneously perfusing the thyroid. In view of later experiments, which have established that the thyroid gland produces a hypocalcemic agent, experiments involving perfusion of the thyroparathyroid complex only cannot be regarded as satisfactory evidence that the parathyroid glands play a part in the control of hypercalcemia. Copp *et al.* (C17) also compared hypocalcemic perfusion of dog thyroparathyroid glands with a similar perfusion through isolated thyroid that had been carefully freed from parathyroid tissue. When the parathyroids were present, the venous blood from the perfused glands elicited a significant hypocalcemic response; blood from the perfused thyroid alone caused no change in the systemic blood calcium. Copp's original findings in the dog were confirmed by Kumar *et al.* (K5), but subsequent experiments in the goat (F4) indicated that the parathyroids were not the source of the hypocalcemic factor. In the goat and the sheep the superior pair of parathyroids is anatomically distinct from the thyroid, in which the inferior parathyroids are usually found. Foster *et al.* (F4) perfused the superior parathyroid in the goat with hypercalcemic blood, but did not observe a fall in the systemic blood calcium. When the thyroparathyroid glands were similarly perfused, a significant hypocalcemia was observed. Similar experiments were performed in lambs and in mature sheep by Copp (C16), but with quite different results. Hypercalcemic perfusion of the sheep thyroid, together with its inferior parathyroid, produced no change in the blood calcium, whereas superior parathyroid perfusion in the same animals produced significant hypocalcemic responses. Care and Keynes (C4), in an attempt to explain the differing results of parathyroid perfusion in the goat and the sheep, perfused the superior parathyroid of a conscious sheep with hypercalcemic blood and collected the venous effluent from the perfused gland.

They obtained a prompt systemic hypocalcemic response, while cross-transfusion of the parathyroid venous plasma to another sheep was followed by a sharp fall in its systemic blood calcium also. These authors considered that the negative results obtained by Copp and Henze (C16) and by Foster *et al.* (F4) in their parathyroid perfusions might have arisen because there was insufficient functional parathyroid tissue to give a measurable response, perhaps from effects of anesthesia and of surgical trauma.

Recently, Care *et al.* (C6) have completed a more extensive investigation into the possible parathyroid origin of calcitonin. Hypercalcemic perfusion of one superior parathyroid gland in seven conscious sheep

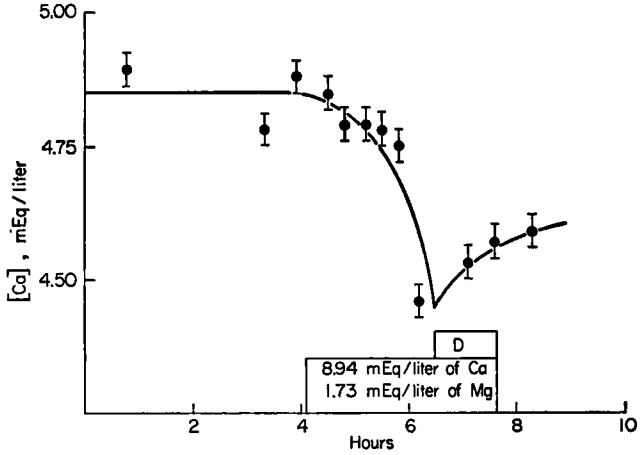

FIG. 1. The effect on the systemic plasma calcium concentration of hypercalcemic perfusion of a parathyroid gland in a conscious, thyroidectomized sheep, followed by drainage (D) of the perfused parathyroid venous blood from the animal. The *vertical lines* represent the standard error. [From Care *et al.* (C6).]

preparations resulted in hypocalcemic responses, in excess of what would have occurred had the perfusions simply resulted in "functional parathyroidectomy." Removal of the parathyroid venous drainage during high-calcium perfusion in a conscious thyroidectomized sheep resulted in a rebound in the falling plasma calcium level (Fig. 1). It was considered that, if the hypocalcemic response to the hypercalcemic perfusion of the parathyroid simply owed to inhibition of parathormone secretion, collection of the parathyroid venous drainage might have been expected to intensify the decrease in the systemic blood calcium level, rather than to reverse it. This rebound effect could not be demonstrated in acute experiments involving perfusion of the parathyroids in anes-

thetized sheep. The same authors' experiments in goats yielded equivocal results. In one, hypercalcemic perfusion of the superior parathyroid caused no significant change in the systemic blood calcium level, whereas in a second experiment a hypocalcemic effect was obtained.

2.1.2. *Evidence from Parathyroid Extracts*

In 1961 Copp and Cameron (C15) found a definite hypocalcemic response to the injection of 1000 IU of parathyroid extract in the dog; the maximum fall in calcium was reached after 20 minutes, but was followed by the expected hypercalcemia typical of potent parathyroid extracts (Fig. 2). A more detailed study of this phenomenon by Copp

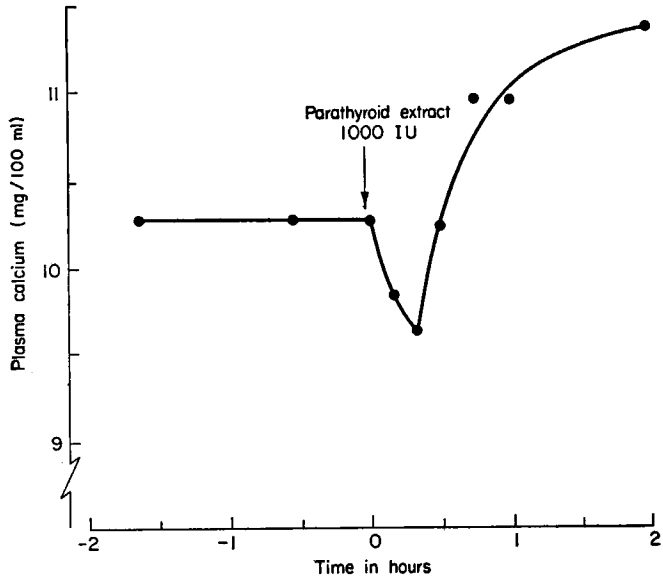

FIG. 2. Change in plasma calcium following intravenous injection of 1000 IU of parathyroid extract in the dog. [Redrawn from Copp and Cameron (C15).]

et al. (C17) revealed that, whereas some parathyroid extracts gave this dual response, others yielded a hypercalcemic response only; some extracts, on the other hand, gave a hypocalcemic reaction without hypercalcemia. This clearly suggested the existence of two distinct hormones, rather than a dual action of the same hormone.

Care *et al.* (C6) tested parathyroid extracts from four sources for their effects on plasma calcium in the sheep, goat, and pig. All four produced hypocalcemia in the sheep, the goat responded to a somewhat lesser degree, and extracts that produced a definite lowering of plasma calcium in the sheep were without effect in the pig.

Tashjian and Munson (T3) were unable to obtain a hypocalcemic response to parathyroid extracts in the rat, a finding also confirmed by Copp at that time (C13, quoted in T3). Subsequently, however, Copp (C14) reported that extracts of beef parathyroids, injected intravenously, consistently produced falls in the plasma calcium within 20–30 minutes in dogs, rats, and sheep. MacIntyre *et al.* (M3) tested crude extracts of bovine parathyroids prepared with acid, alkali, or organic solvents, and could show no hypocalcemic activity of these extracts either in the rat or in the dog. Purified bovine parathyroid extract was similarly shown to be without calcium-lowering activity in the rat.

2.1.3. *The Control of Hypercalcemia after Parathyroidectomy*

Although the importance of the parathyroids in controlling hypocalcemia is well recognized, there is some evidence that these glands can also exert a regulating action on hypercalcemia. Some earlier investigations into the control of induced hypercalcemia after parathyroidectomy are not helpful, since both thyroid and parathyroid glands were removed (C14, S1). Tweedy and Chandler (T6), however, observed that the hypercalcemia resulting from parathyroid extract administration was greater in parathyroidectomized rats (with thyroids intact) than in controls. This was confirmed by Cameron and Copp (C1), who found that a similar degree of hypercalcemia was induced by parathormone in parathyroidectomized rats, whether or not the thyroid was present. A smaller elevation of the plasma calcium level occurred in intact rats, and was attributed to the controlling effect of calcitonin. Gittes and Irvin (G2), using thyroparathyroidectomized, parathyroidectomized, and intact rats, found the greatest response to exogenous parathyroid hormone in the thyroparathyroidectomized animals, an intermediate response in those deprived of parathyroids only, and the least response in intact animals. This finding was not confirmed by Hirsch and Munson (H2) (see Section 2.2.3 below), and Foster (F1) could detect no difference in the ability of intact and parathyroidectomized rats to deal with a calcium challenge.

2.2. The Thyroid Gland

The first indication that the thyroid gland was involved in the control of hypercalcemia arose during parathyroidectomy studies in the rat by Hirsch *et al.* (H3). The mean plasma calcium level of groups of rats parathyroidectomized by hot-wire cautery was much lower 5 hours after operation than was the case in similar animals parathyroidectomized by surgical excision (5.7 and 8.1 mg/100 ml, respectively.) Further, the plasma calcium level of rats parathyroidectomized by excision could be

further depressed by cautery applied at the lower pole of the thyroid gland; the effect was greater after bilateral cautery. Cautery of the lower lobes of the thyroid without parathyroidectomy caused a transient fall in plasma calcium, whereas the fall consequent on parathyroidectomy by cautery was more persistent. Since these results suggested the release of a hypocalcemic factor from the thyroid as a consequence of cautery, relatively crude acid extracts of rat thyroid gland were prepared and found to lower the plasma calcium level both in intact and in parathyroidectomized rats. The responsible agent was named "thyrocalcitonin" to differentiate it from "calcitonin," which Copp (C14) had described as being of parathyroid origin.

The evidence for the existence of a hypocalcemic agent in the thyroid gland will be summarized under headings similar to those used above in the case of the parathyroids.

2.2.1. *Evidence from Perfusion Experiments*

Whereas hypercalcemic perfusion of the thyroparathyroid gland system in the dog had led Copp *et al.* (C17) to the conclusion that parathyroid produces a hypocalcemic agent, similar experiments convinced MacIntyre *et al.* (M3) that the thyroid must be at least partly responsible for the production of the hypocalcemic agent. As was mentioned earlier, hypercalcemic perfusion of the thyroid in the sheep gave negative results, but, in the goat (in which the anatomical relationship between thyroid and parathyroids is similar to that in the sheep), the results clearly indicated that the thyroid was the source of the hypocalcemic substance. In the pig, where the two parathyroid glands are anatomically distinct from the thyroid and have a separate blood supply, hypercalcemic perfusion of the thyroid *in situ* was shown by Care (C2) to bring about a 30% reduction in the systemic plasma calcium level. He also showed that cross-transfusion of venous plasma from the perfused gland resulted in a small but significant hypocalcemic effect in a second pig (Fig. 3). Subsequent experiments by Care *et al.* (C5) confirmed that the maximum hypocalcemic response occurred in the pig after 2 hours of thyroid perfusion, and persisted for as long as the stimulus was applied (Fig. 4). It was also shown that the hypocalcemic response occurred in parathyroidectomized pigs, provided the initial plasma calcium level was held within normal limits by the provision of calcium supplements.

2.2.2. *Evidence from Thyroid Extracts*

There are now numerous reports confirming that thyroid extracts from many animal species are effective in lowering plasma calcium levels. Foster *et al.* (F4) prepared a simple acid extract of goat thyroid, which,

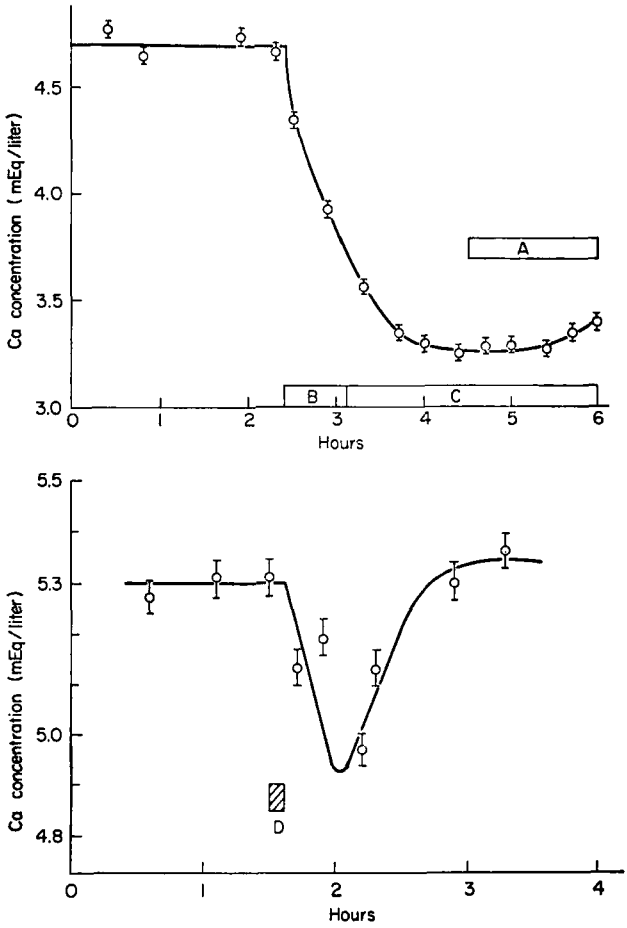

FIG. 3. Effect of hypercalcemic perfusion of pig thyroid on systemic plasma calcium level in donor (upper figure) and recipient (lower figure) animals: (A) collection of donor animal's thyroid venous blood; (B) perfusion calcium concentration, 10 mEq/liter; (C) perfusion calcium concentration, 7 mEq/liter; (D) transfusion of 48 ml of thyroid venous plasma from donor into recipient pig. [Redrawn from Care (C2).]

when injected into the donor animal, produced a definite hypocalcemia. Pig thyroids have been used for the preparation of more highly purified material, which, when injected in amounts corresponding to about 0.2–0.5 μg of protein nitrogen, consistently produced significant hypocalcemic effects in the rat (B1, H4, T5). Calcium-lowering activity has also been demonstrated in extracts from the thyroid of dog, rabbit, ox, monkey, sheep, and deer. Early attempts to show hypocalcemic activity in human

thyroid tissue were not altogether successful, only one sample out of seven tested having significant activity (H4); subsequent studies have shown that human thyroid tissue, whether obtained during surgery or postmortem, yields an extract containing hypocalcemic activity when tested in the rat (A4), monkey (M12), mouse (S2), and pig (C7). Since the pig thyroid contains no parathyroid tissue (C2), this gland has been widely used for the preparation of highly purified thyrocalcitonin preparations free from parathormone and other polypeptides of parathyroid origin.

2.2.3. *The Control of Hypercalcemia after Thyroidectomy*

Talmage *et al.* (T1) studied the effect of calcium loading in rats subjected to parathyroidectomy, thyroparathyroidectomy, and thyroid-

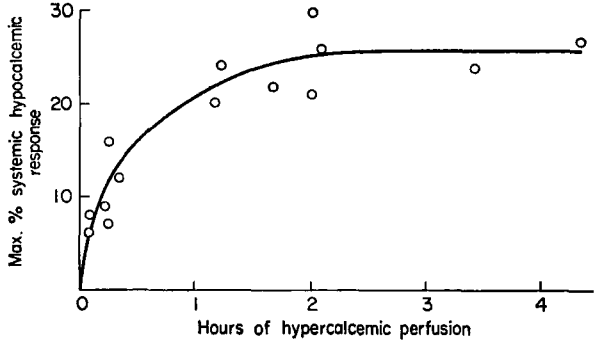

FIG. 4. Relationship between the period of hypercalcemic perfusion of the thyroid *in situ* in 16 anesthetized pigs and the maximum percentage systemic hypocalcemic response. [From Care *et al.* (C5).]

ectomy with parathyroid replacement by transplants. They concluded that the thyroid gland must release the hypocalcemic principle, and found nothing to suggest the existence of a similar principle of parathyroid origin. Gittes and Irvin (G2) obtained evidence in the rat that both the thyroid and parathyroid glands play a role in preventing hypercalcemia. They postulated the existence of a thyrocalcitonin-releasing factor, secreted by the parathyroids in response to hypercalcemia. More recently, Hirsch and Munson (H2) have repeated the earlier work of Cameron and Copp (C1), but with results that indicate that only the thyroid releases a hypocalcemic agent in response to hypercalcemia. Neither these workers nor Foster (F1) could find evidence for the existence of the "thyrocalcitonin-releasing factor" of Gittes and Irvin. In pigs, Care *et al.* (C5) found that hypercalcemia resulting from an intra-

venous calcium infusion was of longer duration and greater extent after thyroidectomy than in the intact animal. They also showed that very rapid isolation and removal of the thyroid gland in the pig, during the intravenous infusion of calcium gluconate, resulted in a marked steepening of the rate of increase in plasma calcium, despite maintenance of the calcium infusion at a constant rate (Fig. 5). Similar infusions without thyroidectomy produced only a very gradual rise in plasma calcium.

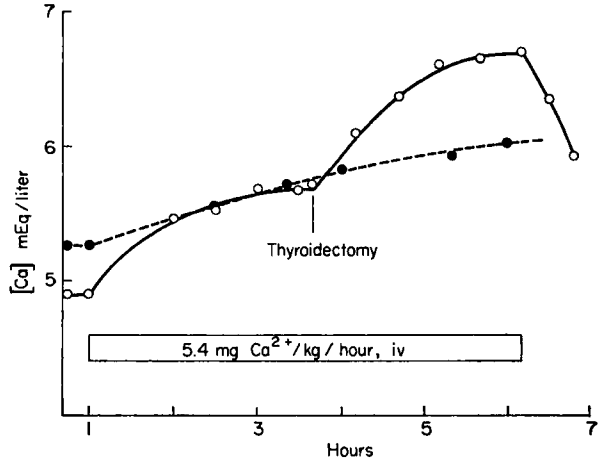

FIG. 5. ○——○: Effect on plasma calcium concentration of thyroidectomy during a constant intravenous infusion of calcium gluconate at the rate of 5.4 mg of Ca^{2+}/kg/hour. Mean results from two anesthetized pigs. ●----●: Effect of the same rate of iv infusion of Ca^{2+} on plasma calcium concentration in another pig with thyroid intact. [From Care *et al.* (C5).]

2.3. OTHER TISSUES

The extraction procedures used for thyrocalcitonin preparation have been applied to many other tissues, in a search for further hypocalcemic agents. Hirsch *et al.* (H4) were unable to demonstrate hypocalcemic activity in extracts of ox thymus, rat pituitary, or rat parathyroid. Rat liver, kidney, and salivary gland likewise yielded inactive extracts on assay in the rat. Gudmundsson *et al.* (G4) found no significant activity in pig liver, bovine parathyroid, or kidney. Extracts of dog lung, kidney, myocardium, skeletal muscle, spleen, brain, liver, and pancreas, when prepared by methods that yield potent extracts from the thyroid, failed to demonstrate hypocalcemic activity (C10). Extracts of sheep liver and of gluteal muscle showed no activity in the sheep, and high-calcium perfusion of the thymus, isolated from parathyroid tissue, produced no

hypocalcemic response (C6). Significant hypocalcemic activity, however, has been found in crude pituitary extracts, but only in the rabbit (N2). The same principle was found in purified ACTH preparations, but there seemed to be no correlation between the ACTH activity and the degree of hypocalcemia produced. This pituitary principle was also found to raise the serum citrate (N3) and free fatty acid levels (F6); its action was potentiated by fasting. Extracts of human and pig pituitary, active in the fasted rabbit, were without effect in normal human subjects, rats, and dogs (N1). Although the hypocalcemic response to pituitary extracts is dramatic (up to a 50% fall has been reported) (N2), its high specificity to the rabbit suggests that only in the light of further evidence of its potency in other species, preferably accompanied by evidence that it is secreted in response to hypercalcemia, could it be considered a factor of general significance in calcium homeostasis.

2.4. Summary

It seems to be fully established that the thyroid gland in many animal species contains a substance or substances (thyrocalcitonin) able to lower the plasma calcium concentration, and secreted in response to the passage of hypercalcemic blood through the gland. Potent extracts can be prepared, and are active when injected or infused into normal and parathyroidectomized animals. This substance is quite distinct from thyroid hormone.

The earlier evidence for a second parathyroid hormone (calcitonin) cannot be said to have been either satisfactorily established or finally disproved. Conflicting reports on the systemic effects of separate perfusions of thyroid and of parathyroid glands, and on the presence or otherwise of consistent biphasic effects on plasma calcium when extracts of the parathyroid gland are injected, have contributed to the atmosphere of uncertainty. There are, however, some observations that cannot be readily explained solely on the basis of a thyroid origin for the hypocalcemic principle, notably the effects of cross-transfusion of parathyroid venous plasma in the sheep and the impaired tolerance to infused calcium that follows parathyroidectomy without thyroidectomy.

With the possible exception of the effects of pituitary extracts in the rabbit, there is no evidence that tissues other than the thyroid and parathyroid glands can store or secrete calcium-regulating hormones.

3. Factors Affecting the Rate of Secretion of Thyrocalcitonin

3.1. Plasma Calcium Level

Perfusion of the thyroid gland with blood containing a raised level of calcium, and the entry of the venous blood from the gland into the

general circulation, result in a fall in the systemic plasma calcium level. This is attributable to the release of thyrocalcitonin into the blood, which in turn exerts an action on one or more target organs, causing the removal of calcium from the circulation. It has been shown (G3) that the plasma calcium concentration in rats prior to thyroidectomy influences the thyrocalcitonin content of the excised gland. After 6 hours of hypercalcemia the amount of thyrocalcitonin that could be extracted from the thyroid was approximately one third of that from the glands of control animals, and after a 5-week period of hypocalcemia following parathyroidectomy the thyrocalcitonin content of the thyroid was nearly 3 times that in control animals. These results clearly suggest that during hypercalcemia the rate of release of thyrocalcitonin exceeds its rate of biosynthesis, and, conversely, that during hypocalcemia the release of thyrocalcitonin is sufficiently slow to result in accumulation of the hormone in the gland.

During hypercalcemic perfusion of the pig thyroid, Care *et al.* (C5) could detect no variation in the magnitude of the hypocalcemic response produced when the calcium content of the perfusing blood varied between 6.4 and 7.8 mEq/l (12.8–15.6 mg/100 ml). At this level of hypercalcemia, the response of the thyroid appeared to be "all-or-none." Perfusion of the gland with normocalcemic blood, and cross-transfusion of the venous drainage blood to a recipient pig, were not associated with a change in the systemic blood calcium level in either animal. However, hypocalcemic perfusion of the pig thyroid *in situ* was associated with a slight rise in the systemic plasma calcium concentration, suggesting an inhibition of thyrocalcitonin secretion. This evidence is consistent with the view that the secretion of thyrocalcitonin is controlled by a negative feedback system operating through the plasma calcium concentration. Until such time as the concentration of hormone in thyroid venous plasma can be readily measured and the hormone production rate assessed, quantitative information on the nature of the thyroid response to hypercalcemia must of necessity be lacking. The amount of hormone released by hypercalcemia, although sufficient to produce a demonstrable rise in plasma calcium in a recipient animal, is insufficient to permit its accurate measurement by present bioassay procedures.

3.2. Other Plasma Constituents

Care *et al.* (C9) have demonstrated that perfusion of the isolated goat parathyroid gland with blood containing an elevated magnesium concentration suppresses the secretion of parathormone in a manner similar to perfusion with hypercalcemic blood. Extension of these studies to the perfusion of pig thyroid with hypermagnesemic blood, however, did not affect the systemic plasma calcium level. It was also shown that per-

fusion of the thyroid with blood rich both in calcium and in magnesium produced a hypocalcemic response similar to that found when high-calcium blood alone was used. Magnesium concentrations in blood, therefore, do not appear to have a direct effect on thyrocalcitonin secretion.

The effects of plasma constituents other than magnesium on the secretion of thyrocalcitonin, either as stimulants of secretion or as modifiers of the response of the gland to hypercalcemia, do not appear to have received much attention as yet.

3.3. Evidence for a Resting Level of Secretion

Hypocalcemic perfusion of the pig thyroid *in situ* results in a slight rise in the systemic blood calcium level, which might be caused by a shutting-off of thyrocalcitonin secretion (C5). The normal level of thyrocalcitonin output might depend on the prevailing level of secretion of parathyroid hormone; this possibility is supported by the observation that hypocalcemic perfusion of the thyroid in parathyroidectomized pigs was unaccompanied by the rise in systemic plasma calcium observed in intact animals. Until sensitive immunoassay procedures become more widely available, however, it is difficult to be sure whether or not thyrocalcitonin is secreted under resting conditions in the intact animal. A preliminary report of a radioimmunoassay technique for thyrocalcitonin, by Arnaud and Littledyke (A5), indicates that all plasma samples examined contained detectable thyrocalcitonin, and that thyroidectomy resulted in a decrease in thyrocalcitonin levels below those found in normal animals.

It is an attractive concept that the uniformity of the plasma calcium concentration in health is attained through balanced low levels of secretion of both parathormone and thyrocalcitonin, and that disturbances of calcium homeostasis may result both in a reduction of the resting level of secretion of one hormone, and in a raised output of the other. Until both hormones can be assayed with precision, the possibility of such a dual mechanism, operating at physiological levels of plasma calcium variation, must remain somewhat speculative.

3.4. Species Differences

Apart from variations in the response of different animal species to thyrocalcitonin injections or infusions, already described, different species might vary widely in the degree of hypercalcemia necessary to stimulate secretion of thyrocalcitonin. Care *et al.* (C6) reported that hypercalcemic perfusion of the sheep thyroid produced a hypocalcemic response similar in magnitude to that obtained by Foster *et al.* (F4) during hypercalcemic perfusion of the thyroid and superior parathyroid in a goat of

similar weight. A smaller response was obtained in the dog by MacIntyre *et al.* (M3) during high-calcium perfusion of thyroid alone and of thyroid plus parathyroids.

Care *et al.* (C7) have compared hypercalcemic perfusion of the isolated thyroid *in situ* in pig, sheep, goat, and calf, and found the percentage fall in the systemic plasma calcium concentration, over the first hour, to be 17%, 2%, 7%, and 0, respectively.

Significant differences in the apparent secretion of thyrocalcitonin might of course result from differences in the rate of destruction of the hormone, rather than from differences in its rate of secretion. A heat-labile factor that inactivates thyrocalcitonin has been found in plasma (T4), but its part in controlling the action of the hormone is as yet uncertain.

3.5. Effect of Thyroid Ablation by Drugs

The question of whether the secretion of thyrocalcitonin can be influenced by experimental hypothyroidism, produced by feeding antithyroid drugs, has been studied by a number of workers. Morii and Talmage (M14) compared the hypercalcemia produced by intravenous infusion of calcium chloride in normal rats with that in rats fed an iodine-deficient diet, and also in rats treated with thiouracil. Although the iodine-depleted rats demonstrated considerable impairment of the ability to deal with hypercalcemia, the thiouracil-treated animals responded in a manner similar to that of the control animals. In rats fed propylthiouracil for 10 days, the thyroid glands were 4 times as large as in control animals, but the amount of thyrocalcitonin which could be extracted from them was not significantly different from that obtained from the smaller control glands (A1). Duncan and Care (D1) have compared the hypocalcemic response to high-calcium perfusion of the thyroid *in situ* in normal and methylthiouracil-treated pigs. The average fall in plasma calcium was $23 \pm 3\%$ for normal animals, and $7 \pm 1\%$ for the methylthiouracil-treated animals. The thyroids of the latter were 5 times larger than normal and histologically showed typical hyperplasia. This considerably reduced output of thyrocalcitonin, in response to hypercalcemic perfusion in the treated animals, could nonetheless be overcome by intravenous administration of exogenous porcine thyrocalcitonin material (Fig. 6). In addition, other methylthiouracil-treated pigs showed a normal hypocalcemic response to exogenous thyrocalcitonin, implying that, although secretion of thyrocalcitonin was diminished, the response of the target organ to the hormone was normal. There was little difference in the reduced ability to deal with hypercalcemia in the methylthiouracil-treated animals before and after thyroidectomy; both re-

sponses were remarkably similar to the impaired response typical of thyroidectomized animals (Fig. 7).

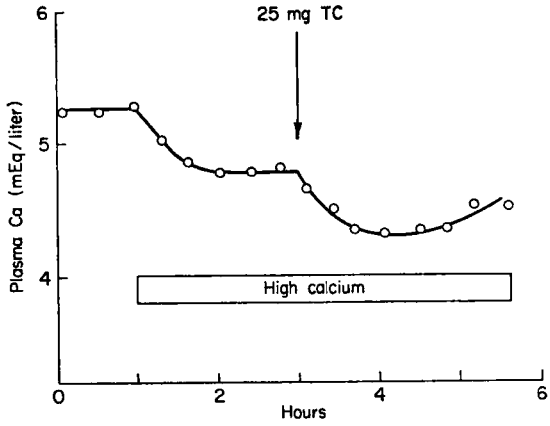

FIG. 6. The effect of exogenous thyrocalcitonin (TC) on the systemic plasma calcium level during hypercalcemic perfusion of the thyroid in a methylthiouracil-treated pig. [Redrawn from Duncan and Care (D1).]

4. Mode of Action of Thyrocalcitonin

4.1. Effects of Exogenous Hormone on Plasma Composition

Hirsch *et al.* (H3) first showed that acid extracts of rat thyroid produce hypocalcemia in recipient animals, and several groups of workers have since confirmed that the injection or infusion of thyrocalcitonin preparations consistently produces significant falls in both the systemic plasma calcium and inorganic phosphate concentrations (C10, G4, H4, K1). The rate of onset of the fall in plasma calcium, and the duration of hypocalcemia, have both been shown to be dose-dependent (A1). Large doses of thyrocalcitonin usually produce a sharp fall in 15–30 minutes, maximal after about 1 hour, and hypocalcemia may persist for up to 4 hours (Fig. 8). There are, however, appreciable species differences in response time, and the route of administration also modifies the time sequence (see Section 5.1 below). With much smaller amounts of hormone, the plasma calcium may fall within 30–60 minutes, and return to normal by 2–3 hours. Changes in the plasma inorganic phosphate level closely parallel the calcium changes, but are less reliable as an index of hormone activity because of the greater variability of control levels of inorganic phosphate.

Little or no change has been observed in the plasma magnesium con-

centration following thyrocalcitonin injections in rats (G4), dogs (C10), and pigs (C5).

Gudmundsson *et al.* (G4) found that thyrocalcitonin produced no fall in calcium in parathyroidectomized rats, although the hypophosphatemic response was still present. If, however, the parathyroidectomized rats were previously fed a diet high in calcium and low in phosphate, so that

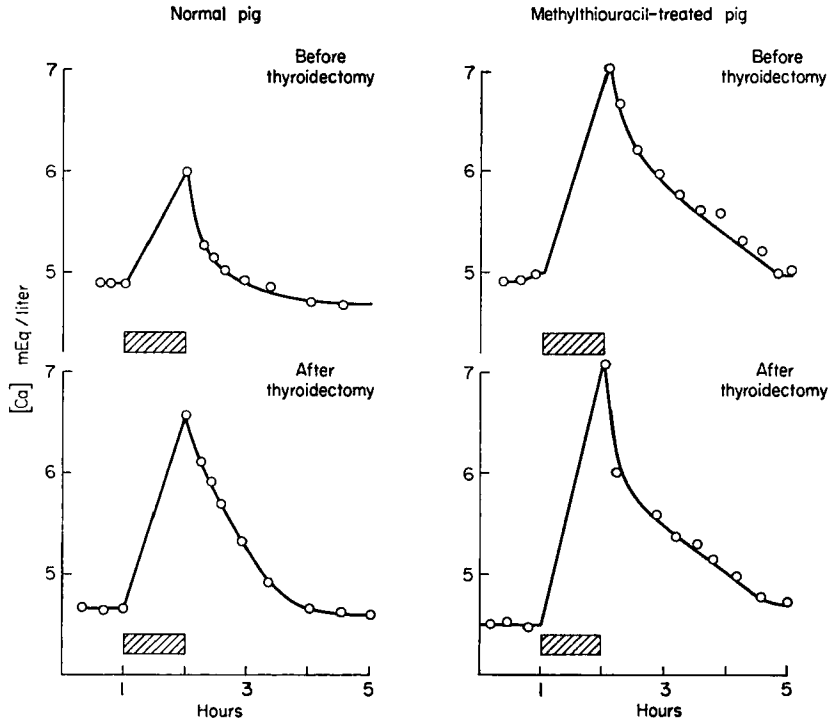

FIG. 7. The effect on systemic plasma calcium concentration of a 1-hour iv infusion of calcium gluconate, before and after thyroidectomy, in normal and methylthiouracil-treated pigs of similar age and weight. The shaded areas represent the periods of infusion (14 mg of Ca/kg of body weight/hour). [Redrawn from Duncan and Care (D1).]

the plasma calcium levels were brought within normal limits, thyrocalcitonin was found to be as effective in these as in normal animals. Aliapoulios *et al.* (A3) found that the low plasma calcium level consequent on parathyroidectomy in the rat could be further reduced by thyrocalcitonin, and that the effect was more persistent than in intact animals. This finding has been confirmed by Tashjian (T2) (Fig. 9). Discrepancies between reports on the action of thyrocalcitonin in para-

thyroidectomized animals may be related to differences in the initial plasma calcium concentration. It has been noted that even in intact animals large doses of thyrocalcitonin do not reduce the plasma calcium level much below 7 mg/100 ml (A3). The effectiveness of thyrocalcitonin in producing a further fall in plasma calcium in parathyroidectomized animals implies that its mode of action does not involve direct inhibition of the effects of parathormone.

Stahl *et al.* (S3) have recently reported that injection of thyrocalcitonin after thyroparathyroidectomy results in a fall in the plasma

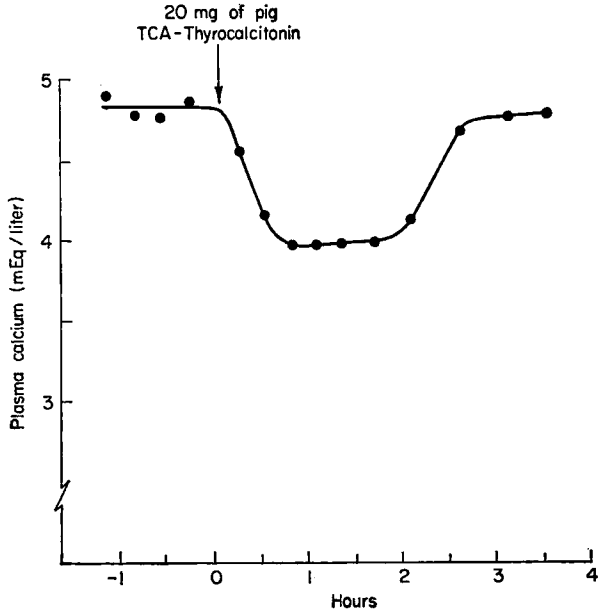

FIG. 8. Effect on plasma calcium concentration of a single injection of porcine thyrocalcitonin into an intact pig.

calcium only; the hypophosphatemic and hyperphosphaturic effects of thyrocalcitonin in the intact animal did not occur. This is in sharp contrast with the finding of Milhaud and Moukhtar (M9) that, in thyroparathyroidectomized rats, thyrocalcitonin and parathormone act antagonistically with respect to plasma calcium levels, but synergistically with respect to plasma phosphate levels. The injection of thyrocalcitonin alone into these thyroparathyroidectomized rats resulted in highly significant falls, both in plasma calcium and in plasma inorganic phosphate levels.

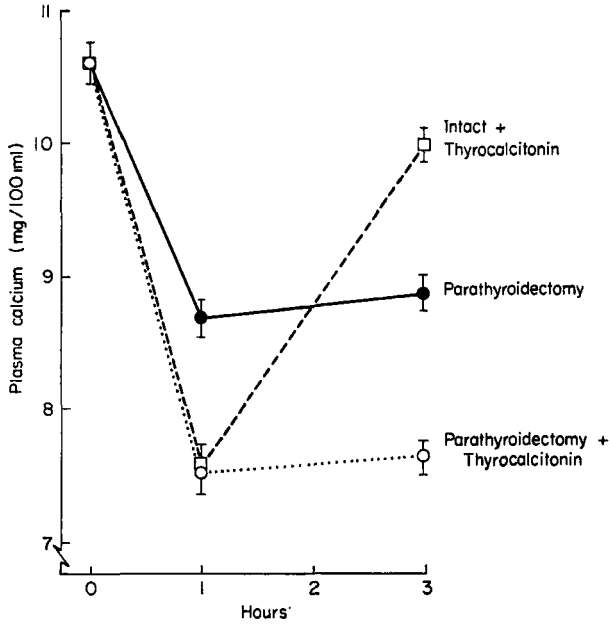

FIG. 9. Effect of parathyroidectomy on the duration of thyrocalcitonin-induced hypocalcemia in the rat. [Redrawn from Tashjian (T2).]

4.2. EFFECTS ON THE KIDNEY

The kidney is obviously a possible site of action of thyrocalcitonin; increased output of calcium in the urine might well account for the rapid fall in plasma calcium levels. Kenny and Heiskell (K1), however, found that thyrocalcitonin produced an increased excretion of phosphate in the rat, but no significant effect on urinary calcium or creatinine output. Milhaud and Moukhtar (M9) obtained similar results in thyroparathyroidectomized rats, although Stahl *et al.* (S3) found that the increased ratio of phosphate to creatinine in the urine of intact animals treated with thyrocalcitonin did not occur if there had been prior thyroparathyroidectomy.

Kenny and Heiskell (K1) regard the phosphaturic effect of thyrocalcitonin as possibly due to secondary stimulation of parathormone release by hypocalcemia; this is not, however, supported by the findings of Robinson *et al.* (R4) and Milhaud and Moukhtar (M9) that the phosphaturic effect of thyrocalcitonin was present in parathyroidectomized and thyroparathyroidectomized rats, respectively. It has been

pointed out that the phosphaturic effect of thyrocalcitonin necessitates considerable caution in the interpretation of phosphate excretion tests, such as may be used in the clinical assessment of parathyroid function (M9, R4). Although the kidney does not apparently alter its output of calcium in response to administered thyrocalcitonin, the calcium content of the kidney itself has been found to be significantly reduced. This, however, may simply reflect a general fall in the calcium content of extracellular fluid (K1). Although the phosphaturic effect of thyrocalcitonin is well established, this cannot be a determining factor in the production of hypocalcemia, since the effect of administered thyrocalcitonin in reducing plasma calcium levels is uninfluenced by nephrectomy (A3, G4, H4, W2).

4.3. Effects on Bone

The generally accepted role of bone as the major target organ for parathormone activity clearly indicated the desirability of investigating whether it played an equally central part in the action of thyrocalcitonin, the more so since hypocalcemia could be produced by the hormone following parathyroidectomy (H3), nephrectomy (G4), or removal of the gastrointestinal tract (A1). The parallelism of the falls in plasma calcium and phosphorus levels (H4) also suggests simultaneous removal of both elements from the extracellular fluid. Chausmer *et al.* (C11), using ^{45}Ca as tracer, found no evidence of increased soft tissue uptake of calcium following thyrocalcitonin, nor were they able to demonstrate an increased uptake of calcium by bone. They did, however, regard their findings as compatible with an acute inhibition of bone resorption, which would not necessarily show an effect on short-term bone uptake of the isotope. Wase *et al.* (W2), however, found that thyrocalcitonin administration to intact rats caused increased uptake of ^{45}Ca both in tibia and in femur. The discrepancy between these two reports has not yet been resolved. The latter workers also demonstrated a markedly positive calcium balance in both young and old rats receiving repeated injections of thyrocalcitonin. On the basis of Kenny and Heiskell's failure to find an appreciable increase in the calcium content of soft tissues (K1), Wase *et al.* feel that the retention of calcium occurs in the skeletal system.

Milhaud *et al.* (M10) studied the turnover of calcium in the plasma pool both in control and in thyrocalcitonin-treated animals. In fasted controls, the release of unlabeled calcium into the pool as a result of bone catabolism caused a fall in plasma calcium specific activity; following thyrocalcitonin treatment, however, the specific activity remained unaltered despite the hypocalcemia, implying a suppression of bone resorption. They claimed good agreement between the experimental find-

ings and those calculated on the basis of an assumed inhibition of bone resorption. Other workers (M11) have found that in prolonged hypocalcemia, produced by repeated injections of thyrocalcitonin into normally fed rats, bone catabolism was markedly depressed, bone anabolism was slightly depressed, and absorption and retention of dietary calcium were greatly enhanced. The influence of thyrocalcitonin on bone resorption *in vitro* has also been studied (A2, F5, G1). Friedman and Raisz (F5), using isotopically labeled bone from rat embryos, showed that thyrocalcitonin *in vitro* inhibited release of ^{45}Ca both from the control bones and from those treated with exogenous parathormone, the latter effect being more pronounced than in the controls. In similar experiments, Aliapoulios *et al.* (A2) assessed bone resorption by microscopic examination of stained preparations, and showed that thyrocalcitonin inhibited parathormone-stimulated bone resorption in mouse bone *in vitro*.

MacIntyre and Parsons (M2) found a small uptake of calcium by cat tibia perfused with thyrocalcitonin, but insufficient to account for the magnitude of the systemic change in calcium level induced by the same concentration of thyrocalcitonin throughout the rest of the body. They postulated a greater uptake of calcium by cancellous bone than by compact bone, and Parsons (P2) suggested that the smaller uptakes observed might be largely explained by a blood shunt through the marrow of the perfused tibia.

Johnston and Deiss (J1) injected rats with ^{45}Ca a few hours before thyrocalcitonin administration. They found the expected reduction in the plasma calcium concentration, although its specific activity was higher in the thyrocalcitonin-treated group than in the controls; this is consistent with an inhibition of release of unlabeled calcium from bone, but could not be explained on the basis of an increased outflow of labeled calcium into tissue from the plasma. When tracer calcium was administered 10–14 days prior to thyrocalcitonin injection, the hormone produced a decrease in both stable and tracer calcium in the blood plasma, indicating an inhibition of calcium release from bone. In these animals having labeled bone calcium, increases in stable and radioactive plasma calcium resulted from the administration of parathyroid extract alone, although the simultaneous administration of parathyroid extract and thyrocalcitonin resulted in no net change in either stable or radioactive calcium. It was concluded that both hormones probably act on a similar metabolic compartment in bone.

Kohler and Pechet (K4) studied the changes in the urinary excretion of calcium, phosphorus, magnesium, ^{90}Sr, and hydroxyproline in intact and thyroparathyroidectomized rats in which the bones had previously

been labeled by the injection of ^{90}Sr. Constant infusions of thyrocalcitonin over periods of 6–18 hours caused in each case a decreased excretion of calcium, phosphorus, magnesium, strontium, and hydroxyproline; similar perfusions of parathormone caused increased excretion in each case, which could be negated by concomitant infusion of thyrocalcitonin. It was concluded that thyrocalcitonin inhibits the action of parathormone on bone resorption, but that it also inhibits bone resorption in the absence of parathormone. Martin *et al.* (M4) also found significant reduction in urinary hydroxyproline output after thyrocalcitonin administration, which they attributed to the direct inhibition of bone resorption.

In studies of the effect of thyrocalcitonin on the removal of calcium, phosphorus, and certain radioisotopes from bone by peritoneal lavage, Klein *et al.* (K3) found a reduction in the rate of removal of calcium, phosphorus, and ^{86}Sr (recently deposited) as well as ^{45}Ca and ^{32}P deposited 3 weeks previously. In intact animals the effect of the hormone was brief, and the reduced rate of removal had returned to normal within 3 hours of the injection; in parathyroidectomized rats the response to the hormone was much slower, of smaller extent, but of longer duration. These authors concluded that thyrocalcitonin inhibits removal both of recently deposited and of longer-standing bone salts, and that its mode of action is not directly antagonistic to parathormone.

In summary, therefore, thyrocalcitonin action on bone appears to be more in the nature of an inhibition of the uptake of bone calcium and phosphorus into the extracellular fluid, rather than an accelerated deposition of these elements. Further careful *in vitro* and *in vivo* studies will probably be necessary before the nature of the relationship between thyrocalcitonin action and parathormone action is clarified.

4.4. Effects on the Alimentary Tract

The actions of thyrocalcitonin on the alimentary tract do not appear to be of major significance. Aliapoulios and Munson (A1) found that administration of the hormone to rats produced the expected hypocalcemia, even when the entire gastrointestinal tract had been removed. Clearly, changes in calcium absorption or fecal loss cannot be relevant to the immediate drop in plasma calcium level. However, previous fasting, or the administration of a low-calcium diet for some days, appears to sensitize the rat to the effects of thyrocalcitonin (H4, K1).

Milhaud *et al.* (M11), using intact rats, found that the prolonged hypocalcemia induced by repeated injections of thyrocalcitonin resulted in significantly increased absorption and retention of dietary calcium; they also noted a reduction in the endogenous and total fecal calcium. These findings have largely been confirmed by Wase *et al.* (W2), who

found that repeated injections of thyrocalcitonin resulted in significantly more positive calcium balances in both young and old rats. During the period of induced hypocalcemia, there was a reduction in the endogenous fecal calcium excretion. These workers, however, noted that there was no effect on the intestinal absorption of calcium.

4.5. Relationship to Other Hormones

The effects of thyrocalcitonin in reducing the serum levels of calcium and phosphate, and in increasing the output of phosphate in the urine, have not at present been shown to be significantly influenced by any hormone other than parathormone. Milhaud *et al.* (M8) found that neither the thyrocalcitonin content of the thyroid gland nor the hypocalcemic activity brought about by administration of thyrocalcitonin was significantly altered by hypophysectomy. The presence or absence of the thyroid gland, and therefore of thyroid hormone, does not appear to influence the sensitivity of an animal to thyrocalcitonin administration, while the interrelationship, if any, to suprarenal cortical activity does not appear to have been studied as yet.

Concomitant administration of large doses of thyrocalcitonin and of parathormone can result in little or no change from normal serum calcium values (M9). However, since administration of thyrocalcitonin to parathyroidectomized rats results in a more prolonged hypocalcemic response than is found in intact animals, thyrocalcitonin apparently does not act by reducing the output of parathormone (see Section 4.1 above). Tashjian (T2) considers that the relatively short duration of hypocalcemia produced in response to a single injection of thyrocalcitonin owes, in part at least, to increased activity of the parathyroid gland. Parathyroidectomized rats and animals rendered unresponsive to the calcium-mobilizing action of parathormone by treatment with actinomycin suffered more prolonged hypocalcemia in response to thyrocalcitonin than did control animals. Tissue culture studies of bone resorption indicate that the action of parathormone is not directly antagonistic to thyrocalcitonin; the net response is dependent only on the relative concentrations of the two hormones present in the medium (A2, F5). Similar conclusions can be drawn from *in vivo* studies in rats previously injected with ^{45}Ca (J1). Thyrocalcitonin administration resulted in reductions in both stable and radioactive calcium in the plasma, parathormone produced an increase in both forms, and concomitant administration of both hormones to the same animals resulted in no net change from controls in either form of calcium. Besides confirming the negating effect of parathormone on the hypocalcemic response to thyrocalcitonin, Milhaud and Moukhtar (M9) show an enhanced hypophosphatemic and

phosphaturic effect when both hormones are administered to the same thyroparathyroidectomized rats.

5. Detection and Measurement of Calcium-Lowering Hormones

5.1. Bioassay Procedures

Several animal species have been used in the biological assay of calcium-lowering hormones. For reasons of size, the rat is most commonly used, although mice have been claimed to be more responsive to human thyrocalcitonin preparations (S2). For studies of the course of thyrocalcitonin action in which serial blood samples were required, both pigs (C5) and goats (F4) have been used. For assessment of the potency of hormone preparations, bioassay in rats is in general satisfactory.

The method described by Hirsch *et al.* (H4) uses intact male rats previously maintained on a low-calcium diet for 4 days. Standard and unknown preparations are injected subcutaneously into parallel groups of test animals, and after 1 hour blood samples are collected by cardiac puncture under ether anesthesia. The plasma calcium levels are measured, and the relative potencies of the preparations are calculated by standard statistical procedures. The mean index of precision of ten consecutive assays was reported as 0.23 ± 0.025.

A similar procedure was used by Kumar *et al.* (K6) in investigations of the differences in response when a standard thyrocalcitonin preparation was injected by different routes. It was found that subcutaneous and intraperitoneal injection resulted in much smaller hypocalcemic responses than were obtained from intravenous administration of the same amount of hormone. An intravenous infusion gave a slightly greater response than a single intravenous injection, although in both cases the maximum response occurred after 30–60 minutes (Fig. 10). It was therefore recommended that blood samples be taken at 50 minutes from the start. Logarithmic dose–response curves, over a 100-fold dose range, were studied for both of these intravenous techniques, and linear responses were obtained. The slopes were similar and statistically highly significant. Bioassay of two thyrocalcitonin preparations by the single intravenous and infusion techniques gave similarly significant log dose–response curves; the potency ratios were comparable although the infusion method was more precise. (The index of precision was 0.26 for the infusion, and 0.42 for the single intravenous injection technique, although later experiments showed a higher degree of precision for the single-injection method, and the authors now recommend the latter for general use.) The full assay procedure requires the use of two standard thyrocalcitonin preparations and two different dose levels of the unknown. The ratio of higher to lower dose for both standard and unknown

should be the same (usually 3:1 or 4:1), and all doses must be given in the same final volume for any one assay.

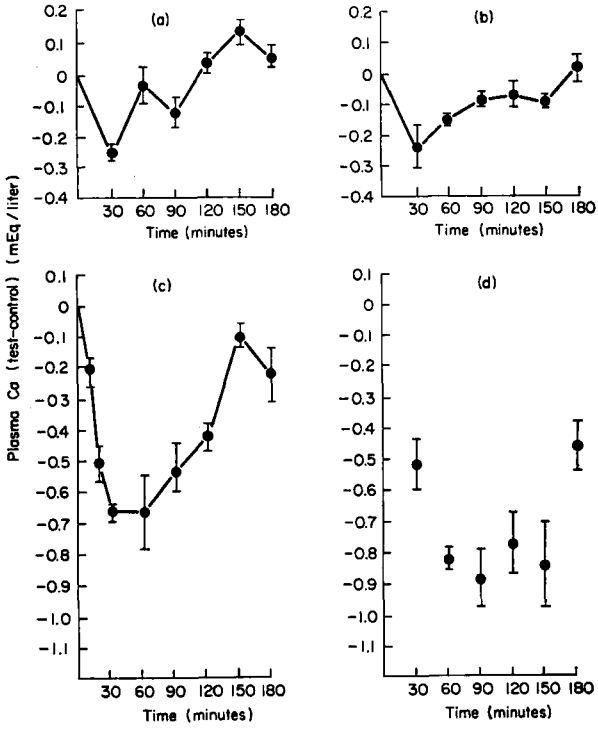

FIG. 10. Effects of administration of the same dose of thyrocalcitonin by different routes in the rat: (a) subcutaneous injection, (b) intraperitoneal injection, (c) single intravenous injection, (d) intravenous infusions over 30, 60, 90, 120, 150, and 180 minutes [From Kumar *et al.* (K6).]

5.1.1. *Strain and Species Variation in Sensitivity*

As was mentioned earlier, there appears to be considerable species variation in the secretion of thyrocalcitonin, and likewise there are reports of species variations in sensitivity to exogenous thyrocalcitonin preparations. Care *et al.* (C8) found sheep to be considerably less responsive to exogenous sheep thyrocalcitonin than pigs to porcine thyrocalcitonin. Smith *et al.* (S2) were unable to demonstrate hypocalcemic activity in human thyroid extracts injected into monkeys or rats, but found the mouse to be responsive. On the other hand, Milhaud *et al.* (M12) demonstrated hypocalcemic activity in human thyroid extracts in the rat and monkey, although these responses were obtained only after injecting much larger doses than would have been required had porcine

thyrocalcitonin been used. Chausmer *et al.* (C10) found the dog to be less responsive than the rat to thyrocalcitonin preparations from pig, lamb, dog, or deer. They also noted that the responsiveness of the dog to porcine thyrocalcitonin was decreased after repeated injections; this fall in response was not observed in dogs after repeated injections of thyroid extract from other animal species. Tashjian and Munson (T4) found that normal human, rat, and rabbit serum contained a factor that when incubated with porcine thyrocalcitonin destroys its activity. They have also shown that an antiserum to pig thyrocalcitonin, produced in the rabbit, is effective in neutralizing the hypocalcemic activity of pig and calf thyroid extracts, but is ineffective against rat and monkey thyrocalcitonin preparations. Implicit in these findings is the possibility that species variation in sensitivity to exogenous hormone might result from different rates of inactivation of heterologous thyrocalcitonin, as well as from possible structural differences between thyrocalcitonin preparations obtained from different animal species. The variable responsiveness to homologous thyrocalcitonin in sheep and in pigs suggests a possible variation in end-organ sensitivity in these species.

In addition to species variations, there can be definitely variable responses between different strains of rat, and even within the same strain. A strain of hooded Lister rats showed no significant hypocalcemic response to either pig or sheep thyrocalcitonin (W3). Sprague-Dawley rats, by contrast, gave good responses to sheep, bovine, and pig thyroid extracts, and more recently we have found high sensitivity to pig thyrocalcitonin in rats of the Wistar strain. Chausmer *et al.* (C10) found differences in the responsiveness to pig thyrocalcitonin between Wistar and Holtzman rats, the latter being more sensitive. No differences were observed between the responsiveness of these two strains to thyrocalcitonin from dog, lamb, and deer. These authors also record that certain batches of Wistar rats did not respond to pig thyroid extracts, despite the fact that other rats from the same source proved to have a sensitivity to the same thyroid extracts comparable to that displayed by the Holtzman rats. We agree with Chausmer *et al.* that environmental factors alone are not responsible for such variable responsiveness of strains or batches of rats to the same thyroid extracts. Such a resistance might owe to the presence of inhibitory substances, described by Tashjian and Munson (T4), but might also result from a failure of target organ responsiveness to certain thyrocalcitonin preparations.

5.1.2. *Effects of Age and Diet*

Although few details have been published on the effects of age and diet on sensitivity in bioassays of thyrocalcitonin, most workers appear

to consider that the use of young rats that have been fasted or subjected to a low-calcium diet for a few days is necessary for satisfactory results. Hirsch *et al.* (H4) found that rats on a stock diet reacted in a similar manner to those previously on a low-calcium diet for 4 days, but that their responses were more variable; Wase *et al.* (W2) found that fasted rats were consistently more sensitive to, and gave a more uniform response to, thyrocalcitonin than nonfasted animals. Recently Morey (M13) has studied the effects of parathyroidectomy and of cautery of the thyroid in both fed and fasted rats, and from the results concluded that fasted animals, when used in such acute experiments, are subject to less variation and are more sensitive to changes in serum calcium than are fed animals. The consensus of opinion in recent publications in which thyrocalcitonin was assayed in the rat favors the use of male rats, aged 7–8 weeks, weighing around 150 g, and having been fed a low-calcium diet for 4 days prior to use in the bioassay.

5.2. Radioimmunoassay Procedures

The investigation of possible clinical disturbances of thyrocalcitonin secretion is likely to require measurement of the amount of the hormone present in body fluids. Since thyrocalcitonin is a peptide of as yet uncertain structure (see Section 7 below), and since the amounts present in blood are likely to be minute, its direct chemical determination may prove to be impracticable. Other peptide hormones, however, have been successfully measured in blood plasma by radioimmunoassay methods, and similar measurements of thyrocalcitonin have already been attempted (A5).

The method is straightforward in principle. A potent antiserum to the protein or peptide hormone is prepared, and a known slight excess of antibody is made to react for a few days with an aliquot of the plasma to be assayed. The mixture is then incubated, usually for about 18 hours, with a known quantity of ^{131}I-labeled hormone of high specific activity (again an excess is necessary). When the reaction is complete, the unreacted labeled hormone is separated from the hormone–antibody complex by electrophoresis. Isotope counting in the two labeled fractions then permits the amount of hormone originally present in the plasma to be calculated, the procedure being calibrated by parallel runs using known amounts of unlabeled hormone in place of the plasma sample.

Although techniques of this kind have been successfully applied to several peptide hormones in blood plasma, considerable problems must be overcome before a radioimmunoassay for thyrocalcitonin can be established as of adequate specificity and sensitivity for routine use. The relatively small molecular size of purified thyrocalcitonin peptide (see

Section 7.2 below) might be expected to cause some difficulty in exciting the intense immunological response necessary for the production of antiserum of high potency. At a time when the purification and characterization of the active hormone are still in progress, it might seem premature to hope for the easy production of an antiserum which would be both highly potent and completely specific. However, several workers have produced antisera that appear to be satisfactory for preliminary work at least. Tashjian and Munson (T4), using relatively crude porcine thyrocalcitonin as antigen together with complete Freund's adjuvant, have obtained a rabbit antiserum that specifically inactivated porcine thyrocalcitonin, but not those derived from rat or monkey. However, it showed considerable activity against bovine hormone.

Arnaud and Littledyke (A5) and Hargis *et al.* (H1) overcame the problem of antigenicity by conjugating relatively pure porcine thyrocalcitonin to rabbit albumin before injection, and claim that the antisera obtained were specific to thyrocalcitonin, judged by gel diffusion and immunoelectrophoresis results. The former workers have briefly described the use of this antiserum in a radioimmunoassay procedure similar to that devised by Berson *et al.* for parathormone (B4). They claim that the technique will detect 0.5 ng of thyrocalcitonin/ml of serum. These workers found no difference in the immunological behavior of human and porcine hormone, and quote a normal range of 30–85 ng/ml in human plasma. In animal studies, elevated values were found in older animals and in response to hypercalcemia.

Although other workers have prepared and used antisera in immunofluorescence studies, confirmatory reports of the practicability of preparing antisera suitable for radioimmunoassays have not yet appeared. A considerable degree of cross-reactivity between species, both in the effects of administered hormone and in hormone–antibody interactions, appears to be a feature of most thyrocalcitonin preparations so far studied. Although this partial lack of species specificity should facilitate the development of antisera and of isotopically labeled hormone preparations from animals for use in immunoassays in man, it must also leave some doubt with respect to the specificity of such assays when applied to so complex a mixture as blood plasma.

5.3. Detection and Localization of Thyrocalcitonin within the Thyroid Gland

5.3.1. *Histochemical Studies*

Although extraction, purification, and biological assay of calcium-lowering hormones have shown the thyroid gland to be the site where

thyrocalcitonin is stored, and presumably also synthesized, the lack of correlation between abnormal thyroid hormone production and disturbances of calcium metabolism implies that the production and release of the two classes of hormone are separate functions of the gland. That the thyroid contains a type of cell (the C cell or parafollicular cell) that is histologically and histochemically different from the thyroxin-producing cells is well known (N4, P4), and enzyme-histochemical studies of the thyroid before and after perfusion with high-calcium blood (F2) suggest that these cells may indeed be the site of thyrocalcitonin production. The main changes found following hypercalcemic perfusion were an increase in acid phosphatase activity, a rise in the cell content of glycerophosphate, and a reduction in nonspecific esterase activity. Whether these changes are related to the release of stored hormone or to the synthesis of new thyrocalcitonin is uncertain.

5.3.2. *Immunofluorescent Localization*

Specific antisera to proteins or peptides, when coupled to suitable fluorescent dyes and observed under ultraviolet light, can be used to identify the sites of these substances within individual cells. Hargis *et al.* (H1) applied, to sections of pig thyroid, conjugated rabbit antiserum to porcine thyrocalcitonin, and found fluorescence in all thyroid epithelial cells, although not in colloid. They concluded that thyrocalcitonin is produced or stored in the same cells that produce thyroglobulin, and thought from the granular appearance of the fluorescent material that thyrocalcitonin might be stored within some intracellular organelle. Bussolati and Pearse (B5), however, using guinea pig antiserum to pig thyrocalcitonin, reached an entirely different conclusion, that the C cells (which are epifollicular in the pig) are the site of thyrocalcitonin production and storage both in pigs and in dogs. In studies of this kind, a valid result will not be obtained unless the thyrocalcitonin used for inducing antibody production is totally free from other thyroid gland antigens, and although it is not impossible that two or more thyrocalcitonin peptides may exist that have different parent cell types, it seems more likely that inadequate purity of antigen is responsible for the discrepancy between these two reports. Fluorescence confined to one type of cell is more likely to be specific than that found in all types of epithelial cells within the thyroid.

5.3.3. *Cell Fractionation Studies*

Although the bulk separation of different thyroid cell types and the separate extraction of any thyrocalcitonin which they contain are not feasible at present, information about the intracellular localization

of the hormone can be obtained by fractionation of all types of thyroid cells by homogenization and differential centrifugation. The resulting particulate and soluble fractions of rat thyroid have been assayed for hypocalcemic activity by Cooper and Tashjian (C12). Activity was maximal in the supernatant fraction after centrifugation at 8000 *g*, but was concentrated in the sediment at 100,000 *g*, implying that microsomes rather than mitochondria are the intracellular site of thyrocalcitonin storage. Bauer and Teitelbaum (B2), in studies involving fractionation of pig thyroid coupled with ultrasonic disintegration of subcellular particles, have obtained evidence suggesting that the hormone is concentrated within secretory granules; they have pointed out the resemblance between these granules, seen in electron micrographs, and the characteristic electron-dense particles seen in parafollicular pig thyroid cells.

6. Isolation and Purification of Thyrocalcitonin

Most thyrocalcitonin preparations so far described have been obtained from thyroid tissue by one of three procedures. The method of Rasmussen *et al.* (R3) for extraction of parathyroid polypeptides was shown by Tenenhouse *et al.* (T5) to be also capable of producing an active hypocalcemic agent when applied to pig thyroid gland. Baghdiantz *et al.* (B1) extracted defatted pig thyroid tissue with hot acid, and obtained an initial partial purification of this extract by salt fractionation. In the third procedure, Hirsch *et al.* (H4) used cold acid extraction of pig thyroid; the bulk of the inactive material was removed by centrifugation, and they achieved a considerable degree of purification by further high-speed centrifugation. These procedures are outlined below under the designations "Method 1," "Method 2," and "Method 3," respectively.

Method 1 (R3). The procedure originally described for isolation of parathyroid polypeptides, parathormone in particular, requires the inclusion of cysteine as a reducing agent at two steps in the extraction procedure, since parathormone is sensitive to oxidation. Although there is no evidence that the hypocalcemic activity of thyrocalcitonin is affected by mild oxidation, cysteine has been retained in the thyrocalcitonin extraction procedure also.

Dried, defatted thyroid powder is extracted with 8 *M* urea–0.1 *M* cysteine in 0.2 *N* HCl in the cold. Glacial acetic acid and acetone are then added, and the precipitate is filtered off and discarded. Diethyl ether is added to the filtrate, and the resulting precipitate is allowed to settle. After washing, the precipitate is dissolved in 20% acetic acid containing 0.01 *M* cysteine, and solid sodium chloride is added to give a concentra-

tion of 5%. Any precipitate that forms is removed by centrifugation, and 45% trichloroacetic acid (TCA) is added to the supernatant to give a final concentration of 7.5% TCA. The precipitate contains thyrocalcitonin activity, and is collected by centrifugation and washed first with 5% TCA and then with diethyl ether. The washed precipitate is dissolved in 0.02 *N* HCl, the solution is extracted 3 times with peroxide-free ether, and the last traces of ether are removed on a rotary evaporator. Chloride is then exchanged for acetate on an ion-exchange column, and the effluent is lyophilized. The resulting powder, termed "TCA material," is light brown, and the usual yield is about 1.2–1.6 g from 200 g of defatted thyroid powder (equivalent to approximately 1 kg of fresh gland). Extraction to this stage requires about 5 days. Although Rasmussen recommended that this procedure be carried out at 4°C, we have consistently obtained active preparations when the entire process is carried out at room temperature. We have also encountered some difficulty in obtaining a solid precipitate at the ether precipitation stage, but this can be overcome by reducing to half the volume of ether added.

Further purification of this TCA material can be achieved by gel filtration, using 0.2 *M* ammonium acetate (pH 4.6) as the developing buffer. The method as originally described for parathyroid polypeptides utilized a Sephadex G-100 column, but Tenenhouse *et al.* (T5) obtained better resolution of thyroid TCA material by using Sephadex G-75. These authors claimed to have obtained a homogeneous product, indicated by starch gel electrophoresis, after only one passage of the relatively crude TCA material through a Sephadex G-75 column; this was on bioassay 5–15 times more active, weight for weight, than the TCA powder. The main hypocalcemic activity in the eluates from Sephadex columns was located in a small protein-containing peak, well separated from and following after the main protein peaks. When applying Rasmussen's method to pig, sheep, bovine, and human thyroid, we have had some difficulty in dissolving the TCA material in the ammonium acetate buffer, so that the gel filtration step described above could not be applied. With columns of Sephadex G-100 in 0.02 *N* HCl, however, we have found the hypocalcemic activity to be associated with the descending limb of the last protein peak, although the degree of purification that we have achieved is less than that claimed by Tenenhouse *et al.*

Method 2 (B1). This procedure, like that of Rasmussen, utilizes defatted thyroid powder obtained by repeated acetone washing of fresh pig thyroid tissue. This powder is then extracted with 0.2 *N* HCl at 60°C for 5 minutes, followed by 1 hour at room temperature. After filtering through cheesecloth, the filtrate is dialyzed for 24 hours against acetate

buffer (pH 4.6) at 4°C; 3 *M* NaCl is added to give a final concentration of 1 *M*; the protein that then precipitates is mostly inactive. When the NaCl concentration is increased to 2 *M*, a further precipitate forms that contains most of the hypocalcemic activity. This precipitate is collected and freeze-dried. The preparation to this stage requires approximately 6–7 days.

Further purification of the salt-precipitated extract is achieved by passage through Sephadex G-100 in 0.1 *M* acetate buffer (pH 4.6) containing 0.2 *M* NaCl. The main hypocalcemic activity is associated with the descending limb of the last main protein peak. A later communication from the same laboratory (G4) describes the use of 0.1 *M* formic acid in place of the acetate buffer for the Sephadex G-100 stage. A further gel filtration step on Sephadex G-100 in 0.1 *M* formic acid—1 *M* urea–0.2 *M* NaCl is then used, and salt and other contaminants are removed by two or more runs on polyacrylamide beads. The active material is finally eluted as a single symmetrical peak.

Electrophoresis on starch gel, in formate buffer (pH 2.9) in 8 *M* urea, followed by nigrosin staining, indicates that the thyrocalcitonin obtained from the polyacrylamide bead stage runs as a fast-moving diffuse band. Other highly purified preparations yield two bands on electrophoresis, both active, with the slower band predominating in most preparations. Genetic polymorphism was suggested as a possible explanation for the occurrence of these two active fractions, although it seems more probable that some degree of molecular fragmentation could occur during the hot acid extraction without necessarily destroying all biological activity in the fragments. In this context it may be noted that, in the case of parathyroid hormone, the initial extraction procedure influences the approximate molecular weight as well as the specific biological activity of the product, hot HCl extraction giving rise to smaller molecules than either an acetic acid or a phenolic extraction procedure (R2).

Method 3 (H4). This procedure, unlike the others, does not require the somewhat tedious defatting and drying of thyroid tissue, but has the disadvantage that smaller quantities of tissue can be processed during a single run, since a preparative ultracentrifuge is required. Fresh pig thyroid is homogenized with cold 0.1 *N* HCl, and a supernatant fraction is obtained by centrifuging the homogenate at 600 *g* at 4°C. This fraction is then centrifuged at 100,000 *g* for 24 hours. The supernatant is lyophilized prior to further purification. Because of the limited capacity of ultracentrifuge rotors, only some 10–15 g of thyroid gland can usually be extracted per run. The method, however, has considerable advantages in simplicity and speed.

Additional purification of this relatively crude extract can be achieved by gel filtration on Sephadex G-50 equilibrated with 0.05 *M* sodium acetate buffer (pH 3.8). Elution with the same buffer produces a large inactive protein fraction, followed by a smaller protein peak, and finally a "nucleotide" fraction. The hypocalcemic activity is associated with the descending limb of the second protein peak and the ascending limb of the "nucleotide" peak. The most active fractions are further purified by gradient elution with sodium acetate buffer on a carboxymethyl-Sephadex G-25 column. Removal of the protein from the column is effected by gradient elution with sodium chloride in sodium acetate buffer. The hypocalcemic activity is associated with the descending limb of the protein peak. The authors claim that a 500-fold purification of thyrocalcitonin from the initial acid extract can be achieved.

7. Physical and Chemical Properties of Thyrocalcitonin

7.1. Purity and Potency

As was indicated above, purification of thyrocalcitonin has been achieved almost exclusively by gel filtration procedures, accompanied by electrophoresis as a means of monitoring progress. The use of Sephadex in various grades (B1, H4, T5) and polyacrylamide beads (G4) has allowed the production of highly purified preparations, but it is still uncertain whether such procedures are capable of carrying the purification to the stage from which a completely homogeneous product results. Tenenhouse *et al.* (T5) claimed to have produced a homogeneous product, adjudged by starch gel electrophoresis, after only one gel filtration step; our own experience has been quite otherwise. Tenenhouse *et al.* do not appear to have confirmed that the single protein band demonstrated on starch gel did in fact possess thyrocalcitonin activity. Our own experiments, in which highly active Sephadex fractions were submitted to acrylamide disc electrophoresis, in many instances revealed single protein bands. Elution of these areas of gel and of unstained areas, however, revealed that hypocalcemic activity was absent from the zones that gave a positive staining reaction for protein, whereas virtually all the applied activity was recovered from unstained areas. Gudmundsson *et al.* (G4) found that several gel filtration steps were necessary before a preparation homogeneous on starch gel electrophoresis could be obtained, and Hirsch *et al.* (H4) achieved a 50-fold improvement in specific activity after the gel filtration stage by a single ion-exchange step.

While the purification procedures so far described undoubtedly achieve substantial increases in the specific activity of the material, until such time as products of consistent specific activity, homogeneous with re-

spect to molecular size and of reproducible amino acid composition, are obtained, it is premature to talk of "pure" thyrocalcitonin.

Comparison of the potencies of different thyrocalcitonin preparations has been complicated by the use of intravenous or subcutaneous injection or of infusion techniques during biological assays. Subcutaneous injections of hormone have been shown to produce a smaller hypocalcemic response than the same amount of material administered by the intravenous route (K6). Using subcutaneous injection of the supernatant liquid from an ultracentrifuged pig thyroid extract, Hirsch *et al.* (H4) obtained a 10% fall in the plasma calcium in the rat from their standard preparation, containing 10 μg of protein nitrogen. This quantity of the standard preparation was therefore adopted as an arbitrary unit of activity. Their most highly purified preparation was 50 times more potent, i.e., an amount equivalent to 0.2 μg of protein nitrogen was capable of producing a 10% fall in serum calcium in the rat. Tenenhouse *et al.* (T5) do not specify whether subcutaneous or intravenous injection was used in their assays and express the dose in terms of weight of material only. Assuming that their preparations contained protein or peptide material only, we calculate that, to produce a 10% fall of plasma calcium in the rat, their crude and purified materials might contain approximately 3 and 0.5 μg of protein nitrogen, respectively. These values are comparable with our own for similarly prepared material; with relatively crude TCA material, and intravenous injection of an amount containing 1–2 μg of protein nitrogen, we ordinarily find a 10% fall in plasma calcium in the rat. With Sephadex-purified products we have obtained a similar response from amounts equivalent to 0.15–0.2 μg of protein nitrogen.

Using intravenous infusions, Baghdiantz *et al.* (B1) obtained a 10% fall in plasma calcium in the rat with relatively crude salt-fractionated pig thyroid extract containing 8 μg of protein nitrogen. The purest preparation reported by these workers appears to have been about 20 times more potent, i.e., 0.4 μg of protein nitrogen produced an approximately 10% fall.

A standard preparation of thyrocalcitonin, prepared to the salt-fractionation stage by Method 2 above, is available from the Medical Research Council, Department of Biological Standards, National Institute for Medical Research, Mill Hill, London, and is distributed in ampules containing 0.25 MRC unit. When 0.01 MRC unit is injected intravenously into a 150-g rat, it produces a fall of approximately 10% in the plasma calcium after 50 minutes, i.e., this new unit is approximately 100 times larger than that described by Hirsch *et al.* (H4) and 1000 times larger than that of Baghdiantz *et al.* (B1). From the in-

formation given by Kumar *et al.* (K6), we calculate that 0.01 unit of the MRC standard preparation contains approximately 16 μg of protein nitrogen.

7.2. Molecular Size

Estimates of molecular weight of thyrocalcitonin have so far been published from only two sources. Tenenhouse *et al.* (T5) calculated from the amino acid composition of their most highly purified thyrocalcitonin preparation that its molecular weight is of the order of 8600, and equilibrium centrifugation studies with and without mercaptoethanol gave figures of 8700 and 9700. MacIntyre *et al.* (M3) estimated the molecular weight by a gel filtration technique, and obtained a value of less than 3000 for their highly purified product. The same workers (M1) find that ultracentrifugation studies indicate a molecular weight of the order of 2000–6000, i.e., much smaller than was found by Tenenhouse *et al.* while O'Riordan *et al.* (O1), using sucrose density gradient ultracentrifugation and biological assay of the fractions obtained, estimated that there most active preparation of porcine thyrocalcitonin had a molecular weight in the range 5000–6000. It is perhaps not unreasonable to deduce that "pure" thyrocalcitonin has yet to be prepared, and, although the product obtained by Tenenhouse *et al.* may be capable of further purification, that of Gudmundsson *et al.* (G4) might well contain a number of active peptides arising from molecular fragmentation during the rather severe extraction procedure used. The single gel-filtration stage carried out by Tenenhouse *et al.* is unlikely to produce a strictly homogeneous product, and the experience of Rasmussen and Craig (R2) with hot acid extraction in the preparation of parathormone seems relevant to the latter possibility. Ideally, a "pure" hormone should be either in the form in which it is present in the gland that produces it, or in the form in which it is passed from that gland into the circulation. On the other hand, a more practicable molecule for study or for synthesis might be the smallest fragment that possesses the characteristic biological activity. This latter is perhaps a more attainable ideal in the field of protein or peptide hormones.

7.3. Amino Acid Composition

Very little information is available regarding the amino acid composition of thyrocalcitonin. Tenenhouse *et al.* (T5) analyzed their most highly purified preparations, and calculated the following tentative empirical formula: Lys 8, His, Arg 4, Thr 4, Ser 4, Glu 10, Pro 4, Gly 5, Ala 8, Val 4, Meth, Cys ½, Ileu 3, Leu 9, Tyr 2, Phe 2, Try, and

(—$CONH_2$) 7; it was also reported that there was a single N-terminal threonine molecule. Although no detailed amino acid composition has been reported for the purified thyrocalcitonin prepared by Gudmundsson *et al.* (G4), it differs considerably from the formula given above in that no cystine or isoleucine has been detected (M1).

Quite apart from the feasibility or otherwise of synthesizing peptides possessing thyrocalcitonin-like activity, the amino acid composition of the molecule is of interest in that the preparation of iodinated hormone of high specific activity is necessary if radioimmunoassay is to be attempted.

7.4. Stability of Purified Preparations

As has been indicated, we found thyrocalcitonin to be sufficiently stable to allow its extraction from thyroid tissue and its subsequent purification through several gel filtration steps to be carried out at room temperature. Thyrocalcitonin has been shown to be capable of surviving fairly vigorous conditions without serious loss of biological activity. Baghdiantz *et al.* (B1) report that boiling in 0.1 *M* acetate buffer (pH 4.6) for 15, 30, and 120 minutes resulted in losses of activity of 12, 46, and 72%, respectively. Boiling in 1 *N* HCl and 1 *N* NaOH for 1 hour resulted in complete loss of activity. Bauer and Teitelbaum (B3) found no loss of activity on heating to 90°C for 15 minutes, and Tashjian and Munson (T4) found that heating at 60°C for 15 minutes in 0.1 *N* HCl was without effect on the hypocalcemic activity. Incubation with pepsin or with trypsin, at pH 3 or 7, respectively, resulted in complete loss of activity after 6 hours (B1), confirming the protein or peptide nature of thyrocalcitonin. MacIntyre *et al.* (M3) found considerable loss of activity of crude thyroid extracts during storage at 4°C with increasing pH of the solution. Thus storage below pH 6 resulted in little change in activity in 14 days, whereas similar storage at pH 8–9 resulted in almost complete loss of activity. Chausmer *et al.* (C10) report that the adjustment of the pH of acid extracts of pig thyroid from pH 1.2 to 7.4 did not result in loss of hypocalcemic activity, while reducing the protein content of the extracts by half.

Hirsch *et al.* (H4) found that their crude acid extract of pig thyroid could be stored frozen for at least 6 months without loss of activity. Our own experience is that both the dry TCA material, as prepared by Method 1, and purified Sephadex fractions stored in 0.02 *N* HCl, retain their potency almost unchanged when stored at 4°C for 6 months.

8. The Clinical Significance of Thyrocalcitonin

Classically, the evidence for the existence of a hormone includes, in addition to the characteristic effects of its administration in normal sub-

jects and of its withdrawal through removal of the secreting organ, the occurrence of clinical syndromes caused by oversecretion or by undersecretion. The former ought to be curable by total or partial removal of the hyperactive gland, and the latter by administration of the deficient hormone. In the case of thyrocalcitonin, therefore, one might expect not only that a state of hypercalcemia would follow surgical ablation of the thyroid gland, but that hypercalcemia might on occasion be attributable to spontaneous hyposecretion of thyrocalcitonin. Hypocalcemia, on the other hand, might arise through hyperplasia or tumor of the cells in the thyroid gland responsible for thyrocalcitonin production. At the time of discovery of thyrocalcitonin, none of these situations had been recognized as a clinical entity. Evidence is gradually accumulating, however, which suggests that thyrocalcitonin may nonetheless have a significant role in human disorders of calcium metabolism.

8.1. Relationship to Thyroidectomy and to Thyroid Hormone Secretion

Total or partial thyroidectomy, so far from being associated with sustained hypercalcemia, is frequently followed by transient or prolonged hypocalcemia, often with tetany (P1, W1). Although this might conceivably be the result of release of thyrocalcitonin into the circulation from the injured gland, the quick onset and short duration of the action of injected thyrocalcitonin (A1) contrast with the delayed appearance of post-thyroidectomy tetany and its occasional persistence for several years after operation. In any case, sustained release of thyrocalcitonin from the damaged gland could hardly be expected to follow total thyroidectomy. The more usual explanation, that hypocalcemia following thyroid surgery results from injury to the parathyroid glands or to their vascular connections, seems unlikely to be revised in consequence of the discovery of thyrocalcitonin. The question should be finally resolved when it becomes possible to make serial measurements of both parathormone and thyrocalcitonin in the blood of thyroidectomy patients. Further studies of the tolerance of human subjects to calcium infusions, before and after thyroidectomy, such as those already carried out by Williams *et al.* (W5), will also help to clarify the position.

Disturbances of calcium homeostasis have often been reported in diseases involving abnormal secretion of thyroid hormone. In thyrotoxicosis (G5, K2) the disturbance, if present, usually manifests itself as hypercalcemia of moderate degree, with loss of calcium from the bones and increased urinary output of calcium, thus suggesting a possible deficiency of thyrocalcitonin output in this type of thyroid disease. Most such observations antedate the discovery of thyrocalcitonin, and there is not yet much information on the response of such patients to an

injected calcium load or on the thyrocalcitonin content of abnormal human thyroid tissue. Aliapoulios *et al.* (A4) found abnormally low activity in extracts of colloid goitrous gland, but none in two cases of thyroid adenoma or in one hyperplastic gland, and Milhaud *et al.* (M12) found activity in extracts of goitrous glands comparable to that found in normal thyroid. Williams *et al.* (W5), in studying totally thyroidectomized patients maintained on exogenous thyroid, found normal resting calcium levels but markedly impaired ability to deal with an infused load of calcium gluconate. If this response reflects the physiological role of thyrocalcitonin, it implies that it plays a part in the control of hypercalcemia rather than in the maintenance of normal levels of calcium.

8.2. Relationship to Parathyroid Hypersecretion and Hyposecretion

If hypercalcemia is the normal stimulus to thyrocalcitonin release, the sustained mobilization of bone calcium occurring in parathyroid adenoma should lead initially to increased output of thyrocalcitonin, and only when this fails to counterbalance the effects of excess parathormone will recognizable hypercalcemia and hypercalcuria occur. One might expect, therefore, that the patient having hypercalcemia of parathyroid origin (or indeed having prolonged hypercalcemia for any reason) will have already exhausted his capacity to secrete additional thyrocalcitonin. In these circumstances the thyrocalcitonin content of the thyroid gland should be abnormally low. One sample of thyroid tissue, obtained postmortem from a patient with hypercalcemia (8.0 mEq of Ca/liter) owing to multiple parathyroid adenomata, yielded only one fifth of the normal amount of thyrocalcitonin (W4).

Where parathyroid secretion is deficient and the serum calcium level is low, inhibition of thyrocalcitonin secretion would also be expected. Gittes *et al.* (G3) found an increasing thyroid content of thyrocalcitonin in rats after parathyroidectomy, and Aliapoulios *et al.* (A4), in one patient diagnosed as suffering from pseudohypoparathyroidism, reported a thyrocalcitonin concentration in the thyroid more than 100 times higher than was found in normal glands. They suggest that abnormal thyrocalcitonin metabolism might be a factor in persistent hypocalcemia without demonstrated hypoparathyroidism. Again, the ability to measure the circulating levels of thyrocalcitonin and of parathormone might greatly help in defining the role of thyrocalcitonin in such clinical disorders of calcium metabolism.

8.3. Thyrocalcitonin-Excess Syndrome

To date, only one case appears to have been reported in which the findings were consistent with hypersecretion of thyrocalcitonin owing to hyperplasia of interfollicular thyroid cells (M5). The signs and symptoms were of tetany and long-standing diffuse nontoxic goiter. The serum calcium level was 8.1 mg/100 ml, and there was no evidence of metabolic bone disease. Biopsy of the thyroid gland revealed an increase in interfollicular cells, and extraction of the tissue yielded approximately 14 times more thyrocalcitonin than was obtained from normal thyroid tissue. Following total thyroidectomy the serum calcium levels rose, although not to normal values. The authors suggest that this may be attributable to persistence of some remnants of thyroid tissue, and possibly also to some damage to the parathyroid glands at operation.

8.4. Therapeutic Possibilities

Apart from the possibility that hypocalcemia and tetany might on occasion result from hypersecretion of thyrocalcitonin, clinical interest in the hormone is at present largely limited to two fields, namely, the possible treatment of hypercalcemia and the promotion of calcium deposition in bone. Foster *et al.* (F3), using purified pig thyrocalcitonin, achieved useful reductions in serum calcium levels in two cases of hypercalcemia occurring in association with bony metastases from breast cancer. A reduction was also achieved in one patient having hypercalcemia associated with metastases and with calcium and vitamin D therapy. Control of hypercalcemia was attained for 6–18 hours after a single injection. The intractable nature of the underlying diseases in most cases of hypercalcemia (other than those of parathyroid origin) obviously limits the therapeutic possibilities of the hormone, but the possibility of obtaining a preoperative reduction in the circulating calcium level in severe hyperparathyroidism deserves further study.

Since thyrocalcitonin appears to act mainly by promoting calcium deposition in bone (see Section 4.3 above), remineralization of bone is an obvious field for study of the hormone's therapeutic usefulness, as Foster and his colleagues have suggested (F3). The large amounts of calcium required for redeposition in, for example, osteoporosis suggest that prolonged treatment will be necessary. For this reason, the peptide nature of the hormone, necessitating parenteral administration, is an obvious drawback, and the evidence from Care and Duncan (C3), that in some species at least the hormone is relatively ineffective in older animals, suggests that only qualified optimism is at present justified.

It is perhaps not unduly speculative to think of the possibility of synthetic peptides being produced with greater potency than the natural hormone, and these might well revolutionize the treatment of patients whose bones require replenishment with calcium.

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References

A1. Aliapoulios, M. A., and Munson, P. L., Thyrocalcitonin. *Surg. Forum, Proc.* **16,** 55–57 (1965).

A2. Aliapoulios, M. A., Goldhaber, P., and Munson, P. L., Thyrocalcitonin inhibition of bone resorption induced by parathyroid hormone in tissue culture. *Science* **151,** 330–331 (1966).

A3. Aliapoulios, M. A., Savery, A., and Munson, P. L., New experiments with thyrocalcitonin. *Federation Proc.* **24,** 322 (1965).

A4. Aliapoulios, M. A., Voekel, E. F., and Munson, P. L., Assay of human thyroid glands for thyrocalcitonin activity. *J. Clin. Endocrinol. Metab.* **26,** 897–901 (1966).

A5. Arnaud, C., and Littledyke, T., The measurement of thyrocalcitonin in human and pig plasma by radioimmunological means. *J. Clin. Invest.* **45,** 982 (1966).

B1. Baghdiantz, A., Foster, G. V., Edwards, A., Kumar, M. A., Slack, E., Soliman, H. A., and MacIntyre, I., Extraction and purification of calcitonin. *Nature* **203,** 1027–1028 (1964).

B2. Bauer, W. C., and Teitelbaum, S. L., Thyrocalcitonin activity of particulate fractions of the thyroid gland. *Lab. Invest.* **15,** 323–329 (1966).

B3. Bauer, W. C., and Teitelbaum, S. L., Thyrocalcitonin activity of particulate fractions of the thyroid. *Federation Proc.* **25,** 496 (1966).

B4. Berson, S. A., Yalow, R. S., Aurbach, G. D., and Potts, J. T., Jr., Immunoassay of bovine and human parathyroid hormone. *Proc. Natl. Acad. Sci. U.S.* **49,** 613–617 (1963).

B5. Bussolati, G., and Pearse, A. G. E., Immunofluorescent localisation of calcitonin in the C cells of pig and dog thyroid. *J. Endocrinol.* **37,** 205–209 (1967).

C1. Cameron, E. C., and Copp, D. H., Parathyroid control of hypercalcaemia due to injection of parathyroid extract in the rat. *Proc. Soc. Exptl. Biol. Med.* **114,** 278–280 (1963).

C2. Care, A. D., Secretion of thyrocalcitonin. *Nature* **205,** 1289–1291 (1965).

C3. Care, A. D., and Duncan, T., Age as a factor in the response to thyrocalcitonin secretion. *J. Endocrinol.* **37,** 107–108 (1967).

C4. Care, A. D., and Keynes, W. M., The secretion of calcitonin by the parathyroid glands of the sheep. *J. Endocrinol.* **31,** xxxi–xxxii (1965).

C5. Care, A. D., Duncan, T., and Webster, D., Thyrocalcitonin and its role in calcium homeostasis. *J. Endocrinol.* **37,** 155–167 (1967).

C6. Care, A. D., Keynes, W. M., and Duncan, T., An investigation into the parathyroid origin of calcitonin. *J. Endocrinol.* **34,** 299–318 (1966).

C7. Care, A. D., Webster, D., and Duncan, T., Species variation to thyrocalcitonin. *Excerpta Med. Intern. Congr.* Ser. **120,** 13 (1966).

C8. Care, A. D., Duncan, T., Webster, D., and Frazer, S. C., Factors affecting the secretion of thyrocalcitonin. *J. Bone Joint Surg.* **48B,** 386 (1966).

C9. Care, A. D., Sherwood, L. M., Potts, J. T., Jr., and Aurbach, G. D., Perfusion of the isolated parathyroid gland of the goat and sheep. *Nature* **209,** 52–57 (1966).
C10. Chausmer, A., Mittleman, R., and Wallach, S., Studies of thyrocalcitonin action. *Endocrinology* **79,** 131–137 (1966).
C11. Chausmer, A., Weiss, P., and Wallach, S., Effect of thyrocalcitonin on calcium exchange in rat tissues. *Endocrinology* **77,** 1151–1154 (1965).
C12. Cooper, C. W., and Tashjian, A. H., Subcellular localization of thyrocalcitonin. *Endocrinology* **79,** 819–822 (1966).
C13. Copp, D. H., Personal communication to Tashjian and Munson, quoted in T3 (1962).
C14. Copp, D. H., Parathyroids, calcitonin, and control of plasma calcium. *Recent Progr. Hormone Res.* **20,** 59–77 (1964).
C15. Copp, D. H., and Cameron, E. C., Demonstration of a hypocalcaemic factor (calcitonin) in commercial parathyroid extract. *Science* **134,** 2038 (1961).
C16. Copp, D. H., and Henze, K. G., Parathyroid origin of calcitonin—evidence from perfusion of sheep glands. *Endocrinology* **75,** 49–55 (1964).
C17. Copp, D. H., Cameron, E. C., Cheney, B. A., Davidson, A. G. F., and Henze, K. G., Evidence for calcitonin—a new hormone from the parathyroid that lowers blood calcium. *Endocrinology* **70,** 638–649 (1962).
D1. Duncan, T., and Care, A. D., The effect of anti-thyroid drugs on thyrocalcitonin secretion. *Brit. J. Surg.* **54,** 196–198 (1967).
F1. Foster, G. V., Thyrocalcitonin: Failure to demonstrate a parathyroid releasing factor. *Nature* **211,** 1319–1320 (1966).
F2. Foster, G. V., MacIntyre, I., and Pearse, A. G. E., Calcitonin and the mitochondrion-rich cells of the dog thyroid. *Nature* **203,** 1029–1030 (1964).
F3. Foster, G. V., Joplin, G. F., MacIntyre, I., Melvin, K. E. W., and Slack, E., Effect of thyrocalcitonin in man. *Lancet* **I,** 107–109 (1966).
F4. Foster, G. V., Baghdiantz, A., Kumar, M. A., Slack, E., Soliman, H. A., and MacIntyre, I., Thyroid origin of calcitonin. *Nature* **202,** 1303–1305 (1964).
F5. Friedman, J., and Raisz, L. G., Thyrocalcitonin: Inhibitor of bone resorption in tissue culture. *Science* **150,** 1465–1466 (1965).
F6. Friesen, H., Hypocalcaemic effect of pituitary polypeptides in rabbits. *Endocrinology* **75,** 692–697 (1964).
G1. Gaillard, P. J., Bone culture studies. *J. Bone Joint Surg.* **48B,** 386 (1966).
G2. Gittes, R. F., and Irvin, G. L., Thyroid and parathyroid roles in hypercalcaemia: evidence for a thyrocalcitonin-releasing factor. *Science* **148,** 1737–1739 (1965).
G3. Gittes, R. F., Munson, P. L., and Toverud, S. U., Effect of plasma calcium on the thyrocalcitonin content of rat thyroid glands. *Federation Proc.* **25,** 496 (1966).
G4. Gudmundsson, T. V., MacIntyre, I., and Soliman, H. A., The isolation of thyrocalcitonin and a study of its effects in the rat. *Proc. Roy. Soc.* **B164,** 460–477 (1966).
G5. Guyer, P. B., Hypercalcaemia in thyrotoxicosis. *Brit. Med. J.* **I,** 169 (1965).
H1. Hargis, G. K., Williams, G. A., Tenenhouse, A., and Arnaud, C. D., Thyrocalcitonin: Cytological localization by immunofluorescence. *Science* **152,** 73–75 (1966).
H2. Hirsch, P. F., and Munson, P. L., Importance of the thyroid gland in the prevention of hypercalcaemia in rats. *Endocrinology* **79,** 655–658 (1966).
H3. Hirsch, P. F., Gauthier, G. F., and Munson, P. L., Thyroid hypocalcaemic principle and recurrent laryngeal nerve injury as factors affecting the response to parathyroidectomy in rats. *Endocrinology* **73,** 244–252 (1963).
H4. Hirsch, P. F., Voelkel, E. F., and Munson, P. L., Thyrocalcitonin: Hypocalcaemic hypophosphataemic principle of the thyroid gland. *Science* **146,** 412–413 (1964).

J1. Johnston, C. C., Jr., and Deiss, W. P., Jr., An inhibitory effect of thyrocalcitonin on calcium release *in vivo* and on bone metabolism *in vitro*. *Endocrinology* **78,** 1139–1143 (1966).

K1. Kenny, A. D., and Heiskell, C. A., Effect of crude thyrocalcitonin on calcium and phosphorus metabolism in rats. *Proc. Soc. Exptl. Biol. Med.* **120,** 269–271 (1965).

K2. Kleeman, C. R., Tuttle, S., and Bassett, S. H., Metabolic observations in a case of thyrotoxicosis with hypercalcaemia. *J. Clin. Endocrinol.* **18,** 477–491 (1958).

K3. Klein, D. C., Morii, H., and Talmage, R. V., Effect of thyrocalcitonin on removal of calcium, phosphate, and certain radioisotopes by peritoneal lavage. *Federation Proc.* **25,** 496 (1966).

K4. Kohler, H. F., and Pechet, M. M., The inhibition of bone-resorption by thyrocalcitonin. *J. Clin. Invest.* **45,** 1033 (1966).

K5. Kumar, M. A., Foster, G. V., and MacIntyre, I., Further evidence for calcitonin. *Lancet* **II,** 480–482 (1963).

K6. Kumar, M. A., Slack, E., Edwards, A., Soliman, H. A., Baghdiantz, A., Foster, G. V., and MacIntyre, I., A biological assay for calcitonin. *J. Endocrinol.* **33,** 469–475 (1965).

M1. MacIntyre, I., Personal communication (1966).

M2. MacIntyre, I., and Parsons, J. A., Blood-bone calcium equilibrium in the perfused cat tibia and the effect of thyroid calcitonin. *J. Physiol. (London)* **183,** 31P-32P (1966).

M3. MacIntyre, I., Foster, G. V., and Kumar, M. A., The thyroid origin of calcitonin. *In* "The Parathyroid Glands: Ultrastructure, Secretion and Function" (P. J. Gaillard, R. V. Talmage, and A. M. Budy, eds.), pp. 89–102. Univ. of Chicago Press, Chicago, Illinois, 1965.

M4. Martin, T. J., Robinson, C. J., and MacIntyre, I., The mode of action of thyrocalcitonin. *Lancet* **I,** 900–902 (1966).

M5. Mazzuoli, G. F., Coen, G., and Baschieri, L., Thyrocalcitonin-excess syndrome. *Lancet* **II,** 1192–1193 (1966).

M6. McLean, F. C., The parathyroid hormone and bone. *Clin. Orthopaed.* **9,** 46–60 (1951).

M7. McLean, F. C., and Hastings, A. B., Clinical estimation and significance of calcium-ion concentrations in the blood. *Am. J. Med. Sci.* **189,** 601–613 (1935).

M8. Milhaud, G., and Moukhtar, M. S., Hypophysectomie et thyrocalcitonine. *Compt. Rend.* **260,** 3179–3182 (1965).

M9. Milhaud, G., and Moukhtar, M. S., Antagonistic and synergistic actions of thyrocalcitonin and parathyroid hormone on the levels of calcium and phosphate in the rat. *Nature* **211,** 1186–1187 (1966).

M10. Milhaud, G., Perault, A. M., and Moukhtar M. S., Étude du mécanisme de l'action hypocalcémiante de la thyrocalcitonine. *Compt. Rend.* **261,** 813–816 (1965).

M11. Milhaud, G., Moukhtar, M. S., Cherian, G., and Perault, A. M., Effet de l'administration de thyrocalcitonine sur les principaux paramètres du calcium du rat et du rat thyroparathyroïdectomisé. *Compt. Rend.* **262,** 511–514 (1966).

M12. Milhaud, G., Moukhtar, M. S., Bourichon, J., and Perault, A. M., Existence et activité de la thyrocalcitonine chez l'homme. *Compt. Rend.* **261,** 4513–4516 (1965).

M13. Morey, E. R., Endogenous thyrocalcitonin: Effect on serum calcium in fed and fasted rats and in parathyroid transplanted rats. *Endocrinology* **79,** 191–196 (1966).

M14. Morii, H., and Talmage, R. V., Effect of iodine-deficiency on thyrocalcitonin secretion. *Proc. Soc. Exptl. Biol. Med.* **122,** 382–384 (1966).

N1. Natelson, S., Pincus, J. B., and Rannazzisi, G., Dynamic control of calcium, phosphate, citrate, and glucose levels in blood serum. *Clin. Chem.* **9,** 31–62 (1963).
N2. Natelson, S., Pincus, J. B., and Rannazzisi, G., A rabbit serum calcium lowering factor from the pituitary. *Clin. Chem.* **9,** 631–636 (1963).
N3. Natelson, S., Rannazzisi, G., and Pincus, J. B., Effect of ACTH and vasopressin on serum calcium and citrate levels in the rabbit. *Endocrinology* **77,** 108–113 (1965).
N4. Nonidez, J. F., The origin of the 'parafollicular' cell, a second epithelial component of the thyroid gland of the dog. *Am. J. Anat.* **49,** 479–505 (1931).
O1. O'Riordan, J. L. H., Tashjian, A. H., Munson, P. L., Condliffe, P. G., and Aurbach, G. D., Thyrocalcitonin: ultracentrifugation in gradients of sucrose. *Science* **154,** 885–886 (1966).
P1. Painter, N. S., The results of surgery in the treatment of toxic goitre—a review of 172 cases. *Brit. J. Surg.* **48,** 291–296 (1960).
P2. Parsons, J. A., Studies on isolated bone. *J. Bone Joint Surg.* **48B,** 387–388 (1966).
P3. Patt, H. M., and Luckhardt, A. B., Relationship of a low blood calcium to parathyroid secretion. *Endocrinology* **31,** 384–392 (1942).
P4. Pearse, A. G. E., 5-Hydroxytryptophan uptake by dog thyroid 'C' cells, and its possible significance in polypeptide hormone production. *Nature* **211,** 598–600 (1966).
R1. Rasmussen, H., Parathyroid hormone. *Am. J. Med.* **30,** 112–128 (1961).
R2. Rasmussen, H., and Craig, L. C., Isolation and characterization of bovine parathyroid hormone. *J. Biol. Chem.* **236,** 759–764 (1961).
R3. Rasmussen, H., Sze, Y., and Young, R., Further studies on the isolation and characterization of parathyroid polypeptides. *J. Biol. Chem.* **239,** 2852–2857 (1964).
R4. Robinson, C. J., Martin, T. J., and MacIntyre, I., Phosphaturic effect of thyrocalcitonin. *Lancet* **II,** 83–84 (1966).
S1. Sanderson, P. H., Marshall, F., and Wilson, R. E., Calcium and phosphorus homeostasis in the parathyroidectomized dog; evaluation by means of ethylenediamine tetraacetate and calcium tolerance tests. *J. Clin. Invest.* **39,** 662–670 (1960).
S2. Smith, R. N., Laljee, H. K. C., and Dorrington, K. J., Studies of human thyrocalcitonin. *J. Bone Joint Surg.* **48B,** 388 (1966).
S3. Stahl, P. D., Clarke, J. D., Waite, L. C., and Kenny, A. D., Effects of crude thyrocalcitonin in the dog. *Federation Proc.* **25,** 496 (1966).
T1. Talmage, R. V., Neuenschwander, J., and Kraintz, L., Evidence for the existence of thyrocalcitonin in the rat. *Endocrinology* **76,** 103–107 (1965).
T2. Tashjian, A. H., Homeostasis of plasma calcium: Effects of actinomycin D, parathyroidectomy and thyrocalcitonin. *Endocrinology* **77,** 375–381 (1965).
T3. Tashjian, A. H., and Munson, P. L., Chemical nature and properties of parathyroid hormones. *J. Chronic Diseases* **16,** 269–281 (1963).
T4. Tashjian, A. H., and Munson, P. L., Antibodies to porcine thyrocalcitonin: Effects on the hypocalcaemic activity of calf, rat and monkey thyroid extracts. *Endocrinology* **77,** 520–528 (1965).
T5. Tenenhouse, A., Arnaud, C., and Rasmussen, H., The isolation and characterization of thyrocalcitonin. *Proc. Natl. Acad. Sci. U.S.* **53,** 818–822 (1965).
T6. Tweedy, W. R., and Chandler, S. B., Studies on the blood plasma calcium of normal and parathyroidectomised albino rats. *Am. J. Physiol.* **88,** 754–760 (1929).
W1. Wade, J. S. H., The morbidity of subtotal thyroidectomy. *Brit. J. Surg.* **48,** 25–42 (1960).

W2. Wase, A. W., Peterson, A., Rickes, E., and Solewski, J., Some effects of thyrocalcitonin on the calcium metabolism of the rat. *Endocrinology* **79,** 687–691 (1966).
W3. Webster, D., Hinde, F., and Care, A. D., Personal communication (1966).
W4. Webster, D., Keith, J., and Hinde, F., Personal communication (1966).
W5. Williams, G. A., Hargis, G. K., Galloway, W. B., and Henderson, W. J., Evidence for thyrocalcitonin in man. *Proc. Soc. Exptl. Biol. Med.* **122,** 1273–1276 (1966).

AUTOMATED TECHNIQUES IN LIPID CHEMISTRY

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1. Introduction

In the past 10 years there has been an increasing emphasis on the relationship of various lipids to atherosclerotic heart disease. The clinical laboratory is performing a greater number of lipid determinations as well as a larger variety of procedures. In order to meet these demands, laboratories are increasingly turning to automated instrumentation and methodology. This review will discuss one automated instrumental system, and the lipid techniques that have been adapted to it.

2. Instrumental Approach

Manual analytical techniques are carried out on a step-by-step or batch basis. Sample handling and presentation, delivery of reagent volumes in proper sequence, removal of interfering materials, heating or incubating reaction mixtures, and measurement and recording of data are performed as discrete functions. Each stage must be completed before proceeding to the next, since reactions are continued until equilibrium is reached. In 1957 Skeggs (S1) introduced an automated system of analysis, capable of analyzing constituents on continuously flowing streams, whereby samples automatically pass from one stage to another. Specimens are compared to standards that are similarly treated, and exposed to the same conditions, although the reactions themselves do not necessarily reach equilibrium. Consequently, measurements are made on a ratio basis, i.e., comparison of standard to unknown. A brief description of

the instrumentation, commercially available (from the Technicon Instruments Corp.) under the trade name "AutoAnalyzer," is presented. [More detailed information is given by Marsh (M1), Kessler (K5), and Skeggs (S2).]

2.1. AutoAnalyzer

2.1.1. *Sampler*

This introduces samples, standards, or a wash interval to the analytical system in a preset sequence, and at a controlled rate. An unmeasured volume of specimen is added to a polystyrene cup and placed in the sampler tray. Sampling occurs via a polyethylene catheter connected to the manifold of the analytical system. The catheter dips into the cup at preselected sampling rates, usually 20, 40, or 60 times per hour, and between samples a wash fluid or air is aspirated. Both sampling speed and wash cycle are controlled by a timing cam and can be varied.

2.1.2. *Proportioning Pump*

This continuously delivers reagents or gases, and intermittently samples, on a volume per time basis. The continuously flowing reagents constitute the analytical system for a given procedure. The function of the proportioning pump is analogous to pipeting, measuring, reagent additions, etc., for manual methods.

The pump, which can meter up to 15 separate volumes of fluid or gas, consists of a constant-speed gear motor connected to a hinged roller head. This is raised or lowered against a plate on which the manifolds are placed. The roller head compresses the manifold tubing, pushing fluids or gases forward, while at the rear fluids are drawn into the tubes. The fluid volume, on a per time basis, is proportional to the inside diameter of the tube.

Air is continuously introduced as a component of the analytical train. These air segments exert a continuous wiping action on the tubing, and prevent contamination from one sample to another. They also divide a sample into many aliquots, each being treated as an individual specimen. The final reading of a sample constitutes an averaging effect of all these aliquots.

The constant-speed pump assures the continuous proportioning of all reagents that enter into the performance of a given technic. Precise and reproducible determinations are dependent on maintaining this constant speed of operation. Samples or standards are treated exactly alike, exposed to the same conditions for the same time intervals, and directly compared to each other.

Each methodology has its own manifold that makes up its analytical system. The manifold consists of various types of tubing for delivering solvents and reagents. It has glass parts for proportioning the sample, reagents, and gas in a prescribed sequence. Delivery of different volumes is determined by the bore of the tubing; e.g., the smaller the inside diameter, the less the volume of reagent metered into the system.

2.1.3. *Dialyzer*

Manual techniques employ precipitation, filtration, distillation, dialysis, etc., for separating and isolating interfering material from the substance to be analyzed. This system employs dialysis under a constant set of conditions, with samples and standard exposed to exactly the same conditions.

Factors affecting dialysis rates are time, temperature, area, and concentration. For the automated system the area of the dialyzing plates is constant, exposure time of the reaction streams to the semipermeable membrane is the same for a given volume, and temperature is carefully controlled. Thus the only variable is the concentration of the sample. The dialysis rate of a substance under a given set of conditions is constant, but the absolute amount dialyzed will vary since it is dependent on the original sample concentration.

2.1.4. *Heating Bath*

Many methods require a heating step for color development, or an incubation stage for enzyme reactions. In the AutoAnalyzer, this is achieved as the reaction mixture flows through a glass helix (usually 40 feet in length) kept at the desired temperature.

2.1.5. *Detector–Recorder Systems*

Many analytical procedures produce a colored reaction mixture as the end point. The colorimeter detects and measures changes in absorbance of the continuously flowing reaction stream, giving a permanent record on the strip chart of the recorder. The photometer–recorder forms a null-balancing ratio system whereby factors of light-intensity changes, temperature fluctuations, drift of the photocells, etc., produce negligible drift or noise on the record. Other detector systems that have been adapted for measurements on continuously flowing streams are the fluorometer, spectrophotometer, and flame photometer.

3. Techniques

Many analytical procedures have been adapted to the AutoAnalyzer. This review will cover various automated or semiautomated lipid method-

ologies that have currently been published, as well as some unpublished modifications presently employed in our laboratory. The determinations under discussion are those on cholesterol, phospholipid, triglycerides, and free and esterified fatty acids. The present state of lipid technology does not allow for their complete automation. Most procedures require a manual extraction utilizing a variety of solvents, although several direct cholesterol technics have been published.

3.1. Cholesterol

The quantitative determination of total cholesterol is the most widely requested lipid procedure in the clinical laboratory, since it is implicated in atherosclerotic heart disease.

The first automated lipid technique was described in 1959 (K4), based on the reaction discovered by Zlatkis *et al.* (Z2), in which a mixture of ferric chloride–glacial acetic acid and concentrated sulfuric acid is employed. This automated procedure is directly applicable to serum or isopropanolic extracts of serum. Samples or standards are automatically diluted with glacial acetic acid (aldehyde-free) containing acetic anhydride and mixed, and an aliquot of diluted sample is continuously removed for carrying out the colorimetric analysis. An additional quantity of acetic acid–acetic anhydride mixture is added to the diluted sample and mixed. Sulfuric acid color reagent, containing ferric chloride in concentrated acetic acid, is then added to the reaction mixture. It was observed that the rate of color development is temperature-dependent; therefore, the procedure is carried out at an elevated temperature as the reaction mixture flows through glass coils placed in a hot air chamber at $100 \pm 2°C$. When analyzing sera directly, protein precipitation occurs on addition of the highly acid color reagent. Dissolution of the precipitate is aided by using a continuous flow-through magnetic mixer. The ratio of molar absorbance of cholesterol esters to free cholesterol is similar for these reaction conditions. The color developed is measured at 550 mμ, using a flow cuvette with a 10-mm light path.

Other direct serum cholesterol techniques have been modified for the AutoAnalyzer. Several investigators have adapted the procedure of Pearson *et al.* (P1, P2), which is based on the use of glacial acetic, *p*-toluenesulfonic, and sulfuric acids. The several advantages cited by proponents of this technique include simplicity and speed of operation compared to extraction methods, which are time-consuming and require considerable manipulative skill; greater specificity of the Pearson vs. Zlatkis techniques; stability of the final color; and similarity of absorbances of both free and esterified cholesterol. A disadvantage of the direct methods is that standards in organic solvents do not exhibit the

same absorbances as sera for equivalent amounts of cholesterol, thus control sera must be substituted for standards if direct comparisons are to be made. Another problem results from interfering materials that can be present in sera in elevated amounts, namely, hemoglobin and bilirubin.

In 1960 Boy *et al.* (B6) employed a modified Pearson technique, whereby the sample is added to an air-segmented stream of *p*-toluenesulfonic acid in glacial acetic acid, followed by acetic anhydride. The reaction mixture passes through a series of three mixing coils for mixing and dissipating the heat of reaction. The second coil is jacketed, and has cold water circulating through it for cooling the reaction mixture. Concentrated sulfuric acid is then added, and the reaction is carried out in an air bath at 60°C. Protein precipitation is minimized by incorporating a flow-through magnetic mixer as part of the coils in the air bath. The absorbance is measured at 630 mμ, using a flow cuvette with a 6-mm light path.

The procedure was further modified by Boy in 1963 (B5) with the advent of better tubing for delivering concentrated acids. The *p*-toluenesulfonic acid is made up in a mixture of glacial acetic acid–acetic anhydride. This reagent is heated to 65°C, then serum is added and mixed, while remaining at 65°C for a short period. The reaction mixture is cooled by passing through a jacketed mixing coil through which water circulates at 15°C. Concentrated sulfuric acid is added and mixed, and the reaction mixture temperature is once more raised to 65°C. The color is then read at 630 mμ. Boy recommends the use of commercial sera as a means of standardizing this procedure.

Another modified Pearson technique was published in 1963 and 1964 by Kenny and Jamieson (J1, K2, K3). The procedure differs from that of Boy in respect to composition and volume of reagents, as well as temperature and time for the reaction to take place. Kenny uses a reaction temperature of 45°C, but employs a greater time for color development. Kenny *et al.* found differences between the automated results and those obtained by an extraction procedure (K1) utilizing the Liebermann-Burchard reaction. They feel that the differences result from the presence of water in serum causing a decrease in absorbance, which is not encountered in the water-free acetic acid–cholesterol standard solution. Therefore, these authors have adjusted the cholesterol standards to compensate for the color depression, thus allowing direct reading of results.

Another automated direct cholesterol technique for sera utilizes a modified Liebermann-Burchard reaction, employing a moderately stable reagent consisting of acetic acid–acetic anhydride and concentrated

sulfuric acid. Our experience with this reagent shows it to be usable for a period of 2 weeks. This technique is utilized for the AutoAnalyzer multiple analysis instrument. An aliquot of serum is added to the modified Liebermann-Burchard reagent, mixed, and then kept at 37°C for color development. The absorbance is read at 630 mμ, using a flow cell with a 15-mm light path. Control sera of known cholesterol concentration are used for standardization of this method.

Several investigators have modified the automated Zlatkis method previously discussed (K4). This technique is the basis for the Technicon procedure released in 1960 (C3), using manually prepared isopropanolic extracts and the automated color reaction. In 1963 Bensa (B2) evaluated this technique for the determination of total cholesterol, and adapted the procedure for the colorimetry of free cholesterol after a preliminary digitonide separation. Levine and Zak (L3) have utilized a combined color reagent consisting of ferric chloride in glacial acetic acid plus concentrated sulfuric acid. In this technique, isopropanolic extracts of serum are added to the combined color reagent and mixed. The reaction mixture now passes through a 95°C heating bath for color development. The absorbance is read at 520 mμ in a flow cell of 15-mm light path. Levine and Zak have also automated the preparation of isopropanolic extracts of serum. This is accomplished by adding serum to isopropanol that is being vigorously mixed by a stirrer paddle connected to a motor. The mixture, consisting of solvent and precipitated protein, is deposited on a moving belt of filter paper. It then passes over a filter block (Teflon block with a hole in the middle) that is connected to the pump manifold tubing. An area of negative pressure is created over the hole because of the action of the proportioning pump. Filtrate is aspirated through the paper while the precipitated protein remains behind on the moving strip of filter paper. The extract is then treated as previously described for the manually prepared material. Two approaches are used for calibration of the continuous filtration technic. Standard solutions of cholesterol are analyzed and a correction factor applied to the values, or a serum pool, whose cholesterol value is predetermined, is analyzed along with the samples and used as a standard. A correction factor can be used, since the calibration is always linear. The correction factor is necessary, since the reaction characteristics of serum and standards differ. Serum yields a greater absorbance than standard solutions for the 2-minute sampling period.

Cooper *et al.* (C7) have evaluated the use of the premixed color reagent technique and compared it with a modified Abell-Kendall method (A1). These investigators found that variations in results can arise from sampling, mixing of reagents, failing to control the temper-

ature of the color reaction and failing to protect the chromogen from light, and from reaction of reagents with tubing. The AutoAnalyzer premixed reagent technique used in their laboratory compares favorably with the modified Abell-Kendall procedure for normal and abnormal sera.

In 1966 Block *et al.* (B3, B4) published a modification of the Zlatkis *et al.* and Levine and Zak technics. Manually prepared serum extracts are added to a stream of preheated color reagent, and the mixture is then passed through three mixing coils connected in series. The absorbance is determined at 550 mμ, using a 15-mm tubular flow cell. This technique is currently recommended as the total cholesterol procedure by the Technicon Instruments Corp. (C4, C5).

A fluorometric technique has been proposed by Antonis (A3) for measuring cholesterol concentration at levels corresponding to 20–100 mg/100 ml present in the original sample. The same technique can be used for concentrations corresponding to cholesterol levels up to 500 mg/100 ml of serum. A phospholipid-free extract is prepared as follows. Silicic acid (4 g) is slurried with 25 ml of diisopropyl ether, followed by 1 ml of serum. The mixture is well shaken until free from lumps. The silicic acid is separated by centrifugation, and the supernatant extract taken off into a separate tube. An aliquot (usually 5 ml) is taken to dryness and then reconstituted with the same volume of cholorform or 1,1,2-trichloroethane. This represents a 1:25 serum dilution. The automated fluorometric technique is carried out as follows. Aliquots of extract in either chloroform or 1,1,2-trichloroethane (the latter solvent is preferred because it is less volatile) are placed in glass cups on the sampling tray. Working standards of the appropriate range (0.8–4 or 4–20 mg/100 ml) are prepared in the same solvent and also placed on the sampler. Approximately 0.4 ml is aspirated and added to 3 ml of color reagent and mixed. The color reagent consists of 100 volumes of 1,1,2-trichloroethane and 20 volumes of acetic anhydride. This is cooled to —15°C and then 5 volumes of concentrated sulfuric acid are added. The reagent is prepared just before it is required and stored in a container fitted with a drying tube (anhydrous $CaSO_4$). This reaction mixture then passes through a 40-foot glass time-delay coil at 25°C, and the fluorescence is measured. The fluorometer is adjusted to 100% transmission with the appropriate standard, using a primary filter combination of Corning 4010 +5120 filters and a secondary filter Corning 2424.

The many variations and modifications for measuring total cholesterol attest to the difficulty that this determination apparently presents for both manual and automated techniques. In the author's experience, a reliable and simple automated procedure is obtained by modifying the

technique originally proposed by employing isopropanolic serum extracts that can be related to pure standards. The procedure is carried out as follows:

Reagents

(1) Isopropanol (A.C.S. reagent grade)

(2) Sulfuric acid (concentrated A.C.S. reagent grade)

(3) *Stock* ferric chloride: 12.5 g of $FeCl_3 \cdot 6H_2O$/liter of concentrated phosphoric acid (85%)

(4) *Working* ferric chloride: 50 ml stock ferric chloride plus 950 ml glacial acetic acid

(5) Standards: cholesterol is recrystallized twice from acetone and dried in a vacuum desiccator; standards are prepared in isopropanol at concentrations of 100, 200, 300, and 400 mg/100 ml

Preparation of Extracts. Leffler (L2) and others have previously used isopropanol as a lipid solvent for determining cholesterol. Connerty *et al.* (C6) and Lofland (L4) also showed that triglycerides and phospholipids are extracted quantitatively. Under our extraction conditions, complete recovery of these lipid fractions is obtained at a serum-to-solvent ratio of 1:10 through 1:25. We routinely use 1:20 dilution (0.5 ml of sera + 9.5 ml of isopropanol). We find that the addition of Lloyd's reagent removes bilirubin and other chromogenic material present in serum, as well as approximately 80% of the phospholipids present.

Screw-capped culture tubes (16 × 150 mm) with Teflon-lined caps are used for carrying out the extractions. All tubes contain approximately 200 mg of Lloyd's reagent. Isopropanol (9.5 ml) is placed in tubes for sera, while tubes for standards contain 9.0 ml of isopropanol plus 0.5 ml of 0.9% NaCl. The contents of the tubes are mixed on a Vortex mixer, while adding 0.5 ml of serum or standard. Mixing is continued for 15 seconds. Tubes are capped and centrifuged, and the supernatant is decanted into another tube that is kept capped until used.

Analytical System. The manifold schematic is illustrated in Fig. 1. An unmeasured aliquot of extract is added to the working ferric chloride reagent that has been preheated by passing through a 95°C heating bath. After mixing, concentrated sulfuric acid is added and the reaction mixture again mixed. The absorbance is then measured at 550 mμ, using a tubular flow cell with a 15-mm light path. The sampling rate is 40 determinations per hour.

Results. Values obtained with this technique correlate well with those of both the Sperry-Webb (S3) and Abell-Kendall (A1) procedures.

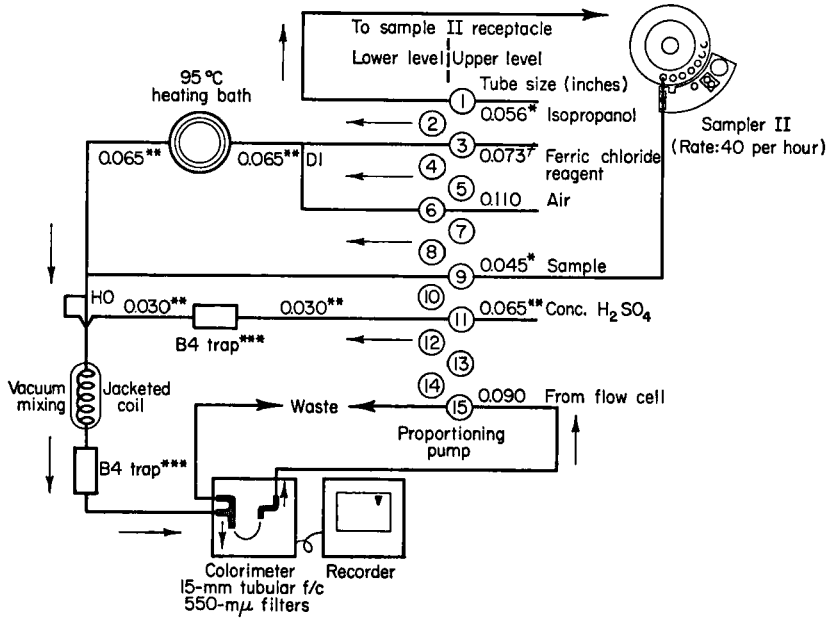

FIG. 1. Analytical system: manifold schematic for total cholesterol. * Solvaflex. ** Acidflex. *** Side arm sealed—used as surge and/or mixing chamber. † Manifold tube replaced daily.

3.2. PHOSPHOLIPIDS

At present all automated techniques for the determination of phospholipids require the manual preparation of extracts. Some investigators carry out the destruction of organic material manually; others have automated this step. The colorimetric analysis for phosphorus is performed on an automated basis.

Whitley and Alburn (W1) have adapted the procedure of Zilversmit and Davis (Z1) for the complete automation of the digestion and colorimetry steps of the phospholipid analysis. The trichloroacetic acid (TCA) precipitation method is carried out by adding 0.2 ml of serum or plasma to 3 ml of 10% trichloroacetic acid, centrifuging, and then discarding the supernatant. The precipitate is dissolved in 6 ml 1 *M* NaOH and transferred to the plastic sample cups for assaying. The digestion medium is a perchloric–sulfuric acid mixture containing vanadium pentoxide as a catalyst (90% H_2SO_4–0.05% V_2O_5–10% $HClO_4$). Oxidation is quite rapid, occurring in a minute or two. The samples or standards are added

to a stream of the hot digestion mixture that has previously passed through a 95°C heating bath. After mixing, the reaction mixture is dropped into the middle of the first heated section of the Technicon helical digestor that is rotated at 4.5 rpm. Following digestion, the acid stream is cooled by an air blower and diluted with water at the exit end of the helix. The mixture is aspirated from the helix into a mixer, and a continuous sample is withdrawn from the mixer by a second pump. Resampling of the diluted digest by use of a debubbler T is essential to prevent air from being pumped through the color assay system. NaOH is added to the acid digest to neutralize the acidity partially. The digest is mixed, cooled, and resampled through a debubbler T. Acid molybdate and hydrazine sulfate are then added and mixed. Color is developed by pumping the solution through a glass coil (2-mm ID $\times$ 40 feet long) that is held at 95°C. The colored solution goes through a cooling coil, then into a 15-mm tubular flow cell colorimeter, using 815-mμ filters and silicon photocells. The rate of analysis is 20–40 samples/hour with a wash between samples, giving a net rate of 10–20 determinations/hour. These investigators have found that the most satisfactory standard is one employing serum TCA precipitates that approximate the composition of the unknown samples. The response to phosphate concentration is linear, although when inorganic phosphate is used as a standard the organic phosphate values must be increased approximately 6% to allow for differences in response.

Antonis (A3) has estimated phospholipids by a procedure for determining fatty acids. This technique requires a total serum extract and a phospholipid-free extract for the measurement of both total and free fatty acids, the difference between them being a measure of the phospholipid content. Free fatty acids (A4) are determined on a phospholipid-free extract by a procedure based on partitioning the fatty acids as copper soaps into chloroform, and subsequent photometric determination of the copper with diethyldithiocarbamate. Phospholipids, as well as the free fatty acids present in the total lipid-extract, are measured by the same method, since they also form a complex with copper that is soluble in chloroform. A criticism of this technique is that equal response is not given by dipalmitoyl lecithin, dipalmitoyl cephalin, or beef brain sphingomyelin.

In 1966 Kraml (K7) published a procedure for determining phospholipids on perchloric acid digests of the lipid extract. This investigator uses the extraction procedure of Bloor or the trichloroacetic acid precipitation technic of Zilversmit and Davis (Z1). Digestions are carried out on a hot plate for 15 minutes at 200°–225°C in the presence of 1.2 ml of 70% $HClO_4$. After cooling, the volume is made up to 5.0 ml. The final perchloric acid concentration is 14%, based on the assumption that

approximately 0.2 ml of perchloric acid is lost during the digestion. The colorimetric measurement of phosphate is adapted from a method reported by Hurst (H1), employing molybdic acid with stannous chloride–hydrazine as the reducing agent. Color development is rapid at room temperature, and the sensitivity is equal to that obtained after prolonged heating when using other reducing agents. This procedure is carried out as follows. The sample in 14% perchloric acid is diluted by addition of 14% $HClO_4$, segmented with air, and mixed. Ammonium molybdate (7.5%, 2 ml) in 3 *N* H_2SO_4 is added and mixed, followed by addition of an equal volume of 0.3% hydrazine sulfate–0.03% stannous chloride in 1 *N* H_2SO_4. The reaction stream is mixed and passed through a 15-mm tubular flow cell, and the absorbance is determined at 660 mμ. The range of phosphate measured is varied by using one of two sample lines, 0.3 ml/minute for a range of 1–15 μg of P/ml or 1.2 ml/minute for a range of 0.25–5 μg of P/ml. The sampling rate is 40 determinations/hour. This semiautomated technique shows excellent reproducibility and recoveries, as well as simplicity and speed of operation.

Our laboratory has been using a similar procedure for the past 2 years. Digestions are carried out on isopropanolic extracts with sulfuric acid–peroxide, after the procedure of Youngburg and Youngburg (Y1). The colorimetric portion of the analysis is automated, utilizing the molybdic acid-stannous chloride technic of Kutter and Cohen (K8). The procedure is performed as follows.

Reagents

(1) Isopropanol (A.C.S. reagent grade)

(2) Sulfuric acid 2.5 *N*

(3) Hydrogen peroxide 30% (phosphate-free)

(4) Molybdic acid reagent: 15 g of $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ is dissolved in approximately 600 ml of water; add, with mixing, 55 ml of concentrated H_2SO_4 and dilute to 1 liter

(5) Stannous chloride reagent. *Stock:* 10 g of $SnCl_2$/25 ml of concentrated HCl. *Working:* dilute 1 ml stock to 1 liter with distilled water; prepare fresh each day

(6) Blank acid reagent: to 1000 ml of water add 70 ml of concentrated H_2SO_4

(7) Standards. *Stock:* (A) 10.0 mg of P/ml; dissolve 4.393 g of KH_2PO_4 in 100 ml water. (B) 50 μg of P/ml; dilute (A) 1:200. *Working:* from (B) prepare standards ranging from 2.5 to 7.5 μg of P/100 ml, and then add 7.0 ml of concentrated H_2SO_4 to each standard. These standards are equivalent to 5–15 mg of P/100 ml or 125–375 mg of phospholipids/100 ml, expressed as lecithin, when based on a 1:20 serum extract

Preparation of Extracts. Screw-capped culture tubes (16 × 150 mm) with Teflon-lined caps are used for carrying out the extractions. For sera or blank, use tubes containing 9.5 ml of isopropanol. While mixing the contents of the tubes on a Vortex mixer, add 0.5 ml of serum or 0.5 ml of 0.9% NaCl. Cap tubes, centrifuge, and transfer to a clean tube if extracts are to be stored, or sample directly if digestions are to be done at once.

Phospholipid Digestions. Transfer 1.0-ml aliquots of the isopropanolic lipid extract to chemical combustion tubes. Add 1.0 ml of 2.5 *N* H_2SO_4 and mix. Place the tubes in a block heater preheated to 100°–125°C. The isopropanol will immediately volatilize without bumping, and oxidation of the organic material occurs immediately. Continue heating until temperature reaches 250°–275°C. The digestion mixture will have now charred, and the tubes are removed from the heating block. Carefully add 3 drops of 30% H_2O_2 at the side of the tube near the bottom, mix, and replace tubes in heating blocks. Continue heating for 5 minutes at 250°–275°C. Remove tubes from block and cool. If the destruction of organic material is not complete, add additional H_2O_2, reheat, and cool. The analysis can be stopped at this point if necessary. Prior to the colorimetry, add 1.0 ml of water and mix contents of the tube well.

Analytical System. The manifold schematic is illustrated in Fig. 2. An unmeasured aliquot is transferred to the sample cups. Samples are aspirated at a rate of 60 specimens/hour and added to an air-segmented stream of molybdic acid reagent followed by mixing. The stannous chloride reagent is then added to the reaction mixture. After mixing and a 3–4-minute time delay, the absorbance is measured at 660 mμ, using a tubular flow cell with a 15-mm light path.

3.3. Triglycerides

Current interest in lipid metabolism and the relationship of serum triglycerides to atherosclerotic heart disease has also created a need for an automated direct triglyceride procedure. Manual techniques can be divided into three stages: extraction of serum lipids and removal of phospholipids, saponification of triglycerides to glycerol and its oxidation to formaldehyde, and the colorimetric measurement of formaldehyde. For the present, the first step has not lent itself to automation. Currently, two semiautomated triglyceride procedures, which pertain to the last two stages of the manual technique, have been published.

In 1964 Lofland (L4, L5) automated the chromotropic acid technique of Lambert and Neish (L1) for the colorimetric measurement of formaldehyde. The extract preparation, evaporation, saponification, and dilution steps are performed manually. This extract can also be used for the

determination of cholesterol and phospholipid. Oxidation of glycerol to formaldehyde and its subsequent measurement by the chromotropic acid reaction are carried out automatically. The saponified sample, which has been dissolved in 0.2 N H_2SO_4, is placed in the sample cups, and an aliquot is added to an air-segmented sodium metaperiodate solution for oxidation of glycerol to formaldehyde. The excess periodate reagent is destroyed by addition of sodium arsenite. After mixing, chromotropic acid reagent is added to the reaction mixture and subjected to additional mixing. The reagent stream then passes through a 95°C heating bath

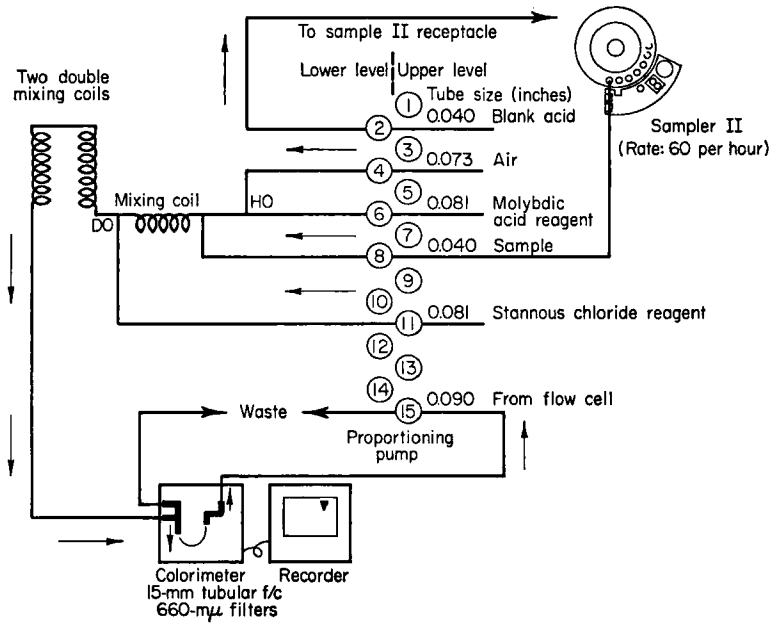

FIG. 2. Analytical system: manifold schematic for phosphorus from phospholipid digests.

containing two 40-foot glass coils. The absorbance is measured at 570 mμ, using a flow cell with a 15-mm light path. The net rate of analysis is 40 determinations/hour.

A semiautomated fluorometric procedure for quantitating triglycerides was presented by Kessler and Lederer in 1965 (K6). This is based on the Hantzsch condensation reaction between an amine, β-diketone, and an aldehyde (B1, N1). Isopropanolic extracts of serum are prepared manually, and at this point can also be used for the determination of cholesterol and phospholipids. Triglycerides can be determined after treatment with Zeolite, as employed by Van Handel and Zilversmit

(V1, V2), for removal of phospholipids. The extracts are then introduced into the automated analytical system for carrying out saponification of triglycerides to glycerol, oxidation to formaldehyde, followed by condensation with diacetylacetone and ammonia to give a fluorescent product, 3,5-diacetyl-1,4-dihydrolutidine.

Reagents

(1) Isopropanol (aldehyde-free, A.C.S. reagent grade)

(2) Zeolite mixture: consists of 200 g of Zeolite (obtainable from W. A. Taylor Co., Baltimore, Md.) that has been finely ground in a Waring Blendor and dried overnight at 110°C, 20 g of Lloyd reagent (obtainable from Hartman-Leddon Co.) 10 g of $CuSO_4 \cdot 10H_2O$, and 20 g of $Ca(OH)_2$; the components are mixed well

(3) Triolein standards: triolein (obtainable from Applied Science Laboratories, State College, Pa.) is prepared in isopropanol at concentrations of 50–300 mg/100 ml; these standards are carried through both the extraction and analytical procedures

(4) Base reagent: 250 ml of 2% KOH plus 750 ml of isopropanol

(5) Sodium periodate 0.025 *M* in 2 *M* acetic acid: 5.35 g of sodium metaperiodate is dissolved in 120 ml glacial acetic acid and then diluted to 1 liter with distilled water

(6) Ammonium acetate 2 *M* (pH 6.0): dissolve 155 g of ammonium acetate in approximately 900 ml of water; adjust to pH 6.0 $\pm$ 0.1 with glacial acetic acid (approximately 7 ml) and then dilute to 1 liter

(7) Acetylacetone reagent: 7.5 ml acetylacetone (2,4-pentanedione) is added to 25 ml of isopropanol and mixed; then 1 liter of 2 *M* ammonium acetate (pH 6.0) is added and mixed well; prepare fresh daily

Extractions. For carrying out the extractions, screw-capped culture tubes (16 $\times$ 150 mm) with Teflon-lined caps are used. For sera and blank, 9.5 ml of isopropanol is placed in the tubes. Standards contain 9.0 ml of isopropanol plus 0.5 ml of water. While mixing the contents of the tube on a Vortex mixer, 0.5 ml of serum, water, or standard is added, and the mixing continued for 15 seconds. Tubes are capped and allowed to stand for 15 minutes. If phospholipids are to be determined, tubes are centrifuged. An aliquot is removed for hot acid digestion, followed by color development and measurement of inorganic phosphate. Cholesterol can also be determined on the extracts at this point. Zeolite mixture (2 g) is added to the extraction tubes, which are recapped, mixed vigorously, and allowed to stand for 30 minutes with occasional mixing. Tubes are centrifuged and supernatants decanted into clean tubes. The extracts are now usable for determining triglycerides. If desired, cholesterol can also be measured in these treated extracts.

Analytical System. Figure 3 illustrates the flow diagram of the triglyceride method. The isopropanolic phospholipid-free extract is added to an air-segmented base reagent, and saponification of triglycerides to glycerol occurs while going through the 50°C heating bath. After saponification, periodic acid is added to the reaction mixture for carrying out the oxidation of glycerol to formaldehyde. Acetylacetone reagent is also added for condensation with formaldehyde to give the fluorescent product. The oxidation and condensation steps are carried out simultaneously during passage through the 50°C heating bath. After heating,

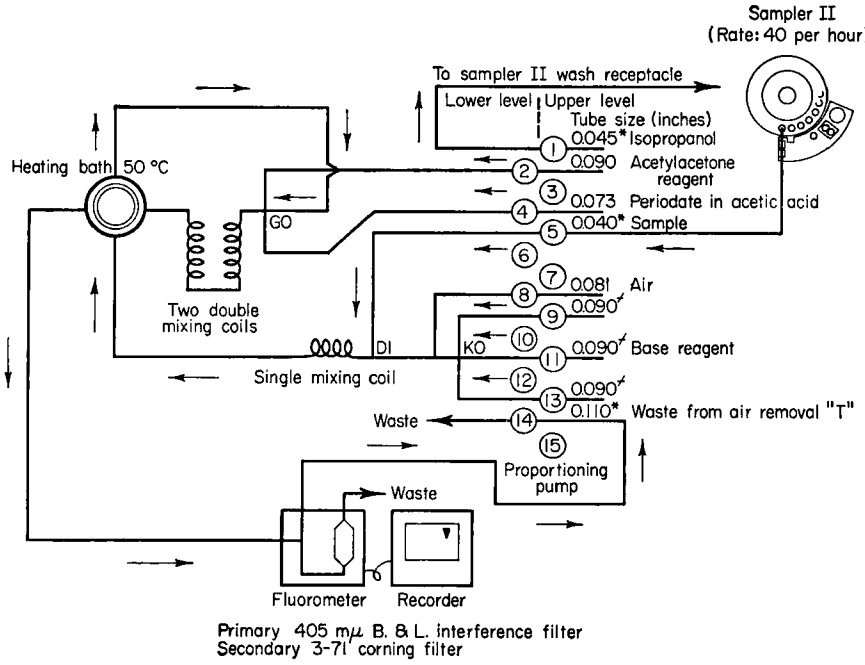

FIG. 3. Analytical system: flow diagram for triglycerides (25–300 mg/100 ml, isopropanolic extracts). * Solvaflex. † Acidflex.

the reaction mixture enters the fluorometer where air is removed and the fluorescence activated, using a Bausch & Lomb 405-mμ interference filter as the primary filter and a Corning 3-71 as the secondary filter.

A blank determination is carried out on the isopropanolic extracts by eliminating the saponification step. The sample-base mixture bypasses the first 50°C heating bath, and is immediately acidified to pH 6.0 by addition of both the periodic and acetylacetone reagents. Sampling speed for both blank and assay runs is 40/hour, giving a net rate of 20 samples/hour.

Results. Under these reagent conditions, the glycerol content of various triglycerides is 92–100% of that present. The recovery of triglycerides added to extracts or serum is 95–104%. An interday precision for a serum pool with a mean value of 119.5 mg/100 ml gives a standard deviation of ±2.9. This technique compares well with the manual procedure of Carlson and Wadström (C1, C2).

3.4. Free Fatty Acids

A semiautomated procedure for the estimation of plasma free fatty acids has been developed by Antonis (A3, A4). The technique requires the preparation of a phospholipid-free plasma lipid extract. The automated colorimetric procedure is based on the methods of Duncombe (D1, D2), and depends on the solubility of copper soaps in chloroform with subsequent complexing of the copper with diethyldithiocarbamate.

Reagents

(1) Diisopropyl ether (reagent grade)

(2) Copper reagent: 9 volumes of aqueous 2 *M* triethanolamine, 1 volume of 2 *N* acetic acid, and 10 volumes of 10% (w/v) $Cu(NO_3)_2$

(3) Diethyldithiocarbamate reagent: 0.2% (w/v) of sodium diethyldithiocarbamate in butanol

(4) Standards: palmitic acid working standards (15–90 μEq/liter) are prepared in chloroform, and correspond to a 1:25 plasma extract at a range of 375–2250 μEq of palmitic acid/liter

Phospholipid-Free Extracts. The preliminary extraction of plasma with diisopropyl ether and silicic acid is based on the procedure of Mendelsohn and Antonis (M2) (described previously under the section for the fluorometric measurement of cholesterol). These phospholipid-free chloroform extracts are stable indefinitely if stored at 4°C.

Analytical System. Glass sample cups are used for the chloroform extracts, while Acidflex pump tubing is necessary for all lines in contact with chloroform. The Sampler II is operated at a rate of 30 samples/hour with a 1:2 cam. This corresponds to a 40-second sampling period followed by an 80-second chloroform wash time.

Extracts, air, and copper reagent are pumped and passed through a special mixing coil filled with glass beads to give a large surface extraction area. The two phases then separate on entering a glass trap. The aqueous copper phase together with a portion of the chloroform phase passes to waste and the remainder of the chloroform phase is redrawn through the pump. This stream is segmented with air; then diethyldithiocarbamate reagent is added, followed by mixing. The extinction is measured at 440 mμ in a tubular flow cell with a 15-mm light path.

Results. The responses of the major serum fatty acids are approximately equal, giving extinction responses of 0.396–0.411. It is necessary to remove phospholipids completely since they will react significantly in this system. The mean difference between duplicate pairs was 12 μEq/liter, with a standard error of a single determination of ±10.5 μEq/liter for the range 220–1300 μEq/liter of plasma free fatty acids.

3.5. Acyl Esters

Antonis (A3, A5) has presented an automated colorimetric procedure for the determination of acyl esters in serum lipid extracts, based on his manual method (A2). The ester groups are subjected to alkaline hydroxylaminolysis to form hydroxamic acids, which react with ferric ion to form highly colored chelates.

Reagents

(1) Isopropanol (reagent grade)

(2) NaOH 10% (w/v) in absolute methanol

(3) Hydroxylamine hydrochloride 10% (w/v) in absolute ethanol

(4) Alkaline hydroxylamine reagent: mix equal parts of reagents (2) and (3); allow to stand 10–15 minutes and filter; prepare fresh daily

(5) Ferric perchlorate reagent. *Stock:* 8.5 g of ferric perchlorate is dissolved in 20 ml of water and then made up to 250 ml with 72–73% perchloric acid. *Working:* 45 ml of stock solution is added to 755 ml of absolute methanol; prepare just before use

(6) Standards. *Stock:* (25 mEq/liter) 0.7878 g triolein is dissolved in isopropanol and diluted to 100 ml. *Working:* (0.5–2.5 mEq/liter) 2–10-ml aliquots of the stock solution are diluted with approximately 80 ml of isopropanol, then 10 ml of water is added and diluted to 100 ml with isopropanol. Standards are equivalent to 5–25 mEq/liter of total ester based on a 1:10 isopropanolic extract

Extractions. A simple isopropanolic extract is prepared by blowing a fine stream of serum (1.0 ml) with swirling into 9 ml of isopropanol. The mixture is well shaken and allowed to stand for 30 minutes with intermittent shaking. After centrifugation the supernatant is decanted. Extracts can be stored at 4°C.

Analytical System. The sampling rate is 30 samples/hour with a 1:2 cam, which corresponds to a 40-second aspiration of specimen followed by an 80-second blank solution. The specimen is segmented by air, joined by the alkaline hydroxylamine reagent, and mixed. The reaction mixture then passes through two 40-foot time-delay coils kept at room temperature. After reacting for approximately 20 minutes, ferric perchlorate reagent is added and mixed by passing through three glass mixing coils.

The absorbance is measured at 520 mμ in a flow cell with a 15-mm light path.

Results. Similar absorbances are found for triolein, cholesterol oleate, dipalmitoyl lecithin, and dipalmitoyl cephalin. Sphingomyelin, which has an amide fatty acid linkage, does not undergo hydroxylaminolysis. The standard error of the method is 0.36 mEq for an acyl ester concentration of 7–20 mEq/liter serum.

4. Summary

A brief description of various automated techniques for total cholesterol, phospholipids, triglycerides, free fatty acids, and acyl esters has been presented. The reagent and analytical systems for a few techniques are given in greater detail. These procedures, in the opinion and experience of the writer, appear to be the simplest to perform, and produce reliable results.

References

A1. Abell, L. L., Levy, B. B., Brodie, B. B., and Kendall, F. E., A simplified method for the estimation of total cholesterol in serum and demonstration of its specificity. *J. Biol. Chem.* **195,** 357–366 (1952).

A2. Antonis, A., The colorimetric determination of ester groups in lipid extracts. *J. Lipid Res.* **1,** 485–486 (1960).

A3. Antonis, A., Automated techniques in serum lipid analysis. Paper presented at "Automation in Analytical Chemistry," Technicon International Symposium, 1964.

A4. Antonis, A., Semiautomated method for the colorimetric determination of plasma free fatty acids. *J. Lipid Res.* **6,** 307–312 (1965).

A5. Antonis, A., Platt, D. S., and Thorp, J. M., Automated method for the colorimetric determination of acyl esters in serum. *J. Lipid Res.* **6,** 301–306 (1965).

B1. Belman, S., The fluorimetric determination of formaldehyde. *Anal. Chim. Acta* **29,** 120–126 (1963).

B2. Bensa, J., Notes sur le dosage du cholestérol par la methode de Zak et Zlatkis automatisée. Adaptation au cholestérol libre. *Ann. Biol Clin. (Paris)* **21,** 303–312 (1963).

B3. Block, W. D., Jarrett, K. J., and Levine, J. B., Use of a single color reagent to improve the automated determination of serum total cholesterol. *In* "Automation in Analytical Chemistry," (Skeggs, L. T., Jr., ed.) pp. 345–347. Mediad, New York, 1965.

B4. Block, W. D., Jarrett, K. J., Jr., and Levine, J. B., An improved automated determination of serum total cholesterol with a single color reagent. *Clin. Chem.* **12,** 681–689 (1966).

B5. Boy, J., Automatic determination of blood cholesterol. *J. Clin. Pathol.* **16,** 178–180 (1963).

B6. Boy, J., Bonnafe, M., and Mazet, J. B., Determination du cholestérol total par une modification de la méthode de Pearson. Son emploi por voie manuelle et automatique. *Ann. Biol. Clin. (Paris)* **18,** 669–675 (1960).

C1. Carlson, L. A., Determination of serum triglycerides. *J. Atherosclerosis Res.* **3,** 334–336 (1963).

C2. Carlson, L. A., and Wadström, L. B., Determination of glycerides in blood serum. *Clin. Chim. Acta* **4,** 197–205 (1959).

C3. "Cholesterol 3–60, AutoAnalyzer—Method File." Technicon Instruments Corp., Chauncey, New York, 1960.

C4. "Cholesterol N-24 P, AutoAnalyzer—Method File." Technicon Instruments Corp., Chauncey, New York, 1963.

C5. "Cholesterol N-24a, AutoAnalyzer—Method File. Technicon Instruments Corp., Chauncey, New York, 1965.

C6. Connerty, H. V., Briggs, A. R., and Eaton, E. H., Jr., Simplified determination of the lipid components of blood serum. *Clin. Chem.* **7,** 37–53 (1961).

C7. Cooper, G. R., Roland, D. M., and Eavenson, E., Determination of total cholesterol with the AutoAnalyzer. Paper presented at "Automation in Analytical Chemistry," Technicon International Symposium, New York, 1964.

D1. Duncombe, W. G., The colorimetric determination of long chain fatty acids in the 0.05–0.5 μmole range. *Biochem. J.* **83,** 6P (1962).

D2. Duncombe, W. G., The colorimetric microdetermination of long chain fatty acids. *Biochem. J.* **88,** 7–10 (1963).

H1. Hurst, R. O., The determination of nucleotide phosphorus with a stannous chloride–hydrazine sulphate reagent. *Can. J. Biochem.* **42,** 287–292 (1964).

J1. Jamieson, A., A method for the rapid determination of serum total cholesterol using a modification of the Pearson reaction. *Clin. Chim. Acta* **10,** 530–535 (1964).

K1. Kenny, A. P., The determination of cholesterol by the Liebermann-Burchard reaction. *Biochem. J.* **52,** 611–619 (1952).

K2. Kenny, A. P., and Jamieson, A., Direct estimation of serum total cholesterol on the AutoAnalyzer. *Proc. Assoc. Clin. Biochem.* **2,** 213–214 (1963).

K3. Kenny, A. P., and Jamieson, A., Automated method for the direct determination of total serum cholesterol. *Clin. Chim. Acta* **10,** 536–543 (1964).

K4. Kessler, G., Continuous automatic determination of cholesterol. *Clin. Chem.* **5,** 381 (1959) (abstr.).

K5. Kessler, G., An automated system of analysis. *In* "Gradwohl's Clinical Laboratory Methods and Diagnosis" Frankel, S., and Reitman, S., eds.), Vol. 1, pp. 301–321. Mosby, Saint Louis, Missouri, 1963.

K6. Kessler, G., and Lederer, H., Fluorometric measurement of triglycerides. *In* "Automation in Analytical Chemistry," (Skeggs, L. T., Jr., ed.) pp. 341–344. Mediad, New York, 1965.

K7. Kraml, M., A semi-automated determination of phospholipids. *Clin. Chim. Acta* **13,** 442–448 (1966).

K8. Kuttner, T., and Cohen, H. R., Microcolorimetric studies. I. A molybdic acid, stannous chloride reagent. The microestimation of phosphate and calcium in pus, plasma, and spinal fluid. *J. Biol. Chem.* **75,** 517–531 (1927).

L1. Lambert, M., and Neish, A. C., Rapid method for estimation of glycerol in fermentation solutions. *Can. J. Res.* **28B,** 83–89 (1950).

L2. Leffler, H. H., Estimation of cholesterol in serum. *Am. J. Clin. Pathol.* **31,** 310–313 (1959).

L3. Levine, J. B., and Zak, B., Automated determination of serum total cholesterol. *Clin. Chim. Acta* **10,** 381–384 (1964).

L4. Lofland, H. B., Jr., A semiautomated procedure for the determination of triglycerides in serum. *Anal. Biochem.* **9,** 393–400 (1964).

L5. Lofland, H. B., Jr., A semiautomated procedure for the determination of triglycerides in serum. *Anal. Biochem.* **10,** 178 (1965) (addendum).

M1. Marsh, W. H., "Automation in Clinical Chemistry." Thomas, Springfield, Illinois, 1963.
M2. Mendelsohn, D., and Antonis, A., A fluorimetric micro glycerol method and its application to the determination of serum triglycerides. *J. Lipid Res.* **2,** 45–50 (1961).
N1. Nash, T., The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. *Biochem. J.* **55,** 416–421 (1953).
P1. Pearson, S., Stern, S., and McGavack, T. H., A rapid procedure for the determination of serum cholesterol. *J. Clin. Endocrinol. Metab.* **12,** 1245–1246 (1952).
P2. Pearson, S., Stern, S., and McGavack, T. H., A rapid, accurate method for the determination of total cholesterol in serum. *Anal. Chem.* **25,** 813–814 (1953).
S1. Skeggs, L. T., Jr., An automatic method for colorimetric analysis. *Am. J. Clin. Pathol.* **28,** 311–322 (1957).
S2. Skeggs, L. T., Jr., Principles of automatic chemical analysis. *In* "Standard Methods of Clinical Chemistry" (S. Meites, ed.), Vol. 5, pp. 31–42. Academic Press, New York, 1965.
S3. Sperry, W. M., and Webb, M., A revision of the Schoenheimer-Sperry method for cholesterol determination. *J. Biol. Chem.* **187,** 97–110 (1950).
V1. Van Handel, E., Suggested modifications of the micro determination of triglycerides. *Clin. Chem.* **7,** 249–251 (1951).
V2. Van Handel, E., and Zilversmit, D. B., Micromethod for the direct determination of serum triglycerides. *J. Lab. Clin. Med.* **50,** 152–157 (1957).
W1. Whitley, R. W., and Alburn, H. E., A semiautomated method for the determination of serum phospholipids. Paper presented at "Automation in Analytical Chemistry," Technicon International Symposium, New York, 1964.
Y1. Youngburg, G. E., and Youngburg, M. V., Phosphorus metabolism. I. A system of blood phosphorus analysis. *J. Lab. Clin. Med.* **16,** 158–166 (1930).
Z1. Zilversmit, D. B., and Davis, A. K., Microdetermination of plasma phospholipids by trichloroacetic acid precipitation. *J. Lab. Clin. Med.* **35,** 155–160 (1950).
Z2. Zlatkis, A., Zak, B., and Boyle, A. J., A new method for the direct determination of serum cholesterol. *J. Lab. Clin. Med.* **41,** 486–492 (1953).

QUALITY CONTROL IN ROUTINE CLINICAL CHEMISTRY

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1. Introduction

1.1. General

The diagnosis and investigation of disease by means of chemical analyses of the blood and other body fluids have become an indispensable part of the practice of medicine. Some measure of the value to the clinician of chemical data may be derived from the increase in the number of calls on the services of chemical laboratories attached to large hospitals; in the United Kingdom the average annual rate of increase from 1958 to 1963 was 18% per annum (L1, W2), and in many countries a similar growth rate is being experienced. It has long been clear that certain diseases are susceptible to chemical investigation; the diagnosis and management of diabetes mellitus, for example, have depended on chemical investigations for nearly 50 years. Other diseases, or categories of disease, have become delineated primarily as a result of developments in analytical techniques; in the last 20 years the ready availability of plasma sodium and potassium analyses, using the flame photometer, has made possible the much better assessment and management of conditions in which there is a disturbance of fluid and electrolyte balance.

With the growth in the number of investigations carried out by clinical chemistry laboratories, there has also come a widening of their scope. A large laboratory may today be able to perform over 100 different analytical procedures, compared with about 20–30 in 1939. Some of these analyses, notably blood glucose, plasma urea, electrolytes, and the assessment of acid–base balance, are of great importance in the management of acutely ill patients, and for such investigations results are usually required on the same day, often within the minimum period set by the time needed for transport of specimens and performance of analyses. Other investigations are less urgent: tests for the investigation of chronic endocrine dysfunction, for instance, come within this category, and the absence of urgency reaches its most marked stage in the biochemical screening of apparently healthy populations. These different categories of work allow different arrangements to be considered for the organization of laboratories, and correspondingly different quality control programs may be appropriate. It must never be felt, however, that the need to provide a result as quickly as possible ever releases the analyst from his obligations to provide data that are reliable and, particularly in urgent situations, when immediate and irreversible decisions may be taken by clinical staff in the light of information supplied by the laboratory, his duty in this respect can be held to be even more demanding

than in nonurgent situations, where there may well be an opportunity to repeat an investigation if this appears necessary.

A further consequence of the greater use of chemical investigations in following the natural history of disease has been the recognition that subtle variation in the levels of certain constituents in the blood may be of significance in revealing the presence of occult disease, or in differentiating one population of individuals from another. If this extra information is to be obtained from the results of chemical analyses, it is essential that the accuracy and precision of the estimations should be such that these small but significant changes can be detected, instead of being lost against a background of experimental error. The time-honored method of carrying out all analyses in duplicate, which was apparently practiced in the more leisurely days of clinical chemistry, besides being impracticable under today's conditions of vast workloads, often results only in the arbitrary selection of one of two values by the application of criteria that at best can be called intelligent guesses. The modern clinical chemist deserves credit for appreciating that he is faced with a quality control problem, and for being prepared to state the accuracy and precision claimed for his results. The purpose of this review is to consider the sources of analytical and associated errors in the clinical chemistry laboratory, and the ways in which these errors may be detected, evaluated, reduced, and subsequently controlled.

Most of the methods in use in clinical chemistry laboratories involve colorimetric analysis; in fewer instances volumetric or gravimetric procedures are still retained. It is not the purpose of this review to enter into a discussion of the errors inherent in colorimetric, volumetric, and gravimetric analysis as such; for a treatment of this subject the reader is referred to standard works on chemical analysis (e.g., V3). Instead, the review will be confined to those sources of error that are particularly likely to affect the work of a clinical laboratory. These errors arise mainly from the need to perform many analyses on large numbers of samples with a variable degree of urgency, and from the fact that most of these analyses have to be conducted on plasma or serum, which are viscous protein-rich fluids available only in restricted quantities (M12).

Although the proper functioning of clinical chemical laboratories is as dependent as that of other departments on the provision of properly designed premises of an adequate size, in which due attention has been paid to the standards of lighting, heating, ventilation, and amenities for use by staff, this important subject will not be considered further in the review, since laboratory design and furnishing have been fully discussed in a number of recent publications (e.g., R3, R4). Likewise, the shortage of trained graduate and technical staff, the difficulties of organizing and

implementing training programs for junior graduate staff and for student technicians, and more purely administrative matters such as financial restrictions on laboratory budgeting will not receive further specific mention, even though they also affect the reliability of laboratory work directly or indirectly.

1.2. The Present Position as Indicated by Surveys

Belk and Sunderman (B1) in 1947 reported the results of a survey of the accuracy of chemical analyses in clinical laboratories in Pennsylvania, U.S.A. This pioneer survey depended mostly on the use of aqueous solutions (glucose, urea, calcium, and chloride), but also included a more limited assessment of serum protein and of hemoglobin methods. The findings were very disquieting, since they all revealed wide variations between the laboratories when reporting results for the analysis of identical specimens, and this pattern of interlaboratory disagreement has since been shown to be a problem in many different countries. Further descriptions of interlaboratory surveys include the findings from an international trial (W10) and reports from single countries, including Australia (H2–H5), Britain (B8, G4, W15), Canada (T4), New Zealand (D1), Norway (R7), and the United States (B2, F2, S10). It is all too clear from a study of these publications that, although improvements have occurred (e.g., H5), the quality of analytical performance in clinical chemistry laboratories still falls far below the levels which might reasonably be required.

Some surveys (e.g., F2, G4, W10) included an assessment of the precision of each laboratory's work by distributing specimens with interrelated compositions or else duplicate samples, and the results of these comparisons showed that individual laboratories could compare the content of two solutions for the substances under evaluation more accurately than they could determine the absolute value of the particular constituent. This led Wootton (W10) to state the very important conclusion that estimations in clinical chemistry laboratories should whenever possible be made by comparing an unknown with a standard solution, since such comparisons would reveal some errors which could otherwise pass undetected for a long time. This need to incorporate both standards and controls into routine use will be reconsidered later (Section 3.1.1).

Another important general finding has been that the introduction of work-simplified techniques and of automatic methods of analysis has not improved the results of interlaboratory surveys as much as might have been forecast. Some of the main differences between the analytical practice at the time of the earlier trials (B1, W10, W15) and the most recent studies (e.g., G4, H5, T4) have been the simplification of manual methods and the introduction of the AutoAnalyzer (Technicon Instruments Corp.,

Ardsley, New York) into routine laboratory use. Some surveys (B8, G4) included a comparison between the quality of results obtained by laboratories that had adopted AutoAnalyzer methods and results reported from other laboratories that were still using manual techniques. It might have been expected that AutoAnalyzers, because of their fundamental property of a high standard of repeatability in prolonged operation, would have collectively yielded much more accurate and reproducible results than the manual findings, and to some extent this was true (B8), but the results of the latest survey (G4) still show the need for much further improvement in both classes of method.

In some quarters a feeling has grown up that the greatly increased work loads carried by clinical chemistry laboratories have resulted in a deterioration in the reliability of the results reported by them, and there is a considerable amount of evidence that repetition of large numbers of analyses by manual methods leads to a progressive decline in the quality of the results (e.g., R6, R6a). On the other hand, data presented later in this review will show how work simplification of manual methods and of automatic methods of analysis can greatly reduce the deterioration in performance brought about by boredom and fatigue.

It is perhaps disappointing that standards of accuracy and precision have not increased more in the 20 years since publication of the first interlaboratory survey in clinical chemistry. On the other hand, although the limited extent of general improvement must give rise to concern, there appears to be little support for the suggestion that the reliability of work has in fact fallen. There are many reasons which could account for the failure as yet of work simplification and AutoAnalyzers to have produced a general rise in standards of analytical performance in clinical chemistry laboratories, and these will be discussed in this review. At this point, all that need be stressed is that the purchase of expensive automatic analytical equipment does not by itself necessarily render chemical investigations on biological material less liable to errors, although the emphasis on the different sources of error may alter from those encountered in manual methods. In the whole of clinical chemistry, therefore, it is essential always to include controls to check the reliability of methods, and it will be disastrous if the main long-term outcome of the introduction of automatic methods of analysis is merely to enable laboratories to carry out more work without at the same time contributing to improved standards of accuracy and precision.

1.3. The Danger of Oversimplifying the Problems of Quality Control in Clinical Chemistry

In recent years a plethora of ways has become available for monitoring the performance of clinical chemistry laboratories, and for simplifying

the performance of some of the more complex techniques, such as enzyme determinations. Commercial control sera have been marketed with manufacturers, accompanying literature that claims that they can serve as primary standards, true controls, reference standards, etc., and sometimes that they can fulfill combinations of these functions at one and the same time. Details about the method of preparation have to be withheld for commercial reasons, but it is clear in some cases from first biochemical principles that the products cannot begin to fulfill the claims made for them. Some of these obvious shortcomings have been reviewed by Radin (R1).

For many difficult quantitative methods, kits are now available. These are as a rule too expensive for large-scale use, and have proved particularly popular for "one-off" determinations. This is potentially a very dangerous situation from the point of view of quality control, since the stability of kit reagents cannot necessarily be taken for granted, and lack of familiarity with these determinations because they are only occasionally performed may mean that proper controls are not undertaken. Variability between kits obtained from different manufacturers for performing the same investigation also gives rise to confusion (C9).

There is in fact no shortcut to the regular reporting of reliable results from a clinical chemistry laboratory. The various features that make the methods of clinical chemistry particularly difficult to control will be emphasized in this review, but it will not be concerned in great detail with the problems of suitable chemical and biological standards. This latter aspect of the subject has been extensively considered recently (R1), and all that need be said here is that it will be many years before the problems of supply of satisfactory standards of properly defined purity and acceptable stability for all methods are solved. The shortcomings of standards previously available seriously aggravate the difficulty of quality control programs. Nevertheless, the operation of a quality control program is essential to the practice of clinical chemistry. Such a program includes the following stages:

(*i*) The appreciation and assessment of the sources of error inherent in the collection, transport, and reception of specimens for analysis and any initial processing steps, such as separation of plasma.

(*ii*) The validation of new procedures introduced into the analytical repertoire of the laboratory with respect to accuracy, repeatability, and sensitivity by appropriate comparison and recovery experiments.

(*iii*) The establishment of the reproducibility for the analytical methods in regular use, and the definition of acceptable limits of variation for control samples.

(*iv*) The calculation of means and standard deviations and the prepa-

ration of quality control charts for displaying the constancy or variability of control data.

(*v*) The continuous independent monitoring of analytical performance by means of appropriate fictitious patient controls, carry-over samples, etc.

(*vi*) When the program has been established, its value will be lost completely if the need for action indicated by its results is ignored.

These various stages in the establishment and maintenance of a quality control program will be discussed. Examples will also be given of ways in which work simplification and automatic methods of analysis can lead to improvements in the reliability of chemical investigations.

The provision of a routine service of chemical analyses on biological material by no means defines the whole scope of clinical chemistry. The analytical service is founded on the application of the results of continuing research and development in this area of science, but, if these advances are to be effectively exploited, the criteria adopted for assessing the performance of the service must be appropriately rigorous.

2. Nomenclature and Definitions

The results of most investigations performed in clinical chemistry laboratories are expressed in quantitative terms, and the characteristics of the methods adopted by different laboratories can therefore be assessed according to the standard criteria of quantitative chemical analysis. In such an assessment, definitions of terminology are important, and this section defines and discusses the following terms used frequently in this review:

Accuracy	(Section 2.1)	Reliability	(Section 2.4)
Specificity	(Section 2.1)	Error	(Section 2.5)
Precision	(Section 2.2)	Standard	(Section 2.6)
Sensitivity	(Section 2.3)	Control	(Section 2.6)

2.1. Accuracy and Specificity

It is difficult to separate these two concepts entirely. The accuracy of a determination is defined as the concordance between it and the true or most probable value of the quantity measured (V3), and the specificity of a determination may be defined as the ability of the method to determine solely the compound it purports to measure. Since the definition of accuracy includes the concept of the true or most probable value, this introduces the need for reference values against which to assess the accuracy of the determination.

The different categories of reference values have been discussed by Mandel (M2), and for clinical chemistry the relevant category is the

assigned reference value, arrived at by agreement among experts. This choice is dictated by practical consideration of the limitations imposed on chemical measurements, particularly in biological specimens; the reference value in a preparation which may be used for standardizing or comparing the performance of assay methods in different laboratories, especially in the case of serum or urine standards, has its composition agreed as a result of estimations carried out in a number of laboratories selected for their apparent ability to undertake the necessary work with high standards of technical performance. This does not mean that the reference values for such preparations are necessarily absolutely correct, but it implies that the values should be taken to represent the most probably correct figures available until evidence is provided (e.g., as a result of methodological developments) to prove that this assumption has been unfounded. One advantage of the assigned reference value, for purposes of investigating accuracy, is that it allows a meaningful statement to be made of the errors of a particular measurement (M2), and we shall return to a consideration of accuracy as part of the discussion on errors.

It is more difficult to secure conclusive evidence of the specificity of a chemical method, when applied to a biological system (B7). Since the constituent will almost certainly have to be determined as one component in a mixture, the possible interfering effects of some (ideally all) of these other substances need to be determined, each over the range of concentrations liable to be met with in practice. Whereas the accuracy of a method can best be determined by recovery experiments, which depend on the availability of a pure sample of the compound under investigation, the specificity of a technique and the effects of possible interfering factors can be more readily investigated by experiments involving radioactive isotopes; these isotopic assessments of specificity have so far not been widely applied in clinical chemistry.

The established specificity of a method may have to be reassessed when, for instance, a new drug is introduced into therapeutics, if this introduction is followed by the observation of unexpected results for the method under discussion; drug interference may arise for many reasons, e.g., from the properties of the drug itself or of one of its metabolites.

Two examples of the interrelation between accuracy and specificity will be briefly considered. Determinations of blood glucose using a glucose oxidase procedure are potentially more accurate since they are more specific than techniques such as the Folin-Wu method, which also measures a number of nonglucose reducing substances in blood; the introduction of glucose oxidase into routine use meant a reappraisal of the hitherto accepted criteria for normal fasting blood glucose values and

for the pattern of response of blood glucose in various provocative tests of carbohydrate metabolism. Similarly, the introduction of an adsorption step onto Lloyd's reagent in the determination of serum and urine creatinine by the Jaffé reaction improved the accuracy of the procedure, since certain noncreatinine chromogenic substances did not adsorb onto Lloyd's reagent and these had therefore been included in previous less specific determinations of creatinine that omitted the adsorption step (O1).

To establish the accuracy of a particular method, results obtained by the repeated application of the method under test are compared with results obtained on identical specimens by the best available reference procedure. The accuracy of the new method should be assessed over the range of concentrations to which the method will be applied, and the values for the reference method either can be obtained in the laboratory or can be based on observations made in accepted reference laboratories. The difference between these two sets of determinations will serve to show whether the new method under test closely parallels the reference method, thereby indicating that its accuracy is comparable, or whether it systematically overestimates or underestimates the compound being measured, or whether there is no demonstrable correlation between the results for the two methods.

2.2. Precision

The precision of a determination is defined as the concordance of a series of measurements of the same quantity (V3). The definition of precision includes the terms repeatability and reproducibility (H11). Repeatability is a measure of the deviation of test results from their mean value, all determinations being carried out by one operator and without change of apparatus, where apparatus can be significant (H11). For reproducibility in clinical chemistry the definition provided by Hughes (H11) is not entirely satisfactory, and an alternative is proposed as follows: reproducibility of a method is a measure of deviation of test results from their mean value, the determinations being carried out over a period of weeks or months, perhaps by different operators, the operators in no case knowing the identity of the analytical specimens that are being used to assess the continued quality of performance of the technique.

The precision of a determination is often expressed in terms of the standard deviation (SD), obtained by analyzing the same specimen at least 20 times. Both repeatability and reproducibility can be determined in this way. The repeatability of the procedure should be investigated first, employing for this purpose a worker who is fully conversant with the technical details for the performance of the analysis, who knows the

purpose of the assessment that is being performed, and who is sufficiently responsible not to introduce observer bias (Section 2.5) when recording the results of his work. If the repeatability of the technique is satisfactory in terms of the clinical requirements for the assay (Section 3.2.3), as expressed by the SD for the replicate analyses, the reproducibility is then determined for the everyday performance of the method under normal working conditions. In general, the SD of the results of the reproducibility assessment will exceed the figure obtained in the trial of the method's repeatability.

Alternatively, precision can be estimated from the results of duplicate determinations (S6), using the formula

$$\mathrm{SD} = (\Sigma d^2/2N)^{1/2}$$

where d is the difference between duplicates, and N the number of duplicate determinations performed; this method of assessing precision is less liable to introduce observer bias, and has the additional advantage that it can be used to estimate the precision for a particular method over a wide range of concentrations.

Both repeatability and reproducibility are fundamentally statistical assessments obtained from study of the performance of a method, and the SD for these two sets of measurements can perhaps be better thought of as defining the lack of precision of a method rather than its precision (M2). This distinction between repeatability and reproducibility has not always been drawn by other authors, some of whom regard the terms as interchangeable (e.g., B7a), but we regard the distinction as an important one relating to the different stages of establishing and then maintaining a technique in operation.

This review will be more concerned with the determination and maintenance of standards of precision, particularly of reproducibility, than with the assessment of accuracy and specificity of methods in clinical chemistry. This is because accuracy and specificity can be determined in a laboratory in advance of introducing a method, and the limitations of each method in these respects defined and allowed for, whereas the precision obtained for the same method may differ markedly from time to time in one laboratory and from one laboratory to another. The trials of interlaboratory accuracy (Section 1.2) have not so far been able to distinguish fully between inaccuracy and imprecision in the performance of individual laboratories for the chemical determinations under evaluation. Imprecision, however, has probably been the main factor underlying the disturbing findings revealed by these trials, and the emphasis placed in this review upon the search for improved precision and its subsequent maintenance is justified by the fact that most of the work in clinical

chemistry has a comparative element. If a method is inaccurate or lacks specificity, e.g., the Kramer and Tisdall (K2) modification of the Clark and Collip (C6) method for estimating serum and urine calcium (M1), this fact will have been allowed for in the normal range of values obtained by the laboratory when using this technique; on the other hand, if the determinations subsequently performed on patients are imprecise, these cannot be evaluated with the necessary degree of certainty in relation to the normal range for the determination, and changes observed in serial determinations performed on specimens from the same patient, if due mainly to laboratory imprecision, can seriously interfere with the clinical management of the patient.

From these definitions it will be seen that a method can have good precision but at the same time be inaccurate, whereas a method inherently accurate necessarily demands some degree of precision in its performance. Worked examples of these statements have been presented in detail elsewhere (V3).

Although it is useful to define accuracy and precision and initially to consider these two important facets of laboratory work separately when evaluating a method, it must be emphasized that precision of operation affects the day-to-day accuracy of a method, not least with respect to results obtained on individual specimens from patients, and errors can render a determination both inaccurate and imprecise. With individual specimens routine analyses are performed singly or, at most, in duplicate; any operational error affecting the analysis of a single specimen (e.g., faulty operation of a pipet) could render the result of that particular analysis grossly inaccurate, or with duplicate analyses could in addition suggest severe falling off in standards of precision, although the accuracy and precision of the method revealed by the analysis of quality control materials included with the batch had failed to demonstrate any fall in the standards set for its performance.

2.3. Sensitivity

This has been defined (B5) as "the smallest single result which, with some assurance, can be distinguished from zero, or, in statistical terms, the smallest single result whose fiducial limits for, say, $P = 0.05$ do not include zero." This review will be primarily concerned with clinical chemical methods that have acceptable levels of sensitivity, and to which therefore statistical methods of quality control can be applied throughout the range of concentrations which may be encountered in physiological and pathological conditions. To take an extreme example, therefore, the statistical methods of quality control discussed in this review would not be fully applicable to determinations of plasma epinephrine or

norepinephrine, since the range of normal values for these two catecholamines, by present methods, appears to include zero. Although sensitivity is important, therefore, it will be assumed that in the initial validation of a technique the required degree of sensitivity has been established, and that any fall-off in sensitivity will be detected as part of the regular program of quality control.

2.4. Reliability

The reliability of a method is a comprehensive term, expressing the ability to maintain its accuracy, specificity, sensitivity, and reproducibility during prolonged use. It will be used, therefore, in the sense adopted by Loraine and Bell (L6), i.e., incorporating these various components inherent in the concept of reliability. Reliability of a method can be assessed only over long periods, and needs to be kept constantly under review since it may change, for instance, with a change in the staff performing the method.

2.5. Error

The error of a method may be defined as the difference between the value obtained and the assigned value (M2), a definition that underlines the close interrelationship between the concept of accuracy and the errors of a determination. Many different classifications of errors have been produced, and these basically subdivide into (1) errors resulting from inaccuracy, alternatively called the error of bias or systematic error of a method, and (2) errors associated with lack of precision.

The error of bias for a new technique is established as part of its initial validation, and thereafter remains unchanged unless the conditions of performance of the determination are altered; when the error of bias is small, the accuracy is high. The special term "observer bias" refers to avoidable factors which may influence an analyst into recording a result different from the one he has observed in the belief that it is the correct or required result; determinations of accuracy and precision of a method should be designed to avoid this possibility. The major concern of the clinical chemist in monitoring the routine performance of a method is to know how closely the result obtained on a certain sample by the method he is using approaches to the "correct" result given by an accepted reference method; since he should have defined the method's accuracy before adopting it for use in his laboratory, this necessarily implies that the clinical chemist's program of quality control is directed thereafter at the regular assessment of precision.

Henry (H7) subdivides the component errors that together combine to make the total error owing to lack of precision of an observation into

(1) the variation between duplicated analyses carried out in the same batch of determinations (P), (2) the variation between days for the same worker analyzing the same sample in one laboratory, i.e., the between-batch variation (D), (3) the variation between workers in the same laboratory when performing the same analysis on identical samples (A), (4) the variation between laboratories, e.g., as revealed by interlaboratory surveys (L). If these subdivisions correctly include all the possible sources of error, and if each category is independent of the others, then the total error for the method is expressed by the formula:

$$\text{Total error} = (P^2 + D^2 + A^2 + L^2)^{1/2}$$

Berry (B2a) has attempted the difficult task of separately assessing the individual contributions of the first three components (P, D, and A) making up the total error in this equation.

Another subdivision of errors, given by Grant (G5), is into (1) avoidable, and (2) unavoidable errors. As avoidable errors, he lists (a) those which can affect whole batches (e.g., faulty preparation of reagents or poor technical performance), and (b) errors which can affect individual specimens without vitiating the results for the batch as a whole; these latter errors, which do not necessarily arise in the laboratory and are some of the most difficult to detect, will be discussed (particularly in Section 3.4.3). As unavoidable errors, he lists (*i*) the precision, and (*ii*) the accuracy of the method. It is difficult to accept this subdivision of errors as applied to clinical chemistry, since lack of precision, a so-called unavoidable error, is included in any comprehensive assessment of quality control which should of necessity attempt to check for avoidable errors, particularly errors capable of affecting whole batches of results. Imprecision is to a considerable degree avoidable and, for this reason, Henry's (H7) subdivision of errors contributing to lack of precision in clinical chemistry is preferred, although it is accepted that a component of imprecision in the performance of every technique is unavoidable even under ideal conditions of analysis—this is the indeterminate or accidental error of a method in Vogel's (V3) classification. Vogel also presents a useful alternative breakdown of "determinate errors" into (a) operational and personal errors (from poor technical performance), (b) instrumental and reagent errors, (c) errors of the method itself, and (d) additive and proportional errors; some or all of these factors may contribute to lack of precision in the routine performance of a method, and each possibility will have to be eliminated when investigating a set of unsatisfactory results for quality control samples.

To summarize, therefore, the word error will be used without qualification in this review to represent the total error of the method owing to

lack of precision, and "error of bias" will be used if errors specifically resulting from inaccuracy are being considered. Sometimes individual components of the total error may be particularly relevant to the subject under discussion, e.g., when the use of duplicate analyses for samples within the same batch or for samples analyzed in different batches are being considered.

2.6. Standards and Controls

Section 3 discusses quality control techniques, together with criteria for selecting satisfactory standards for the evaluation of a method and the subsequent use of standard materials as part of a control program. The tendency to use the word "standard" in several different contexts is potentially confusing, and has given rise to the misconception that control sera can be used at one and the same time both as standard materials and as an objective means of checking the quality of results. Because of the dangers inseparable from such potential confusion, it has been considered advisable to discuss some of the differences between standards and controls in this section on definitions.

Standard substances in clinical chemistry include primary standards, which can be obtained sufficiently pure to be used for the preparation of solutions by weighing or by reference to other definable physical characteristics (e.g., constant-boiling hydrochloric acid). Primary standard chemicals are available for acid–base reactions, precipitation reactions, oxidation–reduction reactions, etc. (V3), and are used in these various categories of analytical determination to validate the preparation of solutions of other chemical substances which cannot be obtained in a form suitable to meet the criteria demanded for a primary standard. Following their calibration in terms of a primary standard, these other chemicals can act as secondary standards.

Standards should be used regularly in all clinical chemistry procedures to check the performance of reagents, the calibration of photometers, etc., and all quantitative results in clinical chemistry are calculated with respect to the performance of a standard (primary or secondary) preparation of the material under test, or of a closely similar material (e.g., dehydroepiandrosterone for group steroid assays) if the exactly corresponding standard material cannot be made available as a pure compound.

A control preparation is a material of established composition (or activity in the case of enzyme determinations), and one that is used to monitor the performance of some, but preferably all, of the stages of a laboratory method. The composition of these control preparations will have been previously established by frequent determination of their constituents (Section 2.2, "Repeatability"), and the values subsequently

obtained during their use as control materials are calculated in terms of the performance of the standard solutions carried through as part of the batch of analyses.

It will be seen from these definitions that, although the analyst necessarily must believe the correctness of the values previously established for the control solution (unless subsequently these are shown to be incorrect), he cannot use the same preparation in a batch of analyses both as a standard and as a control; if he does this, the most that he achieves is an assessment of agreement between duplicate determinations performed as part of one batch of analyses.

3. Quality Control Techniques

3.1. Standards and Controls and Their Use

The criteria which a primary standard chemical substance should satisfy are fully stated in many textbooks (e.g., V3), and few compounds satisfactorily meet all these exacting requirements. Furthermore, the primary standards of quantitative inorganic analysis are, for the most part, made to take part in reactions occurring in aqueous protein-free solution during the calibration of secondary standards; the conditions under which these secondary standardizations are performed are as nearly ideal as possible, and solutions are kept free from contaminants which might interfere with the stoichiometry of the reactions.

In clinical chemistry, the closely controlled reaction conditions of quantitative inorganic and organic chemistry cannot always be obtained, since estimations have to be performed on complex mixtures. This means that both simple aqueous and protein-containing standards have to be considered, and the criteria adopted when choosing a standard for use in clinical chemistry may be unable to meet the criteria of primary standards used in other branches of quantitative chemistry. For some determinations, the accuracy of a method cannot be established at present since pure materials for standardization are not yet available; the best examples of such methods are provided by determinations of enzyme activity in serum, but suitable control preparations exist for monitoring the precision of these assays (e.g., H8).

The inability always to define accuracy of clinical chemical procedures does not necessarily render such analyses any less valuable, as long as the method of performance is adequately validated and criteria of reproducibility are established. Such nonstandardized data can be used in the application of the procedure to the investigation of different disease states, by comparison with findings obtained in normal individuals, and the standards of technique required for the quantitative performance of

these procedures may then be as exacting as in other branches of chemistry. In all chemical work, the fundamental requirements for satisfactory technical performance remain the same, and include the need for reagents of established purity, the use of accurately calibrated balances, the correct grades of glassware, distilled water, etc., by trained operators.

With the introduction of physical methods of analysis into clinical chemistry as alternatives to some of the earlier volumetric or colorimetric techniques, the difficulties inherent in attempting to analyze small quantities of inorganic compounds in the presence of complex protein-containing fluids have in some cases been greatly reduced, although in others new sets of factors that interfere with initial validation and subsequent day-to-day operation have been revealed. Cotlove (C8), for instance, reviewed the difficulties involved in the determination of chloride in biological materials and discussed the advantages offered by electrometric procedures. Similarly, MacIntyre *et al.* (A1) considered critically various methods used for the measurement of magnesium, as well as the shortcomings of some of the standards previously adopted, when describing the flame spectrophotometric method of estimation which they advocated as an alternative to previously existing techniques.

Standards should be included with every batch of determinations, and most laboratories base the calculations of concentration for patients' specimens included in each batch of analyses on the readings obtained with the standards that have been processed as part of that batch. Henry and Segalove (H7a), however, have suggested that the standard value which ought to be used for these calculations should be the mean of a previously determined series (say 20) of values for that reference standard. The authors have no experience of this method of calculation, which in some ways is tantamount to using a calibration curve (B1a), and prefer to base their calculations on the performance of standards analyzed as part of the batch of specimens under investigation.

3.1.1. *Types of Standard and Control Material*

Although primary or secondary standards are not yet available for all clinical chemical procedures, within the limitations imposed by the properties of biological specimens attempts have been made at various levels (e.g., by government agencies, commercial firms, or individual departments) to develop preparations that can be used regularly by laboratories to monitor the performance of their analytical methods (R1). Some progress has been achieved toward the goal of securing direct comparability of analytical findings between different laboratories in the same country, and comparisons between laboratories in different countries have also been undertaken; these comparisons have depended ultimately

on the provision of standardizing materials that have allowed the acceptability of analytical findings to be defined in terms of their accuracy and precision.

Standard materials used in clinical chemistry include the following:

(1) Primary standards and secondary standards, especially for inorganic constituents

(2) "Pure" chemical compounds, which may not meet the criteria required for a primary standard, and may not be able to be characterized by reference to a primary standard, but for which other criteria of purity have been established (e.g., physical properties; C, H, N, etc., elementary analysis; group analysis; bioassay, etc.)

(3) Radioactive tracer substances

(4) "Standard sera" (Section 3.1.1.1)

These standard materials are mostly used in protein-free aqueous solution, either to monitor the total performance of the series of steps that often together comprise a clinical chemical method, or to provide a standard solely for the final measurement (e.g., in a process that finishes with a colorimetric reaction), or their principal use may be found during the initial stages of validation of a method, as internal standards, when assessing the specificity, accuracy, recovery factors, and precision of the technique that is under evaluation. If these standards are to be carried through all the steps of a procedure as part of the regular technique for performing that test, they should be prepared in a solution that mimics as closely as possible the specimens to be analyzed: this means that, for many methods, the standards should ideally be dissolved in blood, plasma, or serum or a closely similar protein-containing solution; if this is not done, many discrepancies may pass undetected, for instance in the day-to-day performance of the deproteinization stages. Records are kept of the findings (instrument readings, etc.) obtained for these standards in their daily use. Among the "pure" chemical substances that have found a special place as standards in clinical chemistry, more for evaluating methods than for use in routine day-to-day operation, are compounds such as the steroids available on special request from the Medical Research Council Steroid Reference Collection. These steroids provide good examples of compounds that demand special skills for their preparation, and cannot yet be obtained in sufficient quantity for inclusion as standards every time the naturally occurring compounds are estimated. Other reference materials (not true secondary standards) may be required in the day-to-day performance of the relevant methods—such reference materials may not be identical with the substance being estimated, but must have sufficiently similar chemical or physical properties to act as standards in the final measuring procedure (e.g., methyl red

used as a calibration standard for bilirubin determinations). Radioactive tracer materials have proved particularly valuable in the initial validation of some clinical chemical procedures, but little use has been made of these for routine investigational purposes.

The control materials used in clinical chemistry include simple aqueous solutions of single compounds or mixtures of compounds (e.g., W13), and a wide variety of commercial protein-containing preparations (B6). For analyses of plasma or serum, protein-containing controls should be included among the specimens analyzed and these samples will be referred to as control sera. Under certain conditions, control sera can be used instead as standard preparations, and for some purposes there is no satisfactory alternative to "standard sera" for this (Sections 3.1.1.1 and 3.1.1.3).

Control sera can be prepared in several different ways, but the methods are mostly based on the collection of large volumes of human or animal serum. These pools of sera may or may not receive further treatment before being distributed into small containers and refrigerated or lyophilized for storage purposes. Some laboratories prepare their own pools, but others make use of one or more of the commercially available preparations. The further treatments that control sera may undergo during their preparation include dialysis for the removal of smaller molecular weight constituents followed by the addition of weighed quantities of some of the constituents removed by dialysis, using for this replacement chemicals of known degrees of purity; for these dialyzable constituents, such control sera begin to meet the requirements of primary or secondary standards, depending on the properties of the pure chemical that is available for addition. For the preparation of control sera with artificially elevated amounts of various analyzable constituents, either dialyzed or undialyzed serum pools can be used as solvent, and to these are added chemicals or suitable biological preparations (e.g., for the assessment of serum enzyme determinations): these "reinforced" control sera are then distributed into small containers, analyzed, and prepared for storage.

Even with control sera in which some of the constituents have been removed by dialysis or ultrafiltration and then returned as weighed amounts of pure material, it is essential to check the composition of the final product by the appropriate methods of chemical analysis, unless the use to which the control serum is to be put is distinctly circumscribed (e.g., Section 3.1.1.2). Laboratories that prepare their own control sera establish the "true" analytical values, using the methods they employ, by performing a sufficient number (usually about 20) of replicate determinations under conditions that meet the definition of repeatability.

Some commercially prepared sera are investigated similarly by the manufacturers, but others are submitted to a limited number of laboratories which have agreed to undertake such reference work; in all cases it is desirable for the information accompanying commercially prepared sera to state the methods of analysis used by the reference laboratories, and this is essential for enzyme activity determinations.

When the composition of control sera has been determined, checks must be kept on their subsequent stability, and great care must be exercised in reconstituting them prior to analysis. Control sera, etc., that have been carefully analyzed by the best available methods can be used as secondary standards, but if used in this way other specimens of serum, etc., must be used for control purposes. As commonly used, the main value of control sera is for monitoring the reproducibility of clinical chemical determinations and, for this purpose, they have major advantages over simple aqueous control materials, since the aqueous solutions can easily be recognized by laboratory workers to be different from the other specimens they have been called upon to analyze. The principal advantages of aqueous solutions for control purposes have been stressed by Grant (G5) and include, apart from their cheapness, the relative ease with which they can be prepared in a standardized form and the simplicity thereafter of varying the concentrations of their constituents in the specimens submitted for analysis. The concentration of lyophilized control sera, however, can similarly be varied at the time of reconstitution (T1).

With the AutoAnalyzer, apart from the special requirements of multichannel analysis (Section 3.1.1.3), the confusion between standards and controls has become practically inextricable. The following quotation (L2) provides a good example: "Error control. Known standards accompany samples as controls, interspersed with the "unknown" samples at periodic intervals. In their travel through the system both are subjected to exactly the same conditions from beginning to end, and the standards act as controls in the final reading."

It is true that the AutoAnalyzer sample probe cannot differentiate in its action between a standard, a control, and a patient's specimen, and it might be argued that no observer bias will enter into the reading and recording of the peaks on the chart record. The AutoAnalyzer standard curve itself, however, cannot be used for control purposes unless, as with manual methods, it clearly reveals that the overall system is functioning so imperfectly (e.g., showing such loss of sensitivity) that analyses cannot be interpreted. If, as an independent check of the instrument's performance, one of the standards is itself subsequently analyzed as a true control solution, this will at most serve to show that the within-batch

repeatability with the AutoAnalyzer is proving satisfactory, which should be the case if a system of correction for the control of drift (Section 5.4.3) has been properly instituted. A different aqueous solution (i.e., with composition different from any of the standards) is little better as a control, since it can only serve to check the reliability of interpolation calculations between the appropriate pair of standards.

It is, in fact, necessary to control the operation of the AutoAnalyzer in a manner very similar to the control of the corresponding nonautomated determination; the only difference, in a long run of analyses, might be in the number of controls required. This applies both to automated methods requiring dialysis and to determinations performed on the protein-containing outlet from the dialyzer module. No special comment is needed about the analyses carried out in protein-containing solution, but with the analyses performed on the dialyzate it should be recalled that Skeggs (S4) himself reported that urea and glucose dialyzed at a different rate from whole blood and from water, and both Skeggs (S4) and Marsh *et al.* (M4) were unable satisfactorily to explain the difference at high levels (over 100 mg of blood urea nitrogen/100 ml) between urea determinations performed in a protein-free medium by the diacetyl monoxime method on the AutoAnalyzer and results obtained by urease methods.

The difference between the performance of the AutoAnalyzer when aspirating simple aqueous solutions or viscous samples of plasma, serum, etc., should not be exaggerated, since they must in themselves rarely affect the reliability of the final result. These differences do exist, however, and are sufficient to show themselves in detectable differences between the shapes of peaks of corresponding height for these different types of sample (best seen with sampling rates of 40/hour and below); it is an oversimplification to describe all acceptable AutoAnalyzer peaks as parabolic (B4), even for simple aqueous solutions. Although these differences in behavior occur, and continue unexplained, it is clearly preferable to control the operation of AutoAnalyzers with specimens closely related in physical and chemical properties to the samples also to be analyzed.

3.1.1.1. *Biological Materials Used as Standards.* For some analyses in clinical chemistry, pooled collections of serum or urine offer the only readily available standard materials, and for this purpose their content of these substances is first determined as accurately as possible. The composition of the biological material to be used as a standard is obtained by means of reference methods, but practical considerations thereafter prevent the daily checking of the standard serum, urine, etc., against these reference procedures. For the monitoring of reagent and instru-

mental performance, therefore, the readings obtained with these biological standards are recorded daily and successive readings compared. For control purposes, however, a different specimen must be used.

Examples of possible uses of biological materials (pooled collections of serum, urine, etc.) as standards are in methods for estimating total protein concentration in serum, for measuring enzyme activities in serum and other body fluids, and for determining urinary steroids and their metabolites. The special requirements of multichannel analysis will be considered separately (Section 3.1.1.3), and the reasons why these three different investigations can perhaps best be standardized in relation to biological materials will now be discussed.

Although highly purified bovine serum albumin is commercially available, it does not react in exactly the same manner as all the components of human serum when either the biuret reagent or one of a number of protein-binding dyes is used for the colorimetric determination of serum protein. On the other hand, it provides an acceptable standard material for protein nitrogen determinations by the Kjeldahl technique, although even here it has to be assumed that the different proteins in human serum have the same percentage of nitrogen in their composition as does bovine serum albumin. The uniform application of the conversion factor of 6.25 for converting protein nitrogen values in serum to the equivalent content of total protein has recently been questioned (C4, S12). Because of these various criticisms, a pool of human serum is to be preferred for standardizing serum protein determinations.

With enzyme determinations, standards for calibration purposes and for checking of instrumental performance make use of separately prepared solutions of one of the reactants or the products of the enzyme-catalyzed reaction. For instance, a solution of phenol may be standardized and thereafter used itself as the standard in determinations of acid and alkaline phosphatase, in methods employing phenyl phosphate as substrate and depending on the measurement of the amount of phenol liberated. This standard phenol solution, however, cannot be taken through all the steps in the determination of phosphatase, and a separate control solution must be used to check the performance of the overall technique; if this control were omitted, it would be possible, for instance, for a buffer to be incorrectly prepared and for erroneous levels of phosphatase activity to be found. There is no substitute, therefore, in the control of enzyme determinations for the inclusion of a sample (of serum, urine, etc.) previously investigated for its level of enzyme activity. For long-term monitoring of an enzyme method, the repeated analysis of aliquots of a "standardized" enzyme preparation is most useful, provided

the enzyme is itself stable under the conditions of storage (H8); alternatively, comparisons of methodological performance can be made with unstandardized preparations by including in each new batch of analyses one or more samples that were analyzed among the previous batch of specimens, and repeating the determinations on these samples. These samples from patients, used for interbatch comparisons of analytical performance, are variously called "carry-over" specimens, "scout" samples, and "repeat analysis" and "between-batch" specimens; their use has been much wider than merely in the field of clinical enzymology.

In the third example, dehydroepiandrosterone is widely used for standardizing and for checking the performance of the Zimmermann reaction, which forms the final step in many methods of steroid estimation. There is, however, no suitable standard preparation including the commoner urinary steroids and their metabolites which can be carried through all the previous stages of analysis, and the only satisfactory form of standard material that includes the naturally occurring conjugates of the main steroids and their metabolites is urine collected from normal individuals or from patients treated with cortisol.

3.1.1.2. *Bench Standards.* Every quantitative method in clinical chemistry includes (1) a blank, (2) a pure standard, and (3) for enzyme methods a control determination is also required. In controlling the quality of analytical performance in a laboratory from day to day, it is important to record these basic data in order to detect, for instance, whether deterioration in the standard preparation might account for an unacceptable finding in the control specimens. The technician's record should also include a note of the dates on which new reagents were prepared, and preferably the performance of each new set of reagents should be compared with the older set of reagents before the latter become exhausted, using for this comparison observations of blank and standard values as well as the performance on some specimens from patients for whom sufficient material is available to permit the comparison. A rise in the blank reading may similarly reveal reagent deterioration, and this information is lost if the practice of setting the zero of a colorimeter, etc., on the blank solution is followed.

Having stressed the importance of control sera as distinct from simple aqueous control preparations, it is strongly recommended that the staff responsible for the day-to-day performance of analyses on patients should in addition be provided with their own reliable means of assessing whether all stages of their techniques are probably working satisfactorily. For this purpose, each analyst should have available to him a pool of sera which may be called the bench standard. Although the true composition of this need not be determined, the analyst responsible for the par-

ticular technique, by including a specimen of this bench standard with each batch of analyses, rapidly acquires for himself an assessment of the repeatability with which he is performing the method. As indicated in the section on definitions, the repeatability obtained by the analyst can be expected to be better than the reproducibility of his technique, and the use of the bench standard will not therefore prevent the furnishing of an unacceptable result for the analysis of a control serum, but the advantage of providing the bench standard for regular inclusion in each batch of analyses is that it enables the analyst himself to test the overall procedure, and to be the first to alert himself on many of the occasions when a batch of analyses may require repetition, e.g., because of faulty preparation of a reagent, assuming his recording of results is not subject to observer bias.

In the authors' laboratory, bench standards are made available for many of the techniques, and this has helped greatly to reduce the resentment and suspicion which might otherwise have been experienced if the only nonpatient sera in regular use had been control sera. In addition to providing the staff themselves with this additional check on the quality of their work, the bench standards and the control sera serve as interdependent checks on their stability as stored in the department. When checking the long-term performance of a method, therefore, the technician can compare the results obtained with his bench standards as well as the readings for blanks and pure standards, and the quality control supervisor can evaluate these data as well as the results for the true control samples when the performance of a method goes out of control. Bench standards can conveniently be provided for many determinations by pooling the residues of plasma and serum specimens obtained from patients, after analyses have been completed, if the composition of large volumes of these pooled residues for stable constituents is as constant as has been suggested (S8, W14).

3.1.1.3. *The Special Requirements of Multichannel Analysis.* Most single-channel and limited multichannel (i.e., including the 4-channel electrolyte analyzer) methods of analysis using the AutoAnalyzer depend on simple aqueous standards for calibration purposes. However, with the development of the 8-channel sequential analyzer (S5) and the subsequent extension of these multiple systems of analysis to 12-channel operation (W5), the difficulties inherent in the preparation of a suitable artificial standard became formidable. The first Technicon SMA-12 instrument to be introduced performed the following analyses sequentially on serum: sodium, potassium, chloride, bicarbonate, total protein, albumin, calcium, alkaline phosphatase, bilirubin, urea nitrogen, sugar, and aspartate aminotransferase (GOT); a second model has since been developed that substitutes uric acid, inorganic phosphate, cholesterol, and

lactate dehydrogenase for the first four determinations in the previous list.

Consideration of the analyses performed on the 8-channel and both models of the 12-channel Technicon equipment in relation to the earlier discussion means that these latest developments in the field of Auto-Analyzer instrumentation demand standardized sera for calibration purposes. In fact, the successful operation of the SMA-12 instrument is entirely dependent upon the careful analysis and subsequent stability of the standardizing serum, since this is used both for the initial calibration of the various analytical channels and for the subsequent monitoring for drift and application of any correction needed as a result of instrumental drift. Apart from this large demand for standardizing serum (about 70 ml in an 8-hour day), the performance of the SMA-12 should in addition be checked by means of control sera, as for any other method or combination of methods in clinical chemistry. The expense of the standardizing (and to a lesser extent the control) sera used in SMA-12 operation constitutes an important but nevertheless essential fraction of the operating costs of these instruments.

3.1.1.4. *The Use of Radioactive Isotopes as Internal Standards.* The measurements of individual steroids in blood and urine are good examples of lengthy assay techniques that are usually carried out in small batches. The addition of separate quality control samples can apprcciably reduce the number of determinations that can be carried out. Furthermore, the recovery factors in these long techniques may be such that, at best, only 70–90% of the original steroid is finally assayed; this applies, for instance, to urinary estrogen and pregnanediol assays. Many factors can influence this overall recovery, and these may vary considerably among the individual specimens in a batch. The recovery factor for each sample can be measured, and each specimen provided with its own internal standard for quality control purposes, by addition to the original specimens of known small quantities of the compound to be assayed, using isotopically labeled and radiochemically pure materials for this purpose. At the end of the procedure, an isotope measurement is carried out on a portion of the final product from each specimen and the overall loss is calculated. Results can then be individually corrected for any losses which may have occurred (B3), and the use of modern counting equipment reduces the extra work involved to a minimum.

3.1.2. *The Importance of Stability in Standard and Control Preparations*

It might seem superfluous to have to stress the importance of investigating and subsequently checking the stability of standard and control preparations, but many of the compounds assayed in clinical chemistry

are unstable, even as solids, unless carefully stored (e.g., the nicotinamide-containing coenzymes), and the methods of collection of serum, urine, etc., for preparation of pools for control or standardizing purposes can only with difficulty be kept free from the dangers of bacterial contamination. In the complex mixtures represented by serum, urine, etc., many compounds are stable if specimens are stored in a freezer (—20°C), but lyophilization may be necessary in other instances (e.g., for preservation of some enzymes), and the most satisfactory conditions of storage for each preparation have to be determined. In addition, for specimens stored by refrigeration or by freezing, it is essential that each sample be fully restored to room temperature and properly mixed before being subjected to analysis, and lyophilized specimens must also be carefully reconstituted.

If proper care is not taken for the storage and subsequent preparation of control sera for analysis, confidence of laboratory staff in the potential value of a quality control program can very rapidly be lost. If standards deteriorate, this fact is more readily accepted. With the earlier individual and commercial preparations of control sera, insufficient attention may have been paid to the need for ensuring their stability, and this may have led Grant (G5) to stress the disadvantages of serum pools and extol the advantages of aqueous solutions for control purposes. From our experience, we strongly disagree with Grant's conclusion that the use of pooled serum for all analyses on serum, apart from enzymes stable in the freezer, is "probably more trouble than it is worth," and we shall throughout the review stress the advantages of using controls, which as nearly as possible can pass unrecognized through the same analytical steps as specimens from patients, and contain the same types (known and unknown) of interfering factors as do the patients' samples. The reproducibility of each technique, following its introduction into routine use, is of major interest to clinicians, and aqueous solutions cannot be used to assess this.

3.1.3. *The Introduction of Control Samples into the Laboratory for Routine Analysis*

In a small laboratory, carrying out relatively few determinations of any one kind, the staff probably identify each patient by name in their notebooks or on their work sheets, and may well undertake the preparation of each specimen (separation of plasma, serum, etc.) for analysis themselves. As the workload grows, in terms of both numbers of analyses and range of determinations undertaken by the laboratory, the advantages of identifying each specimen by name diminish and centralization of sample preparation becomes increasingly worthwhile. In this depart-

ment, most samples are now prepared for analysis in a centrally placed area, and specimens are identified throughout the laboratory by means of an accession number (W4) (Section 5.2.2). The preparation area is manned by technical staff, on a rota, and these staff perform no analytical work during their time at the sample-preparation bench.

Although it might be felt that this system of centralized sample processing and identification of specimens solely by accession number would be unacceptable (by removing the personal element of laboratory work which could arise from dealing with named specimens), this has not been found to be true and the present system has distinct advantages for the quality control program. Because of the volume of work currently handled by the laboratory, one of the senior members of technical staff has as a major responsibility the task of inserting control sera among the incoming specimens, at irregular intervals during the day. These control sera are handled by the technical staff exactly as if they were samples from patients, and they only come to realize the true nature of these control specimens when they are entering the results of their analyses on the record cards at the end of the analysis and after completion of any consequential calculations.

The use of anonymous control sera will be referred to as fictitious patient controls, since the staff are given no indication that they differ in any way from other samples in a batch. Even with AutoAnalyzers it is possible to include bias in the handling of samples known to be particularly important (e.g., care in the correct identification and reading of the relevant peak on a chart record), and this danger of bias influencing the results for control sera applies much more to manually performed methods. Fictitious patient controls overcome this tendency to bias, but it would be much more difficult to introduce them with equal certainty of anonymity into a laboratory with a small workload.

Many different types of control serum can be used for the purpose of fictitious patient controls. Depending on the method being evaluated, and the purpose of the control assessments, the authors have at various times used locally prepared pooled sera, commercial sera, and carry-over specimens from patients, and these together constitute the main present objective assessment of the quality of work that is being reported from this laboratory. When the results for the fictitious patient controls have been obtained, these are collected by the senior technician referred to above, recorded, charted, and analyzed (Section 3.3).

3.1.4. *Interlaboratory Comparisons*

The results of interlaboratory comparisons of assays on identical specimens have been mentioned (Section 1.2). These trials involve con-

siderable efforts of organization and are undertaken at wide and irregular intervals. They cannot be regarded as forming part of a laboratory's routine program of quality control. On the other hand, the concept of regular interlaboratory comparisons of analytical results is a valuable one and, following publication of the most recent British assessment of interlaboratory performance of urea, sodium, potassium, and inorganic phosphate determinations (G4), an attempt has been made to initiate a program of nationwide pairing of hospital chemical laboratories. The arrangement suggested is that each laboratory should pair with another department in the vicinity for the regular interchange of specimens that have first been examined in the donor department for an agreed number of constituents (W7a). The specimens are then to be analyzed again in the receiving laboratory and the results compared. In each department, specimens taking part in the interchange should if possible not receive preferential treatment (ideally they should be treated as anonymously as fictitious patient controls), and the results of the paired analyses are then compared. With many departments already overworked, this suggested interchange scheme is unfortunately being only slowly adopted, but it clearly offers an additional regular means for testing the quality of a laboratory's work, by checking its results against the findings of an independent department.

When the paired departments use fundamentally different methods of analysis, as is possible with serum enzyme determinations, for instance, the comparisons may be of only limited value. However, many laboratories now use AutoAnalyzer techniques for 60–80% of their total work, and a start on regular interlaboratory comparisons of these methods should be worthwhile and involve relatively little additional effort. Although the AutoAnalyzer methods used in the different laboratories might themselves show some differences in detail, the information provided by these comparisons should serve to detect in one laboratory the development of some systematic error in the routine performance of a method at an earlier stage than if the laboratory had itself been solely responsible for its assessment of results of quality control programs.

3.1.5. *Summary of Standards and Controls*

3.1.5.1. *Standards*

(a) *Primary standards and pure chemicals:* These form an essential part of each quantitative method in clinical chemistry, and are particularly important in the initial validation of a new technique, in assessing recovery factors, etc.

(b) *Biological materials (standardized sera, urine, etc.)*: These are

classed as secondary standards and have the advantage that they can be taken right through all the stages of a method in the same way as the specimens from patients; they should be used regularly in addition to primary standards and pure chemicals.

3.1.5.2. *Controls*

(a) *Aqueous preparations of pure substances:* These are cheap and easy to prepare but can be singled out for special treatment by an analyst, cannot as a rule be taken through all stages of a method in the same way as specimens from patients, and are not available for all determinations.

(b) *Biological materials (pooled sera, urine, etc.)*: These are available in various commercial forms and can also be prepared locally. Their use as a routine part of each laboratory's quality control program is strongly advocated. They can be used in several ways (e.g., fictitious patient specimens, carry-over specimens, etc.), and their introduction into regular use does not have to involve major expenditure.

(c) *Interlaboratory comparisons:* The frequent interchange of specimens between laboratories for cross-checking of analyses is advocated; specimens from patients will meet local requirements for interchange. National comparisons require special arrangements, and cannot yet be regarded as an important part of a laboratory's control program.

3.2. The Importance of the Initial Determination of Accuracy and Precision

Before introduction into routine use for diagnostic purposes, clinical chemical methods require validation, and the laboratory should wherever possible acquire for its methods statistical data on which subsequently to base opinions about the significance or otherwise of day-by-day alterations in results obtained on specimens from patients for these investigations. The importance of checking the performance of a published method and the dangers of accepting the published account without such local evaluation have been stressed elsewhere (e.g., G5, H7). Henry (H7) also makes a special point of emphasizing the dangers inherent in the use of "precalibrated" photometers.

The initial validation of a method depends principally on the use of pure standard materials, but the performance of the technique has then to be assessed also on the type of sample to which it is to be applied. For instance, with chromatographic methods applied to urine, the importance of the initial extraction and purification procedures may not be apparent from assessments of a method conducted solely on the basis of the analysis of mixtures of pure compounds.

3.2.1. *The Initial Assessment of Accuracy*

The accuracy of a method can be assessed by comparing results using the method under evaluation with results obtained by the most specific method available (e.g., chloride analyses, C8). Some of the best examples of potentially specific methods of analysis are techniques based on the use of enzymes, e.g., uricase for uric acid, urease for urea, and glucose oxidase for glucose measurements; many laboratories use these enzymes as analytical tools for routine purposes. If the enzyme is absolutely specific and is neither activated nor inhibited by the wide range of substances encountered in biological specimens, then the results of an enzyme-dependent determination have the ability to approach the true values for that constituent. Unfortunately, interfering substances are present in most biological materials, and the accuracy of each method can then best be assessed by means of recovery experiments. In addition, the claims that the methods are specific need to be based on extensive studies of the substrate requirements of the enzyme; these have not been performed for many of the enzymes which have been used as analytical tools in clinical chemistry.

The determination of enzyme activity for diagnostic purposes presents a different problem. No estimates of accuracy can be made except where pure preparations of enzymes are available, so that at present the accuracy of most clinical enzyme assays can be expressed only in terms of other methods which are believed, on theoretical grounds, to provide a valid basis for determination of activity.

The procedures for initially assessing a method solely by the use of pure solutions will not be detailed (e.g., whether or not a colorimetric reaction obeys Beer's law, and if so over what range of concentrations), but the principles of recovery experiments will be briefly discussed. In these, the mean figure for a number of replicate determinations of the compound in a pooled specimen of serum or urine, etc., is obtained, under conditions meeting the criteria for assessing repeatability; this mean result is then compared with the means obtained for other sets of similar determinations on samples of the same pooled specimen in which have been dissolved accurately weighed quantities of the pure compound under investigation. In some instances, the basal pooled preparation is dialyzed to remove its endogenous content of the compound, or to reduce its content to a low level, before dissolving weighed amounts of pure substance in the "natural" medium; this applies, for instance, to the commercially available Versatol preparations (General Diagnostics Division, Warner-Chilcott Laboratories, Morris Plains, New Jersey).

For assessments of accuracy based on recovery experiments, pools of dialyzed or ion exchange-treated material, etc., to which known amounts of pure compound have been added, are particularly valuable, since the residual content of the basal pool may be so small as to be negligible by comparison with the levels attained in the reconstituted sample; in any assessment of accuracy on the basis of recovery experiments it is preferable to know the difference between two mean values, one of which is high and the other of negligible proportions (e.g., 140 and 5 mEq/liter for serum sodium) rather than the difference between two means of comparable magnitude (e.g., 170 and 140 mEq/liter for serum sodium), and this principle is said to be applicable to Versatol preparations, at least for those components that dialyze freely and are also available as pure compounds.

Although assessments of accuracy by recovery experiments must have been widely performed by clinical chemists, data from such studies are not often published. Descriptions of accuracy studies include investigations on the validation of sodium and potassium (C1, S7) and chloride and urea (C1) methods, in which Versatol preparations were used; Campbell and Annan (C1) also carried out recovery experiments for these determinations and for bicarbonate measurements, using pooled sera prepared and analyzed in the laboratory. MacIntyre *et al.* (A1) passed plasma and urine specimens through a cation-exchange resin to obtain pools with low magnesium content before estimating recoveries of added magnesium, whereas Thin and Thomson (T3) based their assessments of accuracy of magnesium and calcium determinations on the difference between (a) the mean analytical results for pooled serum and urine specimens, and (b) the values found on repeating these analyses on samples which had been diluted with calcium- or magnesium-containing fluids of known composition instead of being diluted solely with 0.1 *N* hydrochloric acid. It is to be hoped that data on the recoveries obtainable with new analytical methods will be published more regularly in the future.

The initial assessment of the accuracy of a new method should also include a detailed investigation of its specificity to determine the possible effects of interference from other naturally occurring compounds or from drugs and their metabolites; it is possible for two methods, one with a lower degree of specificity than the other, to provide results that have comparable levels of accuracy. For instance, MacIntyre (M1) showed that the differences between the flame spectrophotometric method and the less specific Kramer-Tisdall (K2) method in the determination of calcium were less than might have been predicted; this occurred because losses of calcium revealed by the use of ^{45}Ca in the assessment of the latter

technique were partially offset by coprecipitation of magnesium as its oxalate, and this magnesium oxalate could not then be distinguished from calcium oxalate in the final titration with potassium permanganate.

3.2.2. *The Initial Assessment of Precision*

The assessment of the precision of a method before its introduction into routine use requires a measurement of its repeatability, but the reproducibility cannot be determined until the method has itself been in routine operation for a sufficient period of time (see Section 2 for definitions). Most published descriptions of techniques provide data about their repeatability when referring to precision, but there are some published figures specifically for reproducibility (e.g., C1, S7, T2). Having decided that a method is sufficiently reliable (on the basis of the SD for repeatability) to be introduced into routine practice, the SD for its reproducibility should be obtained as soon as possible, for instance by calculating the SD for the results of analyses on control sera included in the first 20 batches of determinations; this SD reflects the between-batch variation. Subsequent monitoring of the method should then be related to this first value for reproducibility, if it compares favorably with the SD for repeatability.

Any gross discrepancy between the values obtained for the reproducibility of a technique and its repeatability may result from a lack of proper training of the staff who have been performing the routine determinations, or to the pressure of routine work, change in apparatus, reagents, etc. It is therefore valuable to know the repeatability of a method, since this gives a criterion against which to measure the subsequent figures for reproducibility. The figures for repeatability can be used for the preparation of control charts (Section 3.3.1), and subsequent experience of the method, during which the SD for reproducibility is obtained, will show whether the limits drawn on the chart from the initial assessment of repeatability require modification.

3.2.3. *The Degree of Accuracy and Precision Required in Clinical Chemistry*

The findings of interlaboratory surveys of accuracy have highlighted the need for improvements in reliability of day-to-day work. Where estimates of precision have been obtained as part of these surveys, shortcomings have again been revealed, but not as glaring as the variable standards of accuracy. Although it is desirable that each method introduced into routine diagnostic work should as far as possible be both accurate and specific (i.e., should be capable of measuring what it purports to measure), assuming that reasonable standards have been defined

for each method in these two respects it is also necessary to consider the degree of precision, especially reproducibility, which should thereafter obtain in practice. This is a difficult problem to solve, since several factors have to be taken into account; the limits for the reproducibility of one method may differ markedly from the limits required for another, and the limits to be observed for a single method may differ for results that fall in the physiological as distinct from the grossly pathological range.

In determining the standards of precision that the results of a chemical investigation must meet in routine use, it is important not to introduce a method so demanding in terms of the technical skill required for its performance, or so long and complicated, that the staff either cannot regularly undertake its performance, or else can obtain results only at a rate unacceptably slow for clinical purposes. On the other hand, it is unwise to adopt the attitude that the results furnished by the laboratory are "sufficiently accurate and reproducible for clinical purposes," since the standards of reproducibility required by clinical work have not yet been clearly defined, and are in any case likely to become more demanding with the growing interest in early recognition of disease and the detection of small but significant changes in chemical tests carried out at different times on the same individual. There will therefore be a continuing need to keep under particularly close review the standards of reproducibility for a laboratory's methods, whereas accuracy and specificity come up for fresh consideration each time it is decided to introduce a new technique.

Differences in the requirements for reproducibility of methods can best be illustrated by examples. Bilirubin, for instance, may be present in serum at concentrations ranging from 0.1 to 30 mg/100 ml. Reasonable 95% confidence limits for a serum bilirubin level of 1.0 mg/100 ml might be $\pm$0.15 mg/100 ml, but this degree of precision would certainly not be required at concentrations of 15 mg/100 ml and above. In this example, it is common practice to use different methods for investigating these two ranges of concentration and, for the lower levels (assuming that the SD increases proportionally with bilirubin content), the most valuable single figure for reproducibility of the method adopted would be the coefficient of variation (CV); this coefficient would indicate in this particular example that the 95% confidence limits for serum bilirubin in a jaundiced patient with a level of 10 mg of bilirubin/100 ml were $\pm$1.5 mg/100 ml. It is important to emphasize that a different range of confidence limits applies to the methods used at serum bilirubin levels in the 15–25 mg/100 ml range, and decisions are taken on minor alterations in the region of 20 mg/100 ml whether to initiate exchange transfusion in neonatal hyperbilirubinemia. With serum calcium determinations, a 95% con-

fidence limit of ±1.5 mg/100 ml at a level of 10 mg/100 ml would be totally unacceptable, since the clinical requirements for the reproducibility of this analysis are much more demanding; Willis (W8), for instance, obtained a figure of ±0.20 mg/100 ml and this degree of precision, which is justifiably required on clinical grounds, has since been obtained by other workers (S11, T3).

Tonks (T4) has attempted to define the allowable limits of laboratory error in terms of the empirical formula

$$\text{Allowable limits of error (in \%)} = \pm \frac{\text{1/4 of the normal range}}{\text{Mean of the normal range}} \times 100\%$$

and set the maximum limits for any determination at ±10%; similar maximum limits were set by Wootton (W11), although for some methods the limits were much narrower. Sparapani and Berry (S9) have compared the values obtained by applying the above formula with the CV obtained from calculations of the range of normal values obtained as part of their quality control program for several different analyses. Hendry (H3), on the other hand, has put forward limits for the "clinically acceptable errors" of laboratory work, some of which exceed (e.g., serum uric acid) the limits of ±10% set by Tonks (T4). Benenson *et al.* (B1a) based their suggestions for allowable errors on the precision of analyses conducted in the laboratory; since their formula for allowable errors accepted a scatter of ±3 SD from the mean of repeated analyses, on a percentage basis some of the acceptable limits proposed by these workers greatly exceeded ±10%.

It is not possible to comment in detail on these and other similar attempts to define quantitative limits, particularly for the precision, of work in clinical chemistry laboratories, except to say that at present the limits suggested are in general far too lax; this fact was demonstrated, for instance, by calculations made by Campbell and Annan (C1) in the case of plasma sodium values, and acceptance of these limits for serum calcium determinations would be even more fraught with danger as far as patients are concerned. In the authors' opinion, it is too early to define "allowable limits" of error in clinical chemistry, and they suggest that such limits cannot be properly delineated in the present imperfect state of medical knowledge. The only other point they wish to stress is that relaxation of any limits eventually established for quantitative chemical investigations, carried out as part of screening surveys, would probably be most unwise.

We forecast, therefore, that improvements in the quality of standard materials, reagents, analytical methods, instrumentation, data-processing techniques, and control programs will individually and collectively con-

tribute to the better reliability of clinical chemistry, but the attempts along these various lines to achieve improved reliability must always bear in mind the practicability of the resulting method, i.e., its ability to provide reliable results sufficiently quickly in routine operation to be of assistance in influencing the clinical assessment of patients.

3.3. Statistical Analysis

Before a technique is adopted for routine use in any laboratory its accuracy and reproducibility must be investigated. Thereafter the system of laboratory quality control must ensure that the initial criteria for reliability are being maintained, and provide practical proof that each batch of measurements is satisfactory before results obtained on patients' specimens are reported. A decision in each case is needed about the number of controls to be included in each batch of assays, and about the limits to be set for control purposes. These limits are usually expressed in terms of "95% confidence limits" (± 2 SD), meaning that 95% of a sufficiently large number of results will lie within the limits and 5% will fall outside them. If only one control is placed in a batch, 5% of such controls in any sequence of batches can normally be expected to fall outside these limits, and on average 5% of the batches will have to be carefully investigated or repeated for this reason alone. Too many controls are of course wasteful and an optimum number must be chosen. A determining factor in this choice is the amount of variation in standards of performance which might be expected for a particular technique; for instance, with fully automated methods, large random errors are unlikely to occur, and if one control sample is satisfactorily analyzed the rest of the batch is much more likely to be of acceptable standard than with techniques more dependent on human intervention or participation. Consequently, as a first generalization, the greater the degree of automation the smaller will be the number of controls required.

The small number of controls possible to assay conveniently in each batch will at best, when studied in isolation, only supply information that no serious change has occurred. More insidious changes will become evident only by studying results obtained from controls analyzed in successive batches. These insidious changes are best revealed by the maintenance of quality control charts giving a visual display of any changes that have taken place.

Whenever possible, therefore, it is advisable to run duplicate controls, to investigate the variability between readings in a single run (the within-batch error); comparison of the readings for these controls obtained in different runs will give information about the between-batch error.

3.3.1. *Control Charts*

The use of control charts in clinical chemistry has assumed various forms, and selection of types of chart for discussion in this section does not imply that they necessarily all have advantages over alternative forms of charting practiced in other laboratories. It is possible, however, to make a general recommendation to the effect that the graphic presentation of control data is easier to appreciate at a glance (with the consequent implication that the need for action in respect of poor performance is recognized earlier) than is the recording of control data restricted to a series of figures in a notebook.

Levey and Jennings (L3) first suggested the use of control charts in the clinical laboratory. These charts had previously proved their value in other branches of chemistry and industry generally, and since 1950 have become widely used in clinical chemistry. Different methods of preparing the most suitable data for inclusion on these charts have been discussed, as well as methods for setting action limits (G3, G5, H6, H9, L3). Of these, only the "number plus" method (H9) has been shown to be basically unsound (V1), but the shortcomings suggested by these latter authors in the other laboratory control methods they discussed can be overcome if control specimens are introduced into the laboratory's workload (as recommended in Section 3.1.3). Many laboratories have now accumulated experience over several years of the value of control charts, and the present authors cannot disagree too strongly with the conclusion of van Peenen and Lindberg (V1), "The best method presently available for detecting errors is comparison of reported laboratory values with the patients' clinical states." Instead, the regular use of control preparations with graphical presentation of results is held to be an essential part of the daily operation of a clinical chemistry laboratory.

To prepare a control chart for a method the following basic steps are needed:

(1) Investigate the method for its accuracy and practicability. If an acceptable standard of accuracy is attainable without making the method unduly slow or demanding in its performance, proceed to (2).

(2) Evaluate the repeatability of the method. This gives an initial figure for the SD of the determination under the best available working conditions. Repeatability can be obtained from analyzing the same specimen at least 20 times, but preferably from analyzing a similar number of different specimens in duplicate. The analyst should not know the way in which individual numbers of these duplicated determinations pair off, so that the possibility of any observer bias entering into the recording of colorimeter readings, etc., and the calculation of results is

reduced. In addition, the specimens should preferably span the range (physiological and pathological) over which the method is to be applied. If this latter course is adopted, the coefficient of variation (CV) may prove to be the more appropriate figure for plotting on control charts, or it may be decided to monitor the subsequent performance of the method with separate control charts, one for the physiological range and one for control samples chosen to assess the more grossly pathological ranges of results.

(3) Having decided whether the SD or CV is applicable in the particular case, a control chart is drawn (Fig. 1). The reference line

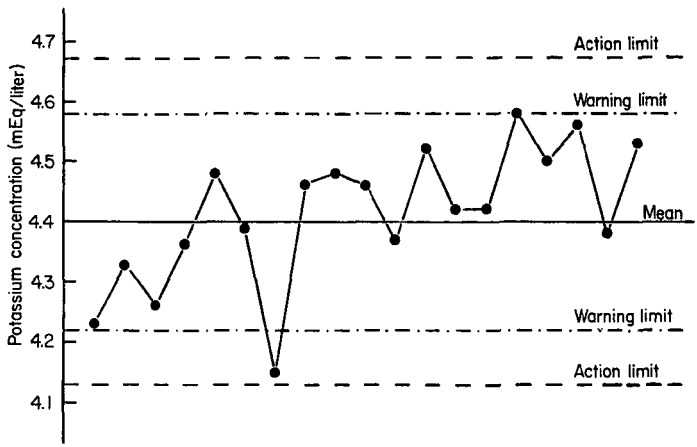


FIG. 1. A control chart showing the results of serum potassium determinations performed daily on the same control serum (previously determined mean value, 4.40 mEq/liter). The warning and action limits are set at ±2 SD and ±3 SD, respectively.

represents the accepted true value for the specimens that are to serve as controls. This may be plotted as zero, and subsequent observations for control preparations recorded as positive or negative values in respect of the amount by which they differ (in mg/100 ml, mEq/liter, etc.) from the true value. Alternatively, for those determinations in which it is desired to monitor on a single chart the performance of a technique using control sera that include values over a wide range (e.g., blood urea nitrogen covering the range 5–150 mg/100 ml), the reference value plotted can be the ratio (ideally, 1.00) between the observed and the "true" value for the particular control preparation.

(4) Lines are then drawn parallel to the reference line, above and below it. If the SD of the repeatability has been chosen for initial monitoring purposes, these lines are drawn at ±2 SD (95% confidence or

warning limits) and ±3 SD (action limits). Alternatively, if the ratio of observed to true concentration or activity has been chosen as the reference value, the lines setting limits for the determination can be based on arbitrary percentages, such as those given by Wootton (W11).

(5) Having drawn on the control chart the lines for the reference value, and warning and action limits (or other warning lines), as based on the results of repeatability experiments, the results subsequently obtained for control preparations are entered on the chart daily. These are plotted as differences (positive or negative) between the observed and the reference values or, for the ratio method of recording results, as the ratio between them. Individual points obtained on successive days or in succeeding batches of analyses are joined by lines on the chart.

(6) As these results accumulate, giving an indication of the reproducibility of the method, the need may arise to revise the warning and action limits, but drastic revision should not be undertaken until the source of any gross discrepancy between results for reproducibility and repeatability has been defined and, if possible, eliminated (e.g., by work simplification or further training of staff).

(7) When duplicate control sera are analyzed in a batch, it may be found advantageous (especially early in the use of a technique) to plot an additional control chart showing the difference between the duplicate samples (G5), plotting means on one and differences on the other. This chart will display the within-batch error more clearly than the standard chart that plots the mean of the duplicates and is of greater use for monitoring between-batch error.

The use of any control chart demands a knowledge of what is the probable limit of nonsignificant fluctuation; inevitable variability must be distinguished from that caused by the existence of trouble. No universal rules can be laid down with respect to when action is required to check the validity or to reject a batch of results outright (J1), but the two sets of limits on the chart (Fig. 1) act as guides. With the uneventful routine operation of a technique having a gaussian distribution of errors in the analysis of the control specimens, the warning limit will be exceeded for only 5% of the control results obtained and the action limit for 0.3%—if more than one control is used in a batch, different warning and action limits may be calculated from a special table (M11). If the frequency distribution curves for the errors of controls do not follow a normal distribution, however, the exact probability of a given error arising from chance cannot be deduced from a small number of readings (C5). Despite this difficulty, in practice, setting the action limit at ±3 SD remains satisfactory; even if the curve for the distribution of values about the mean for a large number of repeated analyses does not conform

to a normal distribution, it is likely that 19 of 20 findings for controls will be within the accepted range (S3), unless there is something wrong with the batch of analyses. When consecutive duplicates are used, the likelihood of the results for both these controls being outside the limit purely from chance will be remote during normal running.

Three methods of producing control charts are shown in Figs. 1–3,

Action		
	4.67	
Warning		
	4.58	
Mean	4.40	4.23, 4.33, 4.26, 4.36, 4.48, 4.39 4.46, 4.48, 4.46, 4.37, 4.52, 4.42, 4.42, 4.58, 4.50, 4.56, 4.38, 4.53
	4.22	
Warning		4.15
	4.13	
Action		

FIG. 2. A control chart showing in tabular form the data for serum potassium assays displayed graphically in Fig. 1. All figures are in mEq/liter.

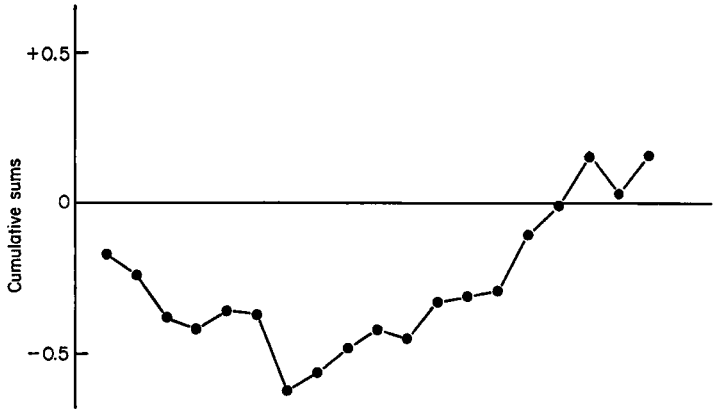


FIG. 3. Cumulative sum chart for the accumulated differences between the individual readings (shown in Figs. 1 and 2) for the potassium content of the control serum and the reference value of 4.40 mEq/liter. The cumulative sums are in mEq/liter.

where a series of results for potassium assays performed on a fictitious patient control serum have been recorded; the assumed true value for its potassium content is 4.40 mEq/liter. Figure 2 shows a numerical recording of the actual findings as a series of figures in a table which has ±2 SD and ±3 SD marked as the warning and action limits. It appears from

this that control has been maintained, although the action limit was almost reached on one occasion. Figure 1 shows a more widely used graphical method of presenting the same information; it is still not easy to detect any trend in the series of results, and inspection of this graph might also suggest that the technique was being maintained in satisfactory control.

3.3.2. *Cumulative Sum Charts*

An alternative method of plotting these same quality control data (Fig. 3) makes use of cumulative sum charts (W9). The preparation and advantages of these charts will first be described in some detail, since they have not yet been widely adopted in clinical chemistry. The basic calculation in their preparation involves subtracting an arbitrarily decided constant value (e.g., 4.40 mEq/liter for serum potassium estimations) from each figure in a series of similar observations (e.g., the daily average for plasma potassium results on patients), and accumulating the differences between each successive set of results and the constant value. The successive accumulated differences are termed the cumulative sums of the original series of results, and these values can be plotted graphically as a cumulative sum or cusum chart.

Cusum techniques have for many years proved valuable in industrial quality control and productivity, allowing the detection of movements away from a target level at much earlier dates than when charts are simply plotted of the basic observations from which cumulative sums are derived. These charts are not of value for displaying the results of a series that exhibits a steady trend; this characteristic is rarely maintained for long in clinical chemistry, however, and cusum charts can be employed in a hospital laboratory's quality control program for the following purposes:

(1) Detecting changes in the average level of a set of figures.

(2) Determining the point of onset of such changes.

(3) Obtaining a reliable estimate for the current average value of the set of figures relating to the method being monitored.

When plotting a cusum chart, the choice of reference value is important, since otherwise the slope of the chart is liable to be too steep for the early recognition of significant changes in slope, and the accumulated sums are liable to go off the limits of the chart inconveniently often. The initial reference value (k) is therefore best taken, in clinical chemistry, as the mean value obtained for the particular technique in the last ten or more batches of analyses. If x_1, x_2, etc., are the mean values obtained for the subsequent sets of observations, the values plotted on the graph (S_1, S_2, etc.) are given by the equations:

$$S_1 = x_1 - k$$
$$S_2 = x_1 - k + x_2 - k = S_1 + x_2 - k$$
$$S_n = S_{n-1} + x_n - k = \Sigma_n x - nk$$

Ways of extracting the maximum information from cusum charts are discussed by Woodward and Goldsmith (W9); guidance is given there about the most suitable relation between distances on the horizontal and vertical axes to adopt when drawing up the charts, and about the various procedures which can be adopted in the event of an obviously freak result. Changes in the mean for the particular set of observations are reflected by changes in the slope of the cusum chart, and quite small changes are quickly detected by alterations revealed in the general slope of the chart (Fig. 3). When examining a cusum chart, a presentation of average values occurring over a considerable time scale is being visually assessed, but in a way that focuses attention on the time when the last change in the average value occurred. So far the authors themselves have practiced only visual assessment of cusum charts, but other more exact methods of deciding when action is required can be applied (W9).

In the example shown in Fig. 3, the reference value for the serum potassium content of the control sample was 4.40 mEq/liter, so the values to be plotted successively on the cusum chart can be derived from the figures given in tabular form in Fig. 2, and are —0.17, —0.24, —0.38, —0.48, —0.40, etc. The cusum chart reveals a change not evident from the data presented in either Fig. 1 or 2, since the downward trend evident in the first seven results plotted on the cusum chart was reversed with the eighth and subsequent entries. The change in slope in Fig. 3 was shown to have coincided with the use of a new manifold on the Technicon electrolyte analyzer.

Cusum charts do not entirely replace earlier graphical methods of presentation, since the severity of error in the analysis of the seventh specimen is less obvious in Fig. 3, whereas it is clearly shown in both Figs. 1 and 2.

In addition to these charts for control sera, charts can be used to display the results obtained with standards and with blanks (H7a) and, by using duplicate standards or controls, charts can be plotted showing the differences between duplicates (L3). These "difference charts" have been found valuable for detecting whether the variation between pairs of analyses performed on different days is significantly greater than the variations between pairs analyzed as part of the same batch.

It would be possible to draw up a long list of different control charts which could be plotted with respect to every determination subjected to quality control of its performance, each chart adding its contribution of

potentially valuable information, but the preparation and maintenance of several control charts for many different techniques can readily become too time-consuming and thereby defeat their own purpose. Not more than two control charts, therefore, are advocated for any one method, and the rest of the control data (e.g., on blanks or standards) can be kept in notebooks for plotting in graphic form if this seems worthwhile when investigating an analysis that has gone out of control. It is recommended that one of the graphical records of data for control sera should be a cusum chart.

3.3.3. *Monitoring of Total Laboratory Error over Long Periods*

The reliability of any result produced by a clinical chemistry laboratory can be deduced only from results found on control specimens such as the fictitious patient controls (referred to in Section 3.1.3). Results for these analyses may be plotted on charts (as shown in Figs. 1–3) where they provide evidence for day-to-day quality control. These charts, however, will indicate only fairly large changes in the reliability of the method and may not reveal a gradual deterioration. To detect this gradual lowering of reliability, the SD or CV for each analysis should be calculated at regular intervals from the figures collected on the charts. It is advisable to carry out this operation at least once a month, and to compare the findings for the previous month with earlier sets of figures for the method.

These findings for the SD at different concentration levels, or for the CV if the SD of the determination changes linearly throughout the overall range for physiological and pathological results, provide valuable statistical information to clinical staff on which to base their assessments of laboratory reports; if the SD or CV for a laboratory method changes significantly from time to time in routine use, as shown by these monthly reviews, the latest set of figures should be made available to users of the laboratory.

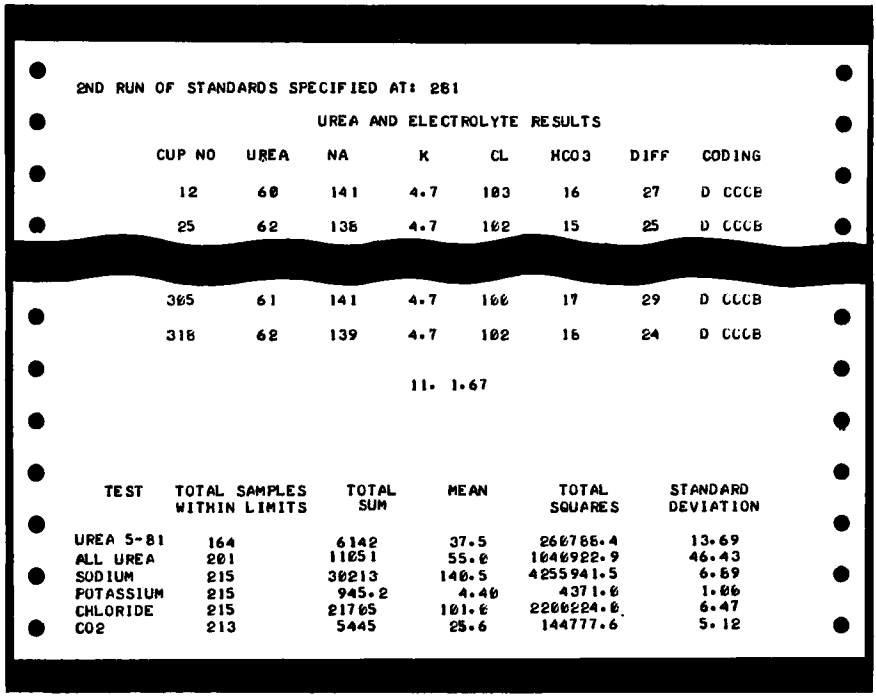
When attempting to control the performance of analyses of relatively unstable components of serum or plasma (e.g., in certain enzyme determinations), it is unwise to depend solely on lyophilized pools (H8) since these may deteriorate at indeterminable rates. Carry-over analyses on patients' samples prove particularly valuable in this connection, and the results plotted on control charts daily, or batch by batch, can also be used collectively on a long-term basis to calculate the SD for the method, using the formula in Section 2.2. Alternatively, the SD can be calculated from the average difference between these pairs of carry-over specimens, by dividing it by 1.128 (M11).

3.3.4. *Computer Analysis*

In most laboratories at present, the results of quality assessments have to be recorded manually, either in notebooks or on control charts, etc., and time is insufficient for extracting all data of potential value from these results. With the introduction of mechanical data-processing equipment and computers, however, additional checks can be introduced. Some of the technical details of these systems will be considered (Section 5.5).

Whitehead (W6) described an IBM-870 system for the recording of laboratory data on punch cards based on an earlier description by Peacock *et al.* (P1). The primary purpose of this system was to improve the presentation of individual laboratory reports, but the cards were subsequently sorted and the data on them subjected to statistical analysis, some of which revealed hitherto unsuspected sources of error as a result of variation in the technical performance of individual members of staff. Whitehead was among the first to apply cusum techniques to clinical chemistry laboratory data for control purposes, but his off-line system was not able to provide quality control information of immediate value to the laboratory.

Another off-line development of computer-dependent data processing has been described by Flynn *et al.* (F3, F4, F5), and this system (which uses equipment supplied by Elliott Medical Automation Ltd., Chenies Mews, London, England) has been further evaluated in the authors' laboratory. As far as the quality control program in this laboratory is concerned, the ALA (Automatic Laboratory Analysis) system, in addition to collecting data from strip-chart recorders onto paper tape and including in the computer program certain correcting steps not readily performed manually on a repetitive basis (Section 5.5.1), presents the printed data from each run of analyses in a form that allows a rapid assessment of the quality of each day's findings. As part of the modification of the original program to meet this laboratory's requirements, the Elliott 803 computer produces two output tapes of computed data. The first of these carries (1) the results for all the control samples included in the batch of analyses and inserted in the analytical run at positions identified for the computer, and (2) statistical information about the results of analyses on patients. These data can be inspected and charted while the longer process of printing out results for individual patients is taking place, and a part of one day's quality control printed output is shown in Fig. 4; the values for the mean results from patients' specimens are used for plotting cusum charts. Flynn (F4) adopted a different statistical evaluation of data from patients, as part of his computer program, confining the assessment to the "average of normals" approach (H10).



2ND RUN OF STANDARDS SPECIFIED AT: 281

UREA AND ELECTROLYTE RESULTS

CUP NO	UREA	NA	K	CL	HCO3	DIFF	CODING
12	60	141	4.7	103	16	27	D CCCB
25	62	138	4.7	102	15	25	D CCCB
305	61	141	4.7	100	17	29	D CCCB
318	62	139	4.7	102	16	24	D CCCB

11. 1.67

TEST	TOTAL SAMPLES WITHIN LIMITS	TOTAL SUM	MEAN	TOTAL SQUARES	STANDARD DEVIATION
UREA 5-81	164	6142	37.5	260785.4	13.69
ALL UREA	201	11051	55.0	1046922.9	46.43
SODIUM	215	30213	140.5	4255941.5	6.59
POTASSIUM	215	945.2	4.40	4371.0	1.06
CHLORIDE	215	21765	101.6	2200224.0	6.47
CO2	213	5445	25.6	144777.6	5.12

FIG. 4. Part of a teletype print-out of one day's quality control assessment of urea and electrolyte (Na, K, Cl, and HCO_3) determinations, using AutoAnalyzer equipment. The results were prepared by a digital computer, using as input punched tape from the Elliott ALA data-acquisition system. The upper part of the record shows the results for four identical control sera, two near the start and two near the end of the run; the left-hand column is the number of each sample's position in the run. Ion difference (Diff) equals $(Na + K) - (Cl + HCO_3)$, and the letter coding on the right gives the classification of individual results into arbitrary groups for normal (C) and various abnormal levels (A, B below and D, E above the normal range). The lower part of the record provides collected statistical data for the analyses on patients, and the patient means are used for plotting on cusum charts.

Blaivas (B4) described the use of an IBM-1710 process-control system, on-line to 20 AutoAnalyzers, in which the computer program included instructions for the acceptance or rejection of standard curves, and a series of checks for monitoring the acceptability of values obtained for control sera. Any results that failed to meet the requisite criteria were indicated within a few seconds of the charting of a peak by a message typed on an on-line typewriter. This system has the ability to include daily statistical assessments of the quality of results, but these possible additional control features were not listed in the description (B4).

For the future, increasing dependence of hospital laboratories on mechanical and electronic data-processing techniques can be forecast,

and their application to process control and data processing, with the facility for rapid statistical evaluations, will contribute more generally to routine quality control in clinical chemistry.

3.4. Use of Assays on Samples from Patients for Control Purposes

3.4.1. *Daily Average*

For any chemical determination regularly performed in sufficient numbers on samples from patients, the average result obtained by the laboratory for these analyses can be calculated daily and made to form part of the quality control program. This is because the characteristics of the population served by the laboratory, when dealing with large numbers of patients, are not liable to change markedly from day to day (S8, W14), and the overall average is little affected by the inclusion in some batches of determinations of the occasional grossly abnormal finding (e.g., plasma sodium of 110 mEq/liter). For the calculation of daily averages for patients' samples, a minimum number of 30 analyses should be being undertaken by the laboratory for the particular method. Table 1 illustrates the constancy of the daily average for plasma electrolyte determinations obtained in this laboratory, and these data lend themselves particularly well to visual presentation on cusum charts.

TABLE 1
The Daily Average for Plasma Electrolyte Results on Patients' Samples (mEq/liter)

Day	Number of samples	Sodium	Potassium	Chloride	Bicarbonate	Ion difference[a]
1	164	139.2	4.28	101.1	26.7	15.7
2	172	139.4	4.23	100.2	25.8	17.6
3	180	139.1	4.32	100.1	25.6	17.8
4	166	139.1	4.26	100.2	26.0	17.2
5	166	140.3	4.36	100.9	26.1	17.7
6	195	139.2	4.39	101.7	25.8	16.1
7	137	137.8	4.07	100.5	25.5	15.9
8	210	140.2	4.46	102.1	25.0	18.6
9	179	140.7	4.48	102.3	24.9	17.9
10	151	141.2	4.42	102.9	24.8	17.9
11	136	141.5	4.40	101.7	25.2	19.0
12	158	141.0	4.36	100.7	26.8	17.9

[a] Difference between sodium + potassium and chloride + bicarbonate.

3.4.2. *Duplicate Analyses on Patients' Samples*

The routine performance of all determinations in duplicate is not advocated, particularly in the many laboratories where the pressure of repetitive work is already a major factor contributing to unsatisfactory quality of results. A limited number of duplicate determinations, however, is valuable, and some of these can depend on the repeated analysis of samples from individual patients. For the investigation of within-batch variation, patients' samples can serve equally well as control sera and do not involve additional expense.

Carry-over specimens from patients have a special part to play in quality control, since these samples will have been stored in the laboratory for much shorter periods than pooled sera and can therefore serve as a source of evidence, additional to the evidence from blanks and standards, that an unsatisfactory series of results for a control may have resulted from deterioration in the specimen of pooled serum itself.

3.4.3. *Patients' Samples Serving as Their Own Controls*

Chemical investigations have an increasingly important part to play in the measurement of a patient's progress during the course of an illness. For these reasons, there is a growing tendency for laboratories to be called upon to perform the same investigations on individual patients on two or more occasions at different times during a single hospital admission, and the results of the latest set of investigations are then considered by the doctor in charge of the patient in relation particularly to the previous findings for similar tests and to any changes in the patient's condition.

In Britain, at least at present, considerable emphasis is still placed on the desirability of careful scrutiny of each set of results before a report is issued from the laboratory (N1); although individual reports can be designed so that they can be inserted in the patient's record and build up there a serial presentation of the laboratory's findings, there are distinct advantages if the first assessment of results of a series of related investigations can take place in the laboratory itself. This is because clinical staff cannot always be relied upon to communicate with their colleagues in the laboratory if a set of results contains unexpected findings which may not be immediately explicable in terms of the patient's clinical progress; instead such unexplained findings are all too often ignored.

For the comparison of the latest set of results for a patient with previous findings to be practicable, recall of previous data must not introduce delays into the reporting process, nor should the system of

data recall involve major additional effort or expense. Assuming the relevant previous findings can be made readily available, however, the latest results can then be considered by the laboratory staff in relation to these records of earlier investigations and to the limited clinical information provided on request forms. If a result appears unacceptable in comparison with previous findings for the patient, the laboratory record can be checked, a repeat of the analysis may be possible, and the doctor in charge of the case can be asked to provide additional clinical information. Several descriptions of manually operated cumulative record systems for results of clinical chemical findings have now been published (e.g., F6, M5, W4).

From the point of view of quality control these cumulative record systems can fill an important gap in the laboratory's program, since the various standards and controls previously discussed cannot allow for errors affecting single specimens from patients. Although the additional expense of such record systems may seem a large price to pay in terms of the occasional error which they may reveal (and cumulative records systems can be applied in practice for the detection of only relatively gross errors), the consequential effects of detecting one erroneous result in a series of findings may be considerable, e.g., if the error detected shows that a series of peaks on an AutoAnalyzer chart record have been incorrectly identified in relation to the corresponding samples.

At present, with manually operated cumulative records systems and the pressure of work experienced in most clinical chemistry laboratories, much of the evaluation in the laboratory itself of individual results in relation to information on request forms is empirical. However, with the development of computer-dependent systems for efficient storage of data and rapid recall, it will be possible to include in the programs predetermined criteria for drawing attention (e.g., by asterisks) to results for investigations that differ by defined percentages from the previous sets of findings for each patient; these marked results can then be assessed particularly carefully, whereas the unmarked results can be reported forthwith—for such computer-prepared results there is, at least in Scotland, no legal requirement for each report to be signed by a member of the laboratory staff before being sent out from the department, with the special exception of forensic work.

3.5. Summary of Quality Control Procedures to Adopt for Monitoring the Routine Performance of a Clinical Chemical Method

So far this review has discussed points to take into account when initially assessing the suitability of a technique for introduction into

diagnostic service, and methods for subsequently monitoring its performance. Because of the wide range of analyses performed by the modern clinical chemistry laboratory and the ever-growing pressure from rising workloads, a balance must be struck between what is ideal and what is practicable in the day-to-day monitoring of a laboratory's quality of performance. The present subsection, therefore, summarizes the different methods of quality control that have been discussed, and indicates those particular practices that are regarded as essential or especially valuable for analyses on plasma or serum. Similar practices should be adopted for other clinical chemical determinations.

3.5.1. *Standards*

These are essential for all clinical chemical methods, and must not be confused with controls. A record of the successive readings obtained with standards used in a technique provides valuable evidence about the continued performance of at least part of the method, or of the final measuring instrument, or both. In some cases, standards can be taken right through all the analytical steps, and a special type of standard—the Bench Standard—has been advocated (Section 3.1.1.2).

3.5.2. *Blanks*

These also form an essential part of all clinical chemical methods, and a record kept of successive readings obtained with blanks may provide a valuable early indication of deterioration in reagents. If the practice of reading test solutions directly against blanks is adopted to simplify repetitive calculations, this should not be at the expense of making an initial reading of the optical density of the blank itself, most often in relation to water.

3.5.3. *Controls*

There are several kinds of control which can be applied. Of these, between-batch controls making use of materials which can be taken through the entire analytical procedure provide the most valuable single method and must be regarded as essential. Other forms of control (e.g., within-batch controls) can provide useful additional information that helps to delineate the cause of a breakdown in a hitherto satisfactory record of quality control. Whenever possible, controls should be introduced anonymously into the laboratory's work (fictitious patient controls, Section 3.1.3). Types of control materials for analyses on plasma or serum which have been discussed include:

(1) Aqueous solutions of pure materials. Limited application, and open to error of bias.

(2) Control sera prepared and analyzed in the laboratory. Various dilutions of these can be prepared to reduce the possibility of error of bias.

(3) Commercially prepared control sera that have had their analytical composition determined by selected reference laboratories. These can also be prepared in different dilutions.

(4) Sera from individual patients in which the only comparison possible is with other results obtained on the same specimen. This provides a very cheap form of control material. When used as between-batch controls, these sera have particular value in monitoring the performance of enzyme determinations.

(5) Sera or aqueous solutions forming part of interlaboratory comparisons. Limited use made of these at present.

(6) Statistical analyses on results obtained for large groups of patients.

All except the last of these examples can be used either for duplicate analyses to assess the within-batch error or for repetition of analysis in a subsequent batch to assess the between-batch error; the regular performance of duplicate analyses on all specimens is regarded as impracticable. The statistical assessments are particularly useful for between-batch comparisons. Although these various checks may indicate that the accuracy of a method is altering in routine use, their main application is in the monitoring of precision.

3.5.4. *Control Charts*

Data collected from the monitoring procedures summarized above can be kept in notebooks, preferably as serial entries that allow direct comparison between successive readings. Visual display is, however, strongly recommended and the particular value of cusum charts in forecasting trends has been mentioned.

3.5.5. *Synopsis*

As a minimum, each batch of analyses in clinical chemistry must include (1) a standard, (2) a blank, and (3) a control that is indistinguishable from the specimens being analyzed. For large batches of analyses, either manually performed or carried out with equipment such as Auto-Analyzers, at least one in every 40 specimens analyzed should be a control. The most important type of control to include in any assessment is one that allows the between-batch reproducibility of the method to be monitored; this can be achieved most simply and cheaply by repeating in the subsequent batch the analysis of a patient's specimen included in the previous batch, due care being taken to ensure the stability of the material being analyzed during the interval between batches of analyses.

4. Sources of Error

4.1. Errors Originating on the Ward or During Transport of Specimens

4.1.1. *Errors during Collection of Specimens*

Some of the errors which can occur at the time of specimen collection on the ward or in an outpatient department may prove impossible to eliminate, and it is almost certain that, when a comprehensive system of controls has become standard practice in any laboratory, the ward will become the major source of error. The overtaxed doctor or nurse is liable to make a mistake over the patient's name when labeling specimen tubes, or accidentally to put the wrong times of collection on sequential specimens. Such errors can be reduced only by training staff responsible for specimen collection to work in a methodical manner regardless of the stress of the moment.

The carrying out of special procedures and the collection of specimens for clinical chemistry investigations entail considerable attention to detail, and all too often errors in collection arise from inexperience, ignorance, or lack of organization. For example, most hospital biochemists will be familiar with the sources of error inherent in such a relatively easy operation as the collection of a 24-hour specimen of urine. A patient cannot be watched throughout the whole 24 hours, and may be attended by many different nurses in a busy ward. Night staff may not be fully informed of all requirements. A well-meaning but uninformed nurse may easily discard a specimen, add it to a previous day's collection, or pour it into another patient's bottle. In many cases the member of the ward staff concerned may be unaware of the simple instructions required to ensure an accurately timed specimen. The regular performance of urinary creatinine estimations or other laboratory measurements to cross-check the completeness of 24-hour urine collections adds unnecessarily to the work of the laboratory, and introduces the possibility of a new set of errors which can affect the interpretation of the final result (C10). In other words, if a 24-hour collection of urine is required for an investigation, proper instructions should be given on the method of collection, to the patient and to the nursing staff, and these instructions should then be carried out to the letter.

Similarly, it might be felt that little skill and experience are required to carry out an oral glucose tolerance test, yet many of these tests are spoiled before the specimens arrive in the laboratory by failing to observe rules regarding the previous diet of the patient and the correct dose of

glucose, or by omitting to note vomiting. If capillary samples of blood are used, it may be necessary to collect accurately measured volumes of blood by capillary pipet. Experience has shown that the last task requires considerable practice before it can be done successfully.

Other reasons which can account for day-to-day variation in results for some analyses from individual patients include (1) the effect of diurnal variation on some plasma constituents (e.g., cortisol, phosphate, iron) (L6, W12), (2) the influence of the phases of the menstrual cycle on serum iron (Z1) or on urinary excretion of estrogens and pregnanediol (L5), (3) changes owing to the posture of the patient (e.g., on plasma cholesterol) (F1), (4) adverse effects of excessive venous stasis (e.g., calcium, protein, pyruvate, etc.) (G2), (5) interference by various foods, drugs, and their metabolites (e.g., iodine-containing compounds on serum protein-bound iodine; vanilla, etc., on catecholamine metabolites in urine), (6) collection of blood specimens from a limb into which an intravenous drip is running, and (7) use of a specimen container in which the wrong anticoagulant or preservative has been placed.

These various sources of error in the collection of specimens from patients, whether physiological in origin or the result of ignorance or carelessness, can each prevent the laboratory from providing a meaningful result for the affected specimen. These errors can grossly undermine confidence in the value of chemical investigations if the faulty specimens are not detected, since results obtained from the analysis of faulty specimens may be later compared with results obtained on specimens properly collected. Many of these potential reasons for unsatisfactory specimens can be overcome, at least in hospital practice, by collecting blood from fasting patients before they are allowed out of bed in the morning, and using a minimum of venous stasis; for timed urine collections, a fixed schedule of times for starting and ending collections can help considerably.

Although this section may not appear immediately relevant to the problem of quality control within a routine hospital laboratory, it has been included to emphasize the fact that, at any stage in the whole procedure of any test, from the time of requesting an investigation to the time of furnishing the report, a mistake can lead to an erroneous and sometimes grossly misleading result, and that the contribution of the ward or out-patient department, etc., to the incidence of these errors has still not been fully appreciated.

4.1.2. *Deterioration of Specimens after Collection and before Analysis*

Some specimens require preservative, such as fluoride to inhibit glycolysis in blood samples, or mineral acid to stabilize catecholamines

and their metabolites in urine, etc., and deterioration of unpreserved specimens can prevent subsequent laboratory work from having practical diagnostic value. Other specimens require a preliminary processing step to be performed within a limited period, and thereafter are stable for relatively long periods; for instance, several analyses on plasma and serum can be adversely affected if separation of these specimens from red cells is unduly delayed (e.g., potassium, phosphate, certain enzyme assays, etc.). Samples for blood gas analysis deteriorate very rapidly, unless carefully refrigerated (S2) when their examination may be delayed for 2–4 hours, and urine samples for assessing the response to a loading test with NH_4Cl are also unstable.

Deterioration of specimens as a result of delays in transport from wards to the laboratory has focused attention on the possible advantages of pneumatic tube communication systems. A technical memorandum (M8) discussed the financial and operational factors to take into consideration before deciding to install such a system, which can be undertaken economically only as part of a building program, and McClellan *et al.* (M6) reported data on the adverse effects of one such transport system on the validity of chemical determinations on blood samples.

4.2. Errors Resulting from Interchange of Samples and Errors of Transcription

Unless properly labeled at all times, specimens from one patient can easily be mistaken for and interchanged with similar specimens from other patients. These opportunities for interchanging samples occur (1) at the time specimens are collected in the wards or outpatient clinics, etc., where they may be labeled with the wrong name, (2) at the sample reception desk, where serial numbers (or other system for identifying specimens during transit through the laboratory) may be put on the wrong specimens, (3) in the laboratory sample-preparation area, where, for instance, large numbers of blood samples are being processed prior to analysis, by centrifugation and subsequent transfer of the supernatant plasma or serum to another container, and this transfer may be made into the wrong receiver unless this is clearly labeled to correspond with the original specimen container, (4) at the time the technician selects a specimen for analysis, either by manual means or by transfer of a portion to an AutoAnalyzer cup, (5) during the actual process of analysis, as a result of interchange of inadequately labeled tubes or of faulty discipline with AutoAnalyzers, leading to unauthorized and unrecorded switching of samples on the sampler turntable after loading has been completed, and (6) at the time of reading the scale on the measuring instrument (colorimeter, spectrophotometer, AutoAnalyzer strip-chart

recorder, etc.), entering the result against the wrong patient in the notebook or other bench record—this last mistake could be classed as an error of transcription.

In addition to the many pitfalls listed above, errors of transcription can occur when entering the results from laboratory work sheets, etc., onto record cards or report forms. These may be mistakes affecting a single patient, or results for two or more patients in a series of analyses may be entered on the wrong forms.

4.3. Laboratory Bench Errors

These errors should be familiar to all trained laboratory workers, and are best classified according to Vogel's (V3) definitions for categories (a) and (b) of determinate errors (Section 2.5). They include such items as dirty or damaged glassware, use of a wrong pipet, and faulty reagents. With measurements depending upon colorimetry, the fact that certain reactions are prone to develop turbidity as well as color must not be overlooked. Fatigue and boredom also undoubtedly contribute to bench errors (R6, R6a).

5. Methods of Improving Reliability

5.1. General Education

5.1.1. *Management*

Surveys have shown (Section 1.2) that many laboratories appear to be unaware of the poor quality of the results which they may be producing. Full awareness of the potential sources of error in the various aspects of laboratory work is undoubtedly the best stimulus to maintaining a high standard of accuracy and precision, and clinical chemistry laboratories should have their quality control data continuously available and under review. Control techniques have come into general use only comparatively recently, and their value may not yet be appreciated widely enough by the heads of laboratories.

5.1.2. *Laboratory Workers*

It will be increasingly possible in laboratory work to eliminate human error by the introduction of automation, at the price of growing capital expenditure on equipment. In order to make the best use of the expensive equipment involved, a trend toward the establishment of large, centralized laboratories that carry the analytical load for as large an area as possible is already being felt (G1, W7). It will not be possible, however,

to automate completely either the smaller laboratories or the more complex techniques and, for these, human sources of error will remain. The more highly skilled the laboratory worker is, the more reliable and precise should be his results. This is shown in Table 4, where the reproducibility obtained by a senior technician operating a manual method is shown to be much better than that of a student technician. The general training and experience of the worker must therefore play a large part in maintaining precision, but greatly improved results can be obtained by relatively inexperienced workers if the importance of good technique is continually stressed during their training.

5.2. Elimination of Ward Errors

Contact between the ward and laboratory staff is the best way of reducing most errors that occur before a specimen reaches the laboratory. The change toward larger laboratories, however, will inevitably mean a considerable decrease in the personal contact that so many of the present small laboratories have with the staffs on the wards they serve. This loss need not increase errors in specimen collection if the possibility of its occurrence is recognized and steps are taken to overcome it.

Each laboratory should make available to all its users a frequently updated set of detailed instructions for the collection of specimens and for the carrying out of all the tests done, together with information on the interpretation of results. No user should then have to rely unduly on his memory, nor should he have to obtain details on the carrying out of tests from textbooks, where many different descriptions of the same test are often to be found. The number of different containers for specimens should be kept to a minimum, and the label and request forms should be as simple as possible to expedite their proper completion; the request form should include a space for entering the time of collection of the specimen. Possible ways of improving and simplifying request forms, and avoiding unnecessary transcription steps in the laboratory itself, have been considered by Lee and Schoen (L2a).

5.3. Elimination of Clerical Errors in the Laboratory

5.3.1. *Laboratory Reception*

Except in a small laboratory, it is unwise to rely on the name of the patient to identify a specimen throughout the process of analysis. Rapidly increasing workloads prevent staff from taking an interest in the personal identity corresponding to each specimen. Many names are easily misspelled or confused, or are inconveniently long, and the hospital record number is always large.

Laboratories employ a wide variety of methods for dealing with the accession and identification of specimens and related documents. It is not possible to cover every alternative, and the following description is based on a summary of the practice in this laboratory.

The simplest identification of specimens for laboratory purposes has proved to be a serial number allocated to them in order of their arrival. The chances of error are reduced if the number of times the identification number has to be written are kept to a minimum, and this can be achieved by having available at the reception desk a reel of perforated gummed paper from which may be torn an appropriate number (e.g., six) of small labels for each new specimen, these labels all having the same number. Labels are stuck immediately on the request form and the specimen container, both of which can then pursue their separate paths through the laboratory, simply and unequivocally identified (W4). The spare labels accompany the specimen and can be used whenever some of it is transferred to a fresh container, or for numbering a bench work sheet.

When a specimen requires analysis only on a single- or multichannel automatic analyzer, and a large number of such specimens are involved, delays occurring in the laboratory may be minimized if the position of the specimen on the machine's sampling plate is used for identification purposes.

Certain specimens require special preservation; for instance, those for acid phosphatase measurement need to be separated and frozen immediately. Specimens for bilirubin determination must be protected from light. Tubes containing specimens requiring special treatment should be clearly marked on arrival at the reception area and suitably dealt with, otherwise gross errors can occur.

5.3.2. *Laboratory Reporting*

Transcription errors can occur whenever a technician transfers figures from his work book to the final report (Section 4.2). A system of cross-checking by a second technician can be adopted but this is extravagant of staff time, and training in a "zero defects" type of program will probably ensure the best results (H1).

It is common practice in many laboratories for a senior member of staff to inspect and sign every report before it is dispatched. He checks whether or not the findings are consistent with the clinical details, which should have been written on the request form. He also checks whether an abnormal result could have resulted from an excessive delay occurring between the time of collection of a specimen and its delivery to the laboratory; if the abnormality is attributable to such an artifact, the result should not be reported without a qualifying comment. When

dealing with single reports, this addition of a signature by a senior member of staff is something of an expensive anachronism, but consideration of serial laboratory records prior to the issue of reports can contribute to the control program (Section 3.4.3).

5.4. Methods of Eliminating Errors in Analytical Work

5.4.1. *Work Organization*

For the maintenance of a high degree of precision, it is most important that work in a laboratory should proceed in an unhurried and well-ordered manner. Every effort must be made to avoid monotony and boredom. For a general laboratory it should be relatively easy to deploy trained staff so that they do not perform the same work for more than 1–2 months at a time. The efficiency of a worker in carrying out any technique usually shows a rapid increase with growing familiarity, followed by a slow decline as boredom has its effect. There is thus an optimum period of operation which will vary from one technique to another, and with the temperament of the worker. In more specialized laboratories, a wide diversity of work is not available and monotony can become a serious problem: in these the possibility of interchanging staff with other laboratories, perhaps on a yearly basis, may have to be considered. Apart from long-term boredom, short-term tedium has for many years been known to affect the performance of factory workers, producing what has been called the "four o'clock phenomenon." Robinson (R6) has shown that tedium can have similar important effects upon analytical precision, and emphasized that positive steps must be taken to guard against it. His data (Fig. 5) show the increase in the CV for duplicate assays of serum potassium, while one worker operating a flame photometer manually made 120 determinations in succession.

Another recognized effect is the slow but insidious deterioration in precision that takes place when a technique is routinely performed by junior staff working without adequate supervision. Too often, in a busy department, junior technicians are allowed to train other juniors who are to replace them at a bench; in this way error-producing habits can be introduced and perpetuated, even though steps were taken to exclude them before the introduction of the technique into the laboratory by more experienced staff. To avoid this, the operation of every method should be checked in detail by a senior person at regular intervals, and the instruction of junior staff should be undertaken only by fully trained persons.

When a junior member of staff is experiencing difficulty with an instrument or piece of apparatus, a trained person may not be readily available for consultation with the result that uninformed action may

be taken that leads to error. Although in an adequately staffed laboratory it is possible and desirable to ensure that senior personnel are not too preoccupied with other duties to exercise this close supervision, it is still necessary for the junior technician to recognize that the difficulty is beyond his own competence to put right. Here the appropriate remedy is to define closely the changes from normal performance that are considered to demand consultation with a more senior colleague.

A hospital clinical chemistry service laboratory may be able to undertake more than 100 different types of assay, but not all these are performed every day, or even every week. To avoid reliance on memory or

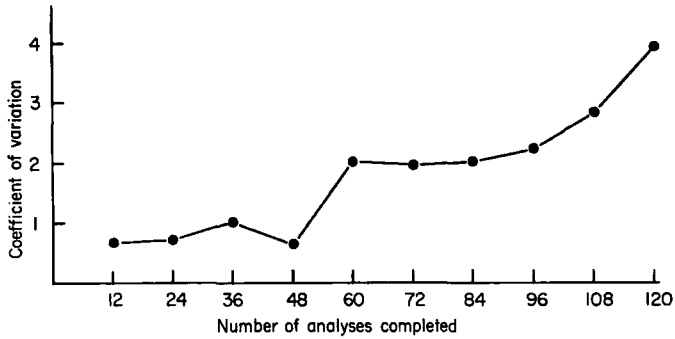


FIG. 5. The effect on the coefficient of variation of continuous performance of serum potassium determinations carried out by one technician and assessed on the basis of duplicate determinations, performed in batches of six pairs of analyses [redrawn from Robinson (R6)].

frequent recourse to textbooks when a method is to be performed, a file containing detailed instruction sheets for every assay should be readily available for consultation.

5.4.2. *Work Simplification*

After 30–40 years of methodological development and particularly the rapid changes of the last 10 years, many of the routine assays in use in clinical chemistry may be expected to continue in similar form for some time to come. From the standpoint of work study and work simplification, however, techniques are often still far from perfect, but, if the help of a work-study consultant is enlisted, he finds difficulty in giving advice on the simplification of bench techniques because of his lack of knowledge of the scientific principles involved. Clinical chemists must therefore, to a large extent, act as their own work-study consultants.

All the automatic machines mentioned in Sections 5.4.3–5.4.5 employ advanced forms of work simplification, but the use of one or more of the

many autodispensers and autodiluters commercially available, in conjunction with other simple devices, can effect major improvements when more fully automatic machines cannot be used or are unavailable, or when their application is not economically justified.

5.4.2.1. *Mechanical and Electrical Aids.* The importance of glassware washing in maintaining the reliability of any laboratory's work cannot be too heavily stressed. Many aids are available for decreasing the drudgery and increasing the efficiency of glass washing, from simple siphon-operated pipet washers to the modern programmed machines incorporating ultrasonic vibration. Several types of washer were reviewed in 1959 (M3); since that time many new machines have been described in the catalogs of laboratory furnishers.

Work-simplification devices on the laboratory bench will be detailed under the headings of automatic dispensers, automatic diluters, and flow-through colorimeters with digital read-out. In each group, examples will be given of equipment of which the authors have had personal experience, but a choice of alternative apparatus will be available in most instances. The instruments mentioned are not necessarily the simplest or cheapest obtainable for performing a particular function.

Automatic dispensers. Many of these function by means of a piston with a suitable valve arrangement, operated either electrically or by hand, or a peristaltic pump may be incorporated. Some simple forms are illustrated in Fig. 6. Movement of the syringe plunger in each case delivers a fixed volume of fluid. Choice of a particular type of instrument partly depends upon whether the syringe is to be held, or operated in a static position. Fully mechanized versions include the Struers (Copenhagen, Denmark) dispenser and the Analmatic proportioning pipet (Baird and Tatlock, Ltd., London, England) which has a rotary peristaltic pump. In the Analmatic pipet, a length of resilient tubing is compressed by a rotating roller, one revolution of which takes up a volume of fluid determined by the bore of the tubing; further turns of the roller cause delivery of the fluid. The instrument can be set to aspirate and deliver any multiple of this volume, by making the required number of revolutions, and can also operate as a diluter by taking up one volume of fluid followed by the requisite number of volumes of diluting fluid; its precision as a dispenser is somewhat limited by changes in the resilience of the tubing, but this criticism does not apply when it is used purely as a diluter. The features in the design of dispensers particularly relevant to considerations of quality control are (1) their ability to deliver repeatedly a fixed volume of fluid, which helps to maintain a constant standard of reproducibility, since they are not liable to operator fatigue, and (2) the fact that the volume of fluid delivered can be varied by

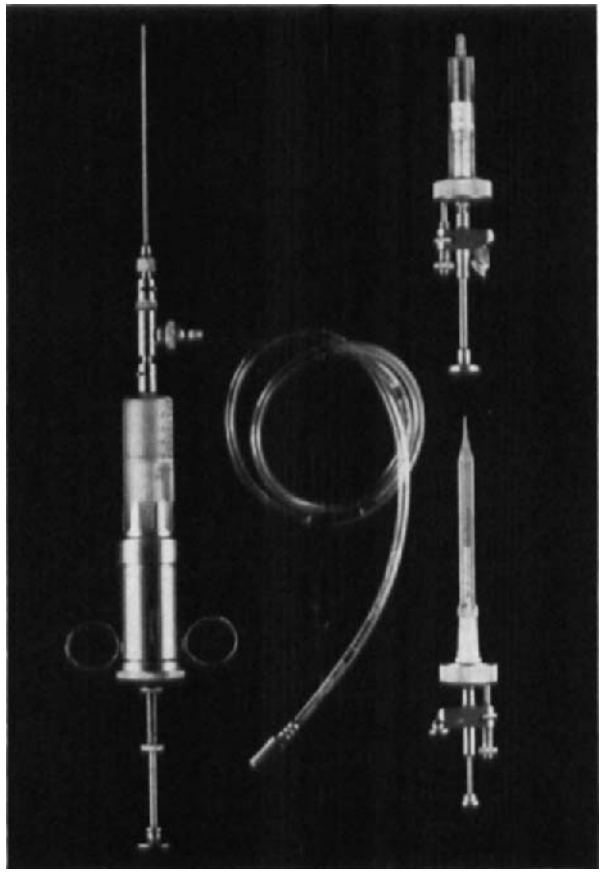


FIG. 6. Four types of simple hand-operated automatic dispenser.

means of the setting of the instrument, which means, however, that they must be recalibrated whenever the setting is adjusted to ensure an acceptable standard of accuracy.

The advantages of dispensers can be vitiated if they are used carelessly, for instance by failure to ensure that the barrel of the syringe, or equivalent chamber, is regularly filled to capacity.

Automatic diluters. The simplest form of automatic diluter was described by Seligson (S1) and this type of instrument is now widely used. Seligson diluters (or pipets) can be constructed in the laboratory, or complete precalibrated instruments can be obtained commercially. An important feature of the operation of all diluters is the wash-out of the

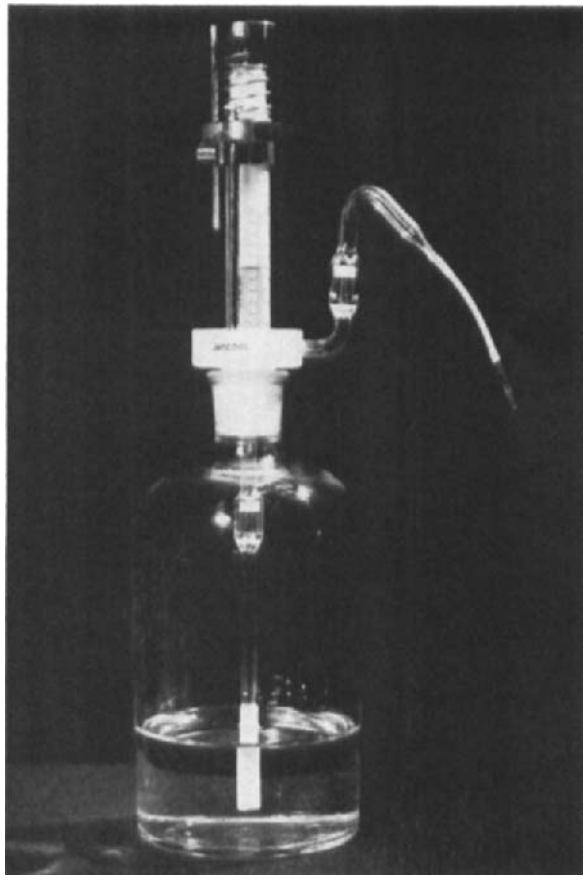


FIG. 6a (*Continued*).

sample from the calibrated chamber by means of the diluent; this eliminates the error from variable draining that is always found with simple delivery pipets, especially when used with viscous fluids. When the calibrated chamber is filled with the next specimen, the first portion is aspirated through the chamber and is discarded, so that only an undiluted sample is finally metered. For the maintenance of precision, it is most important that neither the tip nor the bore of the stopcock sustain damage: a sharp cut-off of sample in the stopcock has always to be ensured, but with normal wear small pieces of glass can fracture off the edges of the bore hole.

Most mechanical automatic diluters incorporate two syringes suitably connected via a valve mechanism. One syringe is calibrated to meter the

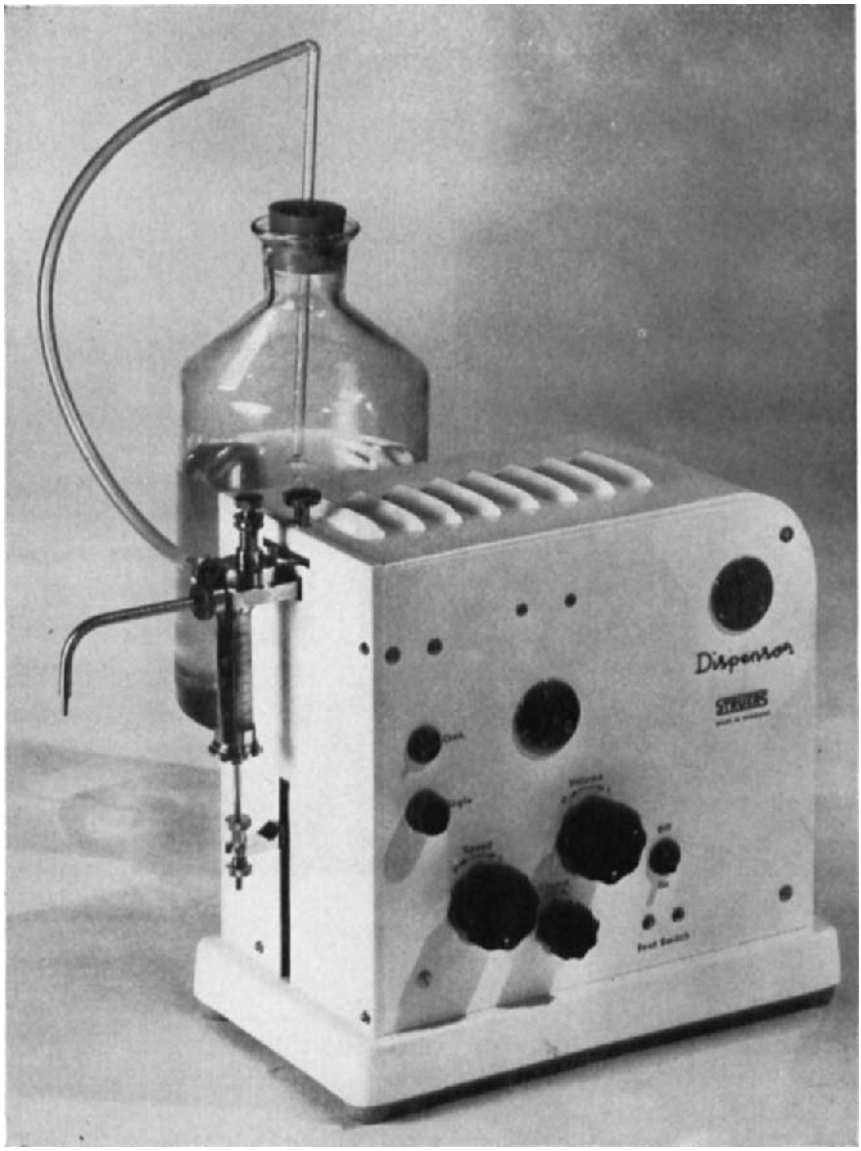


FIG. 6b. Struers automatic dispenser, hand- or foot-operated (by courtesy of Struers, Copenhagen, Denmark).

fluid to be diluted, and the other to measure the diluent. The Filamatic diluter (National Instrument Co., Baltimore, Md.), illustrated in Fig. 7a, is fully mechanized, and microadjusters restricting the stroke of each syringe allow the choice of a wide range of dilutions. The container

with the sample for dilution is placed under the pipet and, on pressing the foot pedal, the preset volume enters the pipet. The container is removed and replaced by an empty tube. Further operation of the pedal then forcefully ejects the sample, followed by the required volume of diluent. Figure 7b shows the Diluspence (Griffin and George Ltd., Wembley, Middlesex, England); this is a less expensive machine which performs a function similar to that of the Filamatic, but with some loss of versatility. The Diluspence operates by compressed air and is controlled by a single hand-operated valve.

Most dispensers and diluters can operate to a high degree of precision but, since they contain mechanical parts liable to wear, should be regularly overhauled and their accuracy checked at frequent intervals. A major source of error in diluters, particularly when very small volumes of sample are being diluted, is that any variation in the shape and size of the meniscus at the tip of the sampling pipet, when the sample container is removed, will impair both the accuracy and precision of the dilution. Any damage to the tip of the pipet must be rectified immediately, and necessarily implies replacement of the pipet and recalibration of the instrument.

Flow-through and digital read-out colorimeters. Flow-through cells are available for installation into most colorimeters and many spectrophotometers. These flow cells considerably shorten the time taken to "read" a large batch of solutions, but are liable to the following sources of error: (1) the draining or wash-out of the previous specimen may be insuffi-

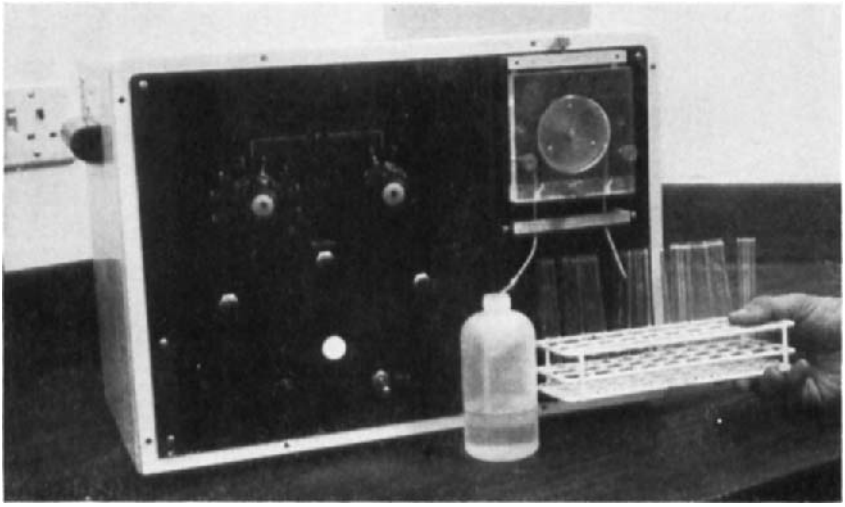


FIG. 6c. Analmatic proportioning pipet, hand-operated.

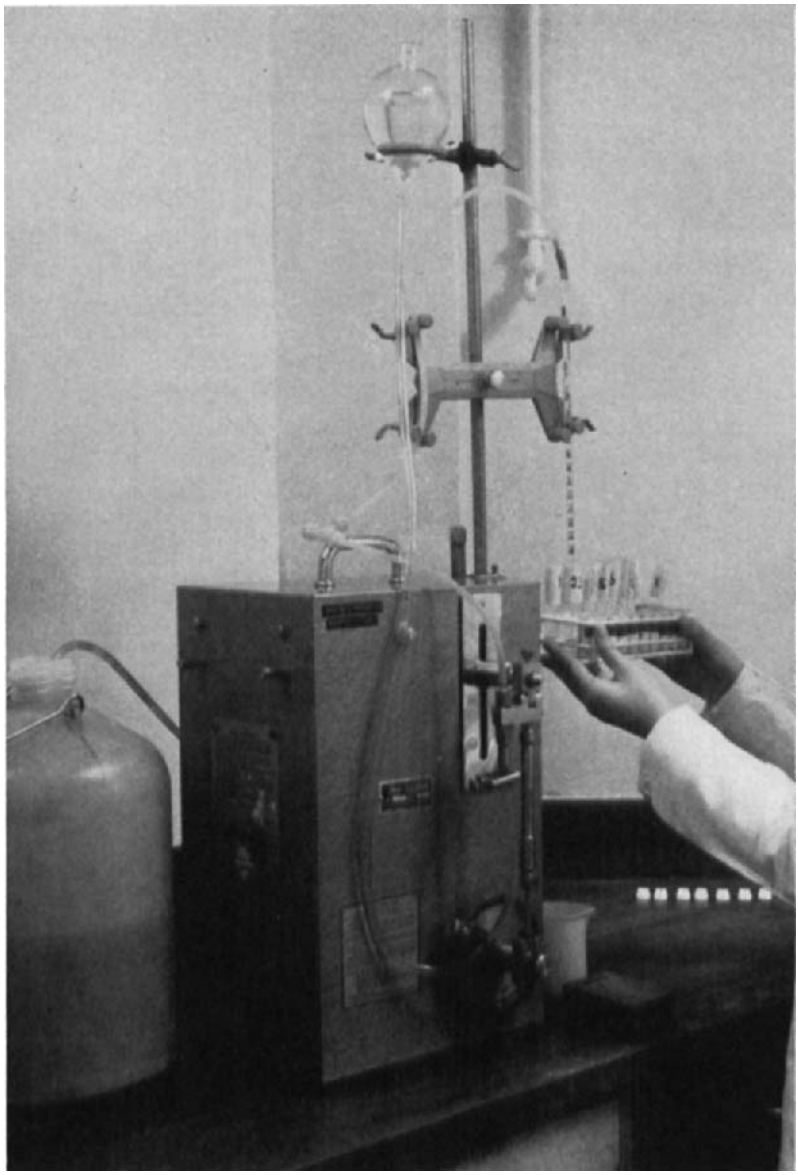


FIG. 7a. Filamatic diluter, hand- or foot-operated.

cient to ensure a negligible "carry-over," and (2) since the solution cannot normally be viewed in the cuvette, it is difficult to ensure that (a) bubbles are absent from the light path, and (b) sufficient liquid has entered the cell to fill it above the top of the light path. The use of flow

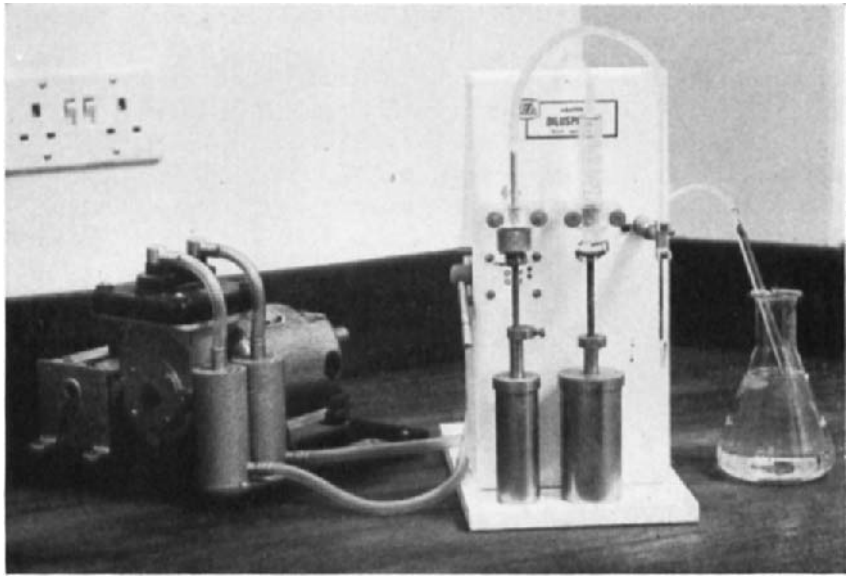


FIG. 7b. Diluspence hand-operated diluter.

cells usually involves discarding the specimen once a reading has been taken, and often insufficient solution is available to repeat a measurement if an error in taking the reading is suspected. Furthermore, unless an instrument has a special cuvette for holding the blank for the series of measurements, it is tempting (because of wastage and inconvenience) to carry out adjustment for drift much less frequently than is usual when operating with normal cuvettes.

All the difficulties mentioned above are surmountable, however, and one big advantage in the use of flow cells is that the same cell is used for all measurements, thereby eliminating errors from the use of unmatched cuvettes. The flow cell is not exposed to frequent handling and therefore errors from finger marking and scratching are not introduced.

Flow-cell spectrophotometers with digital read-out of concentration have recently been introduced; an example of these is the Model 300 microsample spectrophotometer (Gilford Instruments Inc., Oberlin, Ohio). The advantage of such instruments is that digital display of concentration eliminates the manual recording of absorbance with subsequent calculation (or reading from a graph) and the consequent chance of error. The Gilford instrument is so easy to use that a technician can perform 250 readings per hour with a CV ranging from 0.58% at an absorption of 0.140 to 0.27% at an absorption of 1.306 (W7a).

A wide range of other automatic or semiautomatic aids are available

for many specialized operations. It is impossible to discuss them all in detail, but they include several different multiple-sample recording spectrophotometers (particularly useful for the kinetic measurement of enzyme activities), and various types of electrometric titrator.

The adoption of the various devices discussed in this section, at the appropriate place in any particular technique, will eliminate sources of human error, but care must be taken to ensure that these are not replaced by instrumental error, which could at times be much greater. When each machine is installed, the limits of its performance should be determined. The importance of rechecking performance at frequent intervals thereafter cannot be stressed too heavily.

5.4.2.2. *The Application of Work-Simplification and Mechanical and Electrical Aids.* To illustrate the application of work simplification, its

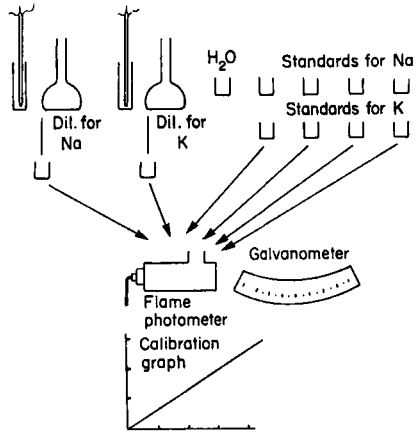


FIG. 8. Diagrammatic representation of the flame-photometric technique for the assay of Na and K in a single specimen of serum, before work simplification [Mitchell *et al.* (M10)].

use will be discussed in detail only for the determination of serum sodium and potassium by flame photometry, but its advantages both in this technique and in certain others will be indicated. The improvements in reproducibility which can be obtained by use of work simplification will be considered (Section 6.1).

Many nonautomated flame photometers are now commercially available. The following study (M10) was carried out on the E.E.L. instrument (Evans Electro-Selenium Ltd., Harlow, Essex, England), but would apply correspondingly to any similar equipment. Some laboratories still use, in whole or in part, the scheme of operation shown diagrammatically in Fig. 8. By inspection of this figure it will be seen that, for the assay

of Na and K in a single specimen of serum, about 50 steps are involved, each one of which could lead to an error. Simplification of the overall procedure will be considered stage by stage in the next four paragraphs.

Serum dilution can be performed rapidly with a Filamatic diluter, with no possibility of contamination and with a CV of less than 1%. To eliminate contamination after dilution, disposable tubes may be used to collect the diluted sample and, to minimize translocation errors, both the serum tube and the tube containing the diluted serum remain in the same rack for the whole procedure, including the flame photometry.

Calibration of the flame-photometer galvanometer scale as normally practiced involves adjustment to zero with water. This is not necessary, because the calibration graph is still linear if the zero is considerably off-set from its mark. This off-setting of the zero enables the useful range of the Na scale to be expanded, and allows a single dilution of serum to be used for the measurement of both Na and K. A feature of flame-photometry is the fact that the slope of a calibration graph does not change unless parts of the instrument are changed, although the graph may "drift" along the axis which shows the galvanometer readings. Because of this constant slope, the galvanometer scale may be calibrated in terms of Na and K, and readings (in mEq/liter) may be made directly. Only one standard need be used regularly to readjust the spots on the scales, in which case the single standard contains both Na and K.

Presentation of the diluted sample to the flame photometer can make use of an automatic device (details available from Evans Electro-Selenium Ltd.), but a preferable alternative is to attach a polyethylene capillary tube (30 cm long) to the atomizer intake; this tube may then be inserted into the diluted samples or into the standard without removing the individual containers from the sample rack. Carry-over from one specimen to the next is negligible.

For the simultaneous assay of sodium and potassium, the installation of a second photocell and filter, on the opposite side of the flame and registering on a second galvanometer, allows readings to be taken for Na and K with a single aspiration of the sample.

Use of the various modifications of the procedure shown in Fig. 8 produces a simplified overall technique that is shown diagrammatically in Fig. 9. The number of separate steps required has been reduced to seven, all of which are relatively simple and free from error, with the exception of reading the galvanometer. A technician can assay 37 sera (74 measurements) in 1 hour. This may be compared with the Auto-Analyzer flame unit, working at the commonly used rate of 60 samples per hour (including standards), where there is the subsequent need to interpret the recorder trace before results can be reported.

Other examples of techniques in which work simplification has proved particularly valuable include enzyme assays. These usually require dilution of a serum sample with a buffered substrate, followed by incubation and subsequent addition of further reagents to develop a color. As one example, Table 2 shows in simplified form a method of assay for aspartate aminotransferase. The first three steps, entailing the addition of multiples of a constant volume, may be rapidly and accurately performed with an Analmatic proportioning pipet.

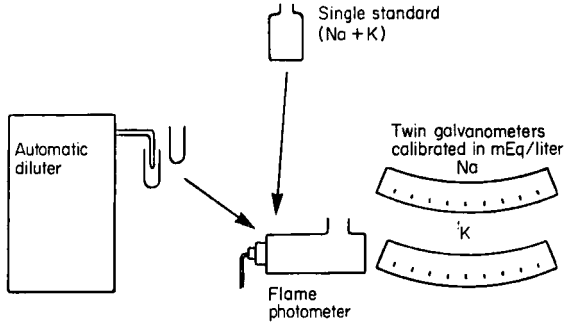


FIG. 9. Diagrammatic representation of the flame-photometric technique for the assay of Na and K in a single specimen of serum, work simplified [Mitchell *et al.* (M10)].

TABLE 2
SIMPLIFIED LIST OF MEASUREMENTS REQUIRED FOR THE ASSAY OF ASPARTATE AMINOTRANSFERASE

1 vol. serum (approx. 0.2ml)
5 vol. substrate
(60-minute incubation)
5 vol. dinitrophenylhydrazine
(20-minute color development)
10 ml 0.4 *N* NaOH
Measure OD at 510 mμ

A further application for sample diluters arises when an initial extraction or dilution is required before the normal sequential operations of an AutoAnalyzer can be started. Examples of this can be considered in relation to the assay of serum cholesterol and blood glucose. For the AutoAnalyzer serum cholesterol method an initial extraction is required before the extract can be placed in the AutoAnalyzer cup, and difficulties can arise if an automatic diluter is used to perform this, since the extracting fluid precipitates the serum proteins at the interface in the diluter

tube, and this precipitate is not easily washed out. These difficulties can be overcome by using a wide-bore tube, a large volume of extracting fluid, and a forceful ejection; the Griffin Diluspence meets these requirements. With glucose determinations on capillary samples of blood, using the AutoAnalyzer, the sample as a rule has first to be diluted so that a reasonable volume of fluid can be presented to the AutoAnalyzer probe; this dilution may be performed with the Filamatic diluter or other similar instrument.

5.4.3. *Automatic Analysis: General Considerations*

There can be little doubt that the introduction of automatic systems of analysis into the clinical chemistry laboratory has had more far-reaching consequences than any other single recent development in the practice of this branch of scientific medicine. Automatic analysis is defined for the purposes of this review as the mechanization of manual methods of analysis, without the result of the analysis necessarily being used for control of the process (R5). It has influenced the quality control requirements of clinical laboratories in several ways; some of these are connected with all forms of mechanization, though others are relevant only to mechanical systems that employ special principles of operation.

Common to all analytical procedures (manual, automatic, etc.) is the initial careful measurement of a volume of fluid (in clinical chemistry usually blood, serum, plasma, or urine) as well as volumes of standardizing solutions; the accuracy and precision of this single operation are probably the factors that most affect the reliability of the whole procedure for any particular type of analysis. Several different sorts of error may be introduced at this stage: the absolute volume of sample measured for each of a batch of replicate analyses may be incorrect; the variation from one member of a batch to another in respect of the volume of sample taken may be outside the limits acceptable for the analysis; and, when batches of specimens are analyzed, there may be cross-contamination of one specimen with material remaining in the system from the analysis of another specimen.

All the automatic systems of analysis that have so far gained acceptance in clinical laboratories, or have been projected, include a device for the successive, mechanical measurement of samples in a batch, and these systems have substantially eliminated variations in volume of sample taken from different specimens. In other words, the precision of these sampling devices falls, or can be made to fall, within the desired limits for the analysis. However, if the absolute amount of sample taken needs to be known, calibration is required and the possibility of a variation of the volume taken with time must be recognized. Mechanical samplers

are also a potential source of carry-over or cross-contamination errors, and the extent to which this type of error can occur is a function of the instrumental design.

Subsequent stages in mechanical analysis also offer increased reliability by eliminating variations from sample to sample in the volume of reagents added, a danger always present potentially with repetitive pipeting by a human operator. The need to calibrate all these stages remains, however, and again the possibility of alteration in calibration when a large batch of samples is being processed sequentially (i.e., instrumental "drift") must be acknowledged, and procedures, such as the analysis of standard solutions processed intercurrently with the unknown samples, should be adopted to monitor drift.

All automatic analytical systems for use in clinical chemistry begin with the presentation of a large number of calibration standards, control samples, and patients' specimens to the sampling head in a sequence determined by their relative positions on a turntable or series of racks, and end with the passage of the reactants through a measuring module coupled to a pen-recorder or print-out unit that produces a record of results for the individual samples, etc. Although the correlation of the sequence of specimens with the output of results is, in principle, a straightforward matter, in practice the possibility of misalignment of these sequences is a real one, arising, for example, from the omission from the record of a peak signal or printed result as a consequence of instrumental failure or insufficient sample volume, etc., and the operator or instrument designer must recognize this risk and institute a system of checks to eliminate it. In their simplest form, these safeguards may consist of the operator verifying that the number of results in the record corresponds with the number of samples, and that series of samples in which the values bear a known relationship to each other (e.g., an ascending or descending run of calibration standards) appear in their expected positions. With more sophisticated systems, where the output of the analytical measuring module is processed by a computer, the program provides for the application of checks of this type (B4, F3).

5.4.3.1. *Automatic Continuous-Flow Analysis.* At the time of writing, the only system of automatic analysis to have gained widespread acceptance in clinical chemistry laboratories employs the principle of continuous flow devised by Skeggs (S4) and incorporated into the AutoAnalyzer. Application of the AutoAnalyzer to large batches of samples can materially improve the reproducibility of the results (Section 6.2). The principles of the AutoAnalyzer are by now well known and do not require recapitatulation; relevant features of the functioning of the basic process modules will, however, be discussed. Other more specialized modules

such as a flame photometer, digestor unit, fluorometer, etc., are available, but the structural and operational features that affect reliability can be considered in relation to the basic units.

The volume of sample withdrawn by the sampling probe depends on the period for which the probe is inserted in the liquid, which is inversely proportional to the sampling rate, the bore of the sample-line tubing where it passes through the pump, and the rate of rotation of the pump head. In practice, the rate of rotation is regarded as constant, and the volume of sample withdrawn is varied by altering the first two factors. For a given arrangement of individual modules, changes in the absolute value of these variable factors can affect the accuracy and reproducibility of the analyses, even though calibration standards are analyzed as part of the same batch, since any variation during an analytical run will result in a change in calibration and produce instrumental drift. In a recent survey by the Association of Clinical Biochemists (N2), the speed of the proportioning pump head in 29 different pumps was found to be very constant, but it was suggested that variation in speed could occur in a worn pump that had been stressed by the use of large manifolds and by increasing the platen pressure, thereby giving rise to drift. A more likely cause of drift would seem to be changes in the elasticity, and hence the effective bore, of pump tubing during a run. Instrumental drift may be progressive in one direction or may be variable, and its importance is greater in some types of analysis than in others. The corrections required because of drift have been discussed by Thiers and Oglesby (T2).

The volume of sample aspirated by the sampler is independent of the amount of liquid in the sample cup, provided there is more than the minimum required for the analysis. However, Thiers and Oglesby (T2) found a correlation between the depth of sample in the cup and the corresponding peak height on the chart record, and the variation was sufficiently significant to affect accuracy and precision. This effect could result from the reduced period of aspiration of sample when the liquid level is low; in the original form of AutoAnalyzer sampler, the crook carrying the sample probe moved slowly. With the later forms of sampler, the movement of the crook from the raised to the aspirating position is much more rapid, and the effect of variation in sample depth on peak height has been largely eliminated.

Since only a proportion (usually less than 35%) of the diffusible constituents of the sample passes across the dialysis membrane, the main influence of this module, as far as a discussion of accuracy and precision is concerned, is in reducing the sensitivity of the method. This lowered sensitivity may result in a reduction of accuracy if the background against which the analysis is conducted (i.e., "blank" color, instrumental

"noise," etc.) begins to approach the level of the "signal" (e.g., color) given by the constituent being estimated. Because dialysis reduces the sensitivity of several determinations, when performed by continuous-flow techniques, there is a temptation to favor alternative methods that do not require deproteinization; when this is done it is essential to assess the specificity of the modified procedure. Probably all components of the AutoAnalyzer system contribute to a phenomenon characteristic of continuous-flow automatic analysis, namely, the tendency of one sample to influence the result of the analysis of the following sample. This interaction is most marked when a sample containing a high value of the constituent being determined precedes one in which the concentration is much lower (Fig. 10). Thiers and Oglesby (T2) have demonstrated that interaction is directly proportional to the concentration of the preceding sample and is independent of the concentration of the measured sample, and that, as a first approximation, any given sample interacts only with the one immediately following it. These authors suggested a procedure for assessing and correcting for interaction in a given analytical method. As well as depending on the relative concentrations of the samples being analyzed, the degree of interaction is a function of the rate of sampling, and depends on whether a water wash is introduced between samples. The use of a water wash, as adopted in the later models of AutoAnalyzer sampler units, markedly reduces the degree of interaction (Fig. 10).

The introduction of AutoAnalyzer equipment has resulted in a considerable degree of standardization in the practice of clinical chemistry laboratories. However, it is well known that when the same techniques and reagents are used with different AutoAnalyzer modules the results, as evidenced by the standard curves, may vary markedly from one set of equipment to another. From a survey conducted to identify the source of this variation, Northam (N2) concluded that, excluding variation in pump-tube sizes, the most likely cause was the considerable differences found in heating-bath coil volumes; he also recommended that a standard practice should be followed when publishing details about AutoAnalyzer methods, including sufficient information about standard curves in these descriptions to allow other laboratories to compare their own performance directly.

A recent development in the application of continuous-flow analysis has been the grouping of analyses of related diagnostic significance into multichannel equipment, so that a specimen is analyzed simultaneously for several constituents after only a single sampling operation (S5). A more advanced form of this technique is illustrated by the Technicon SMA-12 analyzer (Fig. 11), which performs 12 different analyses

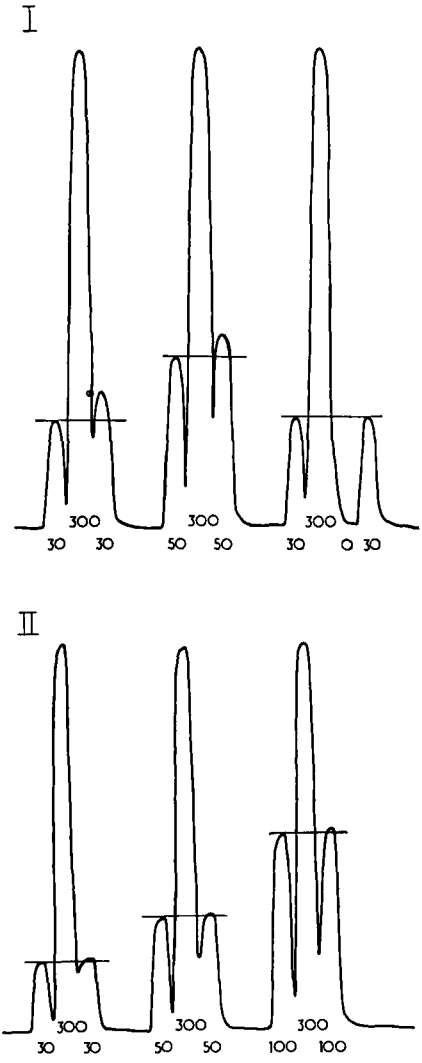


FIG. 10. AutoAnalyzer recorder tracings illustrating interaction between samples when urea solutions of the concentrations indicated (mg/100 ml) are analyzed sequentially at a sampling rate of 40/hour. When a Sampler Type I is used (I) interaction is eliminated by a water wash between samples (right), but with a reduction to half the effective rate of sampling. The Sampler II, which incorporates a water wash in its functioning, virtually eliminates interaction without a reduction in sampling rate (II).

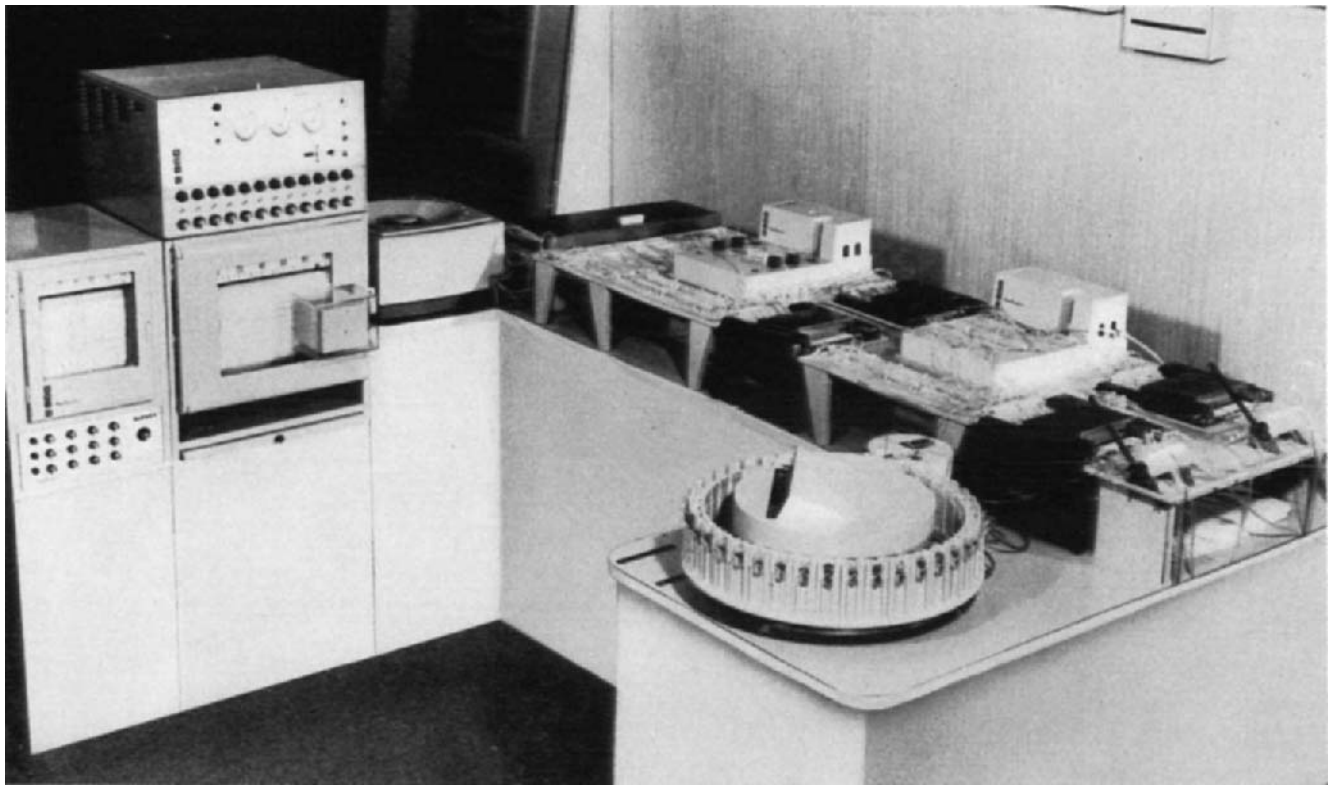


FIG. 11. Technicon 12-channel sequential multiple analyzer (SMA-12). The instrument illustrated is the Survey model equipped with machine-readable (IBM punch card) sample identification (by courtesy of Technicon Instruments Co. Ltd., Chertsey, Surrey, England).

simultaneously on each specimen (W5). Multichannel analyzers present problems in the field of quality control somewhat different from those associated with the operation of single-channel machines, in view of their special requirements for appropriate calibration and drift-correction standards (Section 3.1.1.3). Since a single sampling operation is carried out for all 12 analyses of the unknown samples, a logical step is to provide calibration and drift-correction standards that contain known amounts of each of the constituents being determined. Compared with the use of two or more standards, containing fewer than the 12 constituents, this procedure is more economical of spaces on the sampler plate and helps to retain the maximum throughput of unknown samples. A multiple standard of this nature, placed in every tenth position on the sampler plate, is used to correct for drift on the SMA-12 machine. Standards of this nature are usually secondary standards, since their composition is determined by analysis rather than by weighing-in of pure constituents. Initially, therefore, they are only as good as their calibration, and it is essential also to take account of possible differences in the rates of deterioration of the several constituents of the standard serum.

A further problem in multichannel analysis is encountered when establishing calibration curves for each of the constituents being determined, since solutions having a range of known composition must be provided for each of the constituents. This problem becomes increasingly difficult as the number of channels of simultaneous analysis increases, and with the SMA-12 instrument current practice does not favor the daily calibration at several levels of the various analytical channels. Following the initial adjustment of the base line and 100% transmission settings, the recorder settings are adjusted on the basis of readings for a standard serum; thereafter the detection and correction of drift are carried out on the basis of repeat analysis of the standardizing serum every 20 minutes.

When grouping analyses, the temptation to simplify techniques in order to make the maximum use of the apparatus, by eliminating blank or control assays, is particularly strong. An example is the elimination of the blank from the estimation of total serum protein and albumin in the SMA-12 analyzer. Modifications of this nature must be shown not to affect adversely the performance of the analysis concerned (W3).

5.4.3.2. *Automatic Discontinuous and Continuous Discrete Analysis.* Although the continuous-flow system of automatic analysis has fully demonstrated its practicability and gained widespread acceptance, its principle of operation imposes a limitation on the rate of analysis because interaction between successive samples, and the magnitude of the readings

given by each sample at the sensing module (i.e., the sensitivity), are functions of the frequency of sampling. In current AutoAnalyzer practice the maximum sampling rate is considerably less than 100 samples per hour, including calibration and drift standards and quality control specimens.

For this reason the development of discrete systems of analysis has continued to attract interest; in these the analysis of a particular specimen is carried out as in manual methods in a single tube, or perhaps two tubes, and the only stages at which interaction between specimens can occur are the initial sampling step and the final measuring process (e.g., in the colorimeter). At both these stages, it is possible to safeguard against interaction, by adding reagents through the sample probe to act as a wash following pickup of a sample, and by programming the automatic colorimeter to use part of the final colored solution to wash out the cuvette. With the systems now coming into use, rates of analysis of up to 300 samples per hour are anticipated. At the time of writing, little practical experience with these second-generation automatic systems is available. As they are likely to have as profound an effect on the practice of clinical chemistry as continuous-flow analyzers, however, brief details of the developments now taking place are included here.

Discrete automatic analyzers may be divided into two categories (N3). In the discontinuous discrete systems the samples are processed in batches, with mechanized sampling, addition of reagents, measurement, etc., but with human intervention between stages to transfer the racks or turntables carrying the samples from one module to another. An example of this type of analyzer is the Mecolab system (Joyce, Loebl Co. Ltd., Gateshead-on-Tyne, England) (Fig. 12a), which processes batches of 15 samples (including standards, etc.) at one time. The sampler can withdraw as little as 15 μl with a reproducibility of $\pm 1\%$, and can then add as many as three different reagents in measured volumes at the sample-dilution stage. Up to four further reagents can be added in the reagent-addition unit. In the present form of the system, only colorimetric analyses can be carried out; when the various stages of the reaction are complete the solutions are presented on a turntable to a recording, double-beam colorimeter. The solutions are drawn in turn into the photometer cuvette automatically, the first half being used to wash out the cuvette; the colored sample then spends 5 seconds in the light path, and registration of the extinction as an average value over a timed period begins after 2.5 seconds. An analog-to-digital converter transforms the photometric data to values giving the concentrations of the constituents being assayed, and the results are printed out together with sample identification numbers. The characteristics of the analog–digital con-

verter require a linear calibration curve for the method in use; it is possible, however, to fit the colorimeter with a tapped analog potentiometer, and to generate a function which can then be used to correct the results on the print-out for nonlinearity of the calibration curve. No data about the performance of the Mecolab system, and particularly about drift during prolonged operation, are available.

The second category of discrete automatic analyzers consists of those machines in which transfers of the samples from one stage in the analytical process to the next do not require the intervention of the operator. Examples in this class currently available are the Robot Chemist (Warner-Chilcott Laboratories, Instruments Division, Richmond, Calif.)

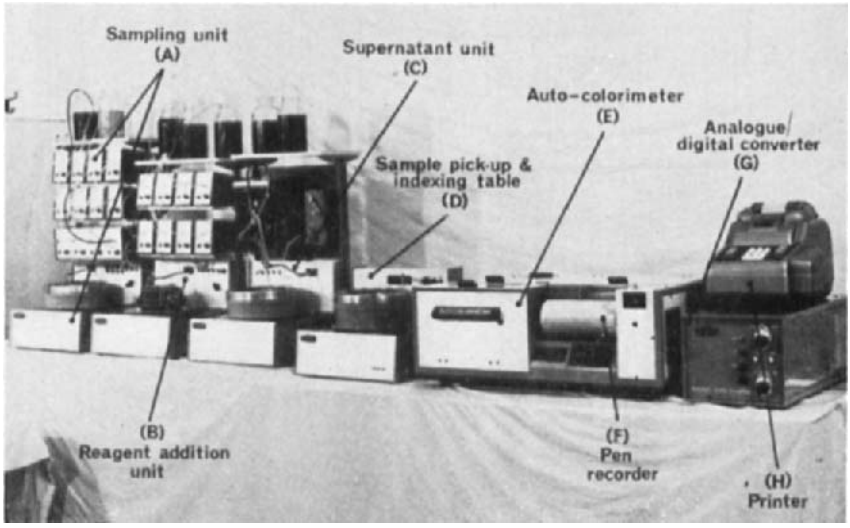


FIG. 12a. Mecolab, discontinuous discrete analyzer (by courtesy of Joyce, Loebl & Co. Ltd., Gateshead-on-Tyne, England).

(Fig. 12b), and the AutoChemist (AGA, Lidingo 1, Sweden). The Robot Chemist can accept batches of up to 100 specimens; after measurement of sample, reagent addition, and (if necessary) incubation, the solutions are read in a spectrophotometer and the results of the analyses are printed out with identification numbers. The manufacturers claim that the rate of analysis is up to 120 specimens per hour; sample volumes between 20 μl and 5 ml can be measured to an "accuracy" of 1%, and up to seven different reagents can be added with 0.5% "accuracy." It is further claimed that interaction between samples has been eliminated.

The AutoChemist is a large-capacity, multichannel machine capable in its basic form of carrying out 20 different analyses on each specimen. The

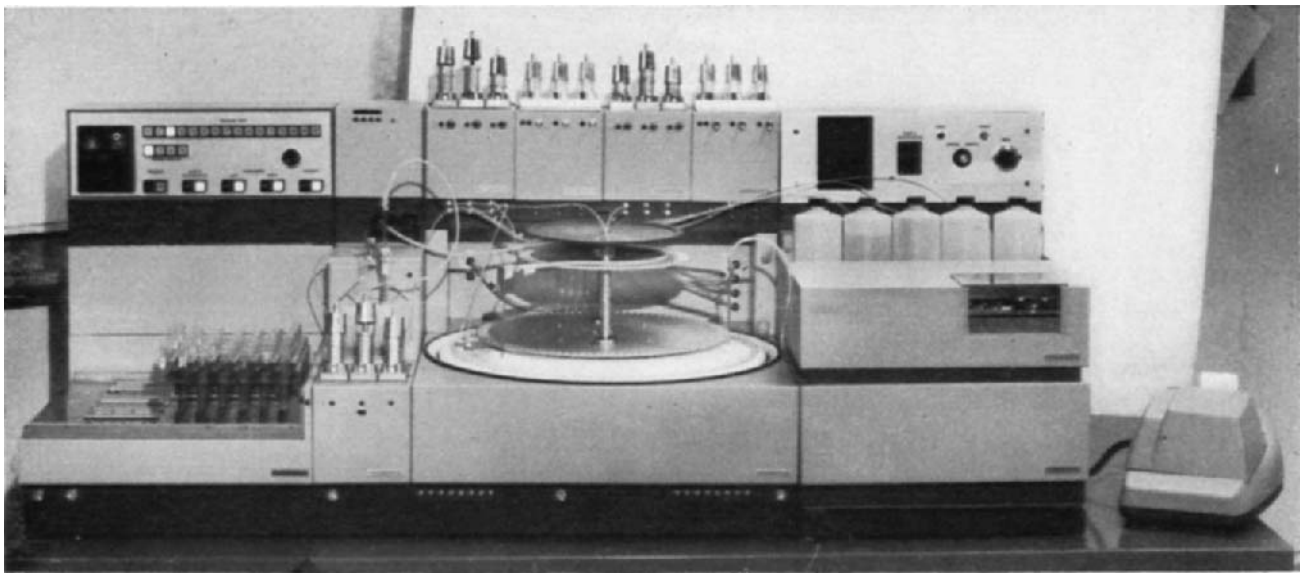


FIG. 12b. Robot Chemist, continuous discrete analyzer (by courtesy of Warner-Chilcott Laboratories Instruments Division, Richmond, Calif.).

samples are loaded in blocks of up to 100 at a time onto belts which feed into two types of analytical channel. The short channel is used for analyses in which heating is not required, whereas the long channel includes heating stages. The analysis time on the short channel is 15 minutes and on the long channel 50 minutes. The sensing device at the end of each channel is typically a colorimeter, but could be a flame photometer or fluorometer. The analytical data are processed by a computer and the results printed out. The sample racks are identified in the machine by a magnetically readable card. Although the Auto-Chemist offers data processing by an on-line computer, the capital outlay is inevitably high (approximately £120,000 or $336,000), and it remains to be seen whether its performance will be so much superior to the less expensive and more flexible modular automatic systems as to justify its greater expense.

The discrete systems of automatic analysis developed so far have not, in general, solved the problem of deproteinization in a way as satisfactory as the dialysis step adopted in the continuous-flow system. An earlier version of the Robot Chemist included a module in which precipitation of proteins followed by filtration was carried out, and the Mecolab system can incorporate a centrifugation step, following which a sample of supernatant can be automatically aspirated, but both these approaches are relatively cumbersome. Consequently attention has been directed to the development of analytical methods for blood serum or plasma that do not involve a deproteinization stage, but although alternative methods not involving the removal of protein may be feasible for many estimations, the responsibility for confirming the validity of such alternative procedures remains with the analyst.

5.5. Data Processing and Computer Techniques

The stages of data handling in clinical laboratories include (1) the computation of instrumental readings to give final results, corrected where necessary for instrumental or other analytical error, (2) the correlation of these results with the corresponding report form and the correct transfer of the findings onto the report, (3) transmission of the results (as reports) to wards, outpatient clinics, and departments, etc., and (4) the classification and storage of the results so that they can be retrieved subsequently by specific or class retrieval in accordance with a number of reference categories—e.g., patient's name, type of analysis, diagnosis, etc. At all these stages, factors can operate that affect the quality of the data produced by the laboratory, and these are particularly relevant in the first three stages of data handling.

Mechanical means of sorting and classifying large numbers of numeri-

cal data are not new and, with the introduction of the electronic digital computer, these possibilities for mechanization of data handling have been greatly extended; they now include the facility for rapid, error-free complex mathematical calculations. Great advances have also taken place in the recording and transmission of data without need for manual transcription. Until comparatively recently, clinical chemists have not felt the need to explore the application of these advances in data handling but, with the continuing increase in output of work from clinical laboratories, the rate of data processing has become a factor limiting the further expansion of the laboratory service. The following sections list some of the solutions to these problems that are currently being explored, with particular reference to their effects on the quality of results.

5.5.1. *Off-Line Systems*

Mechanical systems of sorting and transcribing laboratory data reduce the possibility of errors from incorrect assignment of results to individual patients, and inaccurate copying of results from laboratory work sheets to report forms. Systems of varying degrees of complexity using punch cards have been described (J2, P1, W1, W6). In some of these systems, analytical results, after calculation, are punched into cards which can then be used to produce printed reports of the pay-slip variety by means of an electric typewriter. Another approach is for the analyst to enter readings from instruments directly onto the cards (by a key punch, or by using an electrographic pencil), and the cards are then used as input to a computer for conversion of these data into analytical results.

An extension of these off-line systems dispenses with the manual stage of transcription of instrumental readings, replacing this by a device that translates the output of analytical equipment, such as pen recorders, into a form suitable for subsequent use as input to a computer. In the ALA system developed by Flynn *et al.* (F5), the pen recorders of several AutoAnalyzer channels (urea, Na, K, Cl, and bicarbonate) are monitored by separate peak-picking devices and associated circuits that record the fall-away from a peak value of the recorder trace, together with identifying data relating to the position of the sample on the AutoAnalyzer turntable and the nature of the analysis, all in coded form on 5-hole punched paper tape. This data tape, bearing information about the output from the AutoAnalyzers, and another tape prepared separately carrying details about the patient (name, age, ward, etc.), are input to the computer, where the program first finds the standards and assesses their acceptability, after which it corrects individual readings from patients' samples for the drift-correction standards, and then calculates the concentrations of the various components in the individual samples. In

printing out these final results, which appear on the output tape matched up with the identification data about the patient, the computer has also been programmed to indicate those samples where the analytical findings will have been adversely affected by interaction or carry-over from the previous specimen. As a result of minor programming modifications adopted in this laboratory, the print-out of results (Fig. 4) here is accompanied by an indication of the difference between the total sum for plasma sodium and potassium and chloride and bicarbonate figures, as well as an alphabetical coding for rapid visual assessment of the pattern of results, as suggested by Lindberg *et al.* (L4).

5.5.2. *On-Line Systems*

Data processed by off-line systems have the disadvantage that they provide information retrospectively about an analytical process that has been completed. Any indication of deteriorating performance, as shown for example by control samples, therefore cannot be used to initiate corrective action. On-line systems, in which data are continuously processed as they emerge from the measuring modules of the instruments, provide an opportunity for feedback control of the analytical process, as well as for reducing the overall time lapse between starting the analysis and reporting the results.

Several on-line data-processing systems for use in clinical chemistry have been explored, and the main area of variation between them is in the degree of complexity of the units used to translate instrumental readings into final results. These may range from analog-to-digital converters that are calibrated by known standards (as in the Mecolab and Robot Chemist systems) to data-acquisition systems coupled directly to a digital computer, as with the AutoChemist or the system described by Blaivas (B4) or the Technicon on-line computer control system (G3a). The main advantage of the use of digital computers over the simpler systems lies in the number of simplifying approximations that must be made in each case. When only a simple analog-to-digital conversion unit is employed, the assumption must be made that the shape and slope of a calibration curve remain constant until reset by fresh standard solutions. A digital computer, on the other hand, is able to evaluate and act on changes affecting the calibration curve, as revealed for instance by drift-correction standards, and to correct the intervening unknown samples accordingly. When data are presented in the form of continuously varying signals reaching a peak value, as in the Auto-Analyzer, the criteria applied to digitizing a peak, recognizing its maximum value and accepting the quality of its shape, can be more strict when a digital computer is used (B4). At the present time no on-line

systems have been described in clinical chemistry in which data are fed back from a controlling computer to regulate the analytical process, except through the intervention of a human operator.

6. Results of the Application of Work Simplification, Mechanization, and Automation

6.1. The Advantages and Results of Introducing Work Simplification

The advantages of work simplification of all types may be summarized as follows:

(1) Greater reproducibility: a relatively unskilled person can achieve and maintain the precision of a skilled individual.

(2) Increased speed of operation with less strain on the worker.

(3) The use of smaller samples is often practicable (e.g., for pediatric work).

All these have an impact upon the reliability of the laboratory worker, and many of these advantages may be demonstrated by the results of the examples for work simplification (given in Section 5.4.2.2).

Table 3 shows the results of introducing work simplification in the

TABLE 3
Reproducibility of Results for the Assay of Serum Sodium and Potassium by Flame Photometry[a]

	Coefficients of variation			Variance ratios	
	Manual technique (M)	Work-simplified technique (WS)	Auto-Analyzer (AA)	M/WS	WS/AA
Sodium	4.5	3.3	2.7	1.86	1.56
Potassium	3.3	1.6	1.5	4.26[b]	1.18

[a] Mitchell *et al.* (M10).

[b] Variance ratio significant at the 1% level.

operation of the E.E.L. flame-photometer (M10). There is considerable improvement in the CV when the fully manual method is replaced by the work-simplified technique; the figures obtained by using the Auto-Analyzer flame unit are approached. The required dilution of 1 in 100 is achieved by diluting 0.08 ml of serum to 8 ml; most manual methods in routine use require 0.2 ml of serum.

Table 4 shows the improvement obtained in the assay of serum as-

TABLE 4
REPRODUCIBILITY OF RESULTS FOR THE ASSAY OF SERUM ASPARTATE AMINOTRANSFERASE[a]

Technician	Coefficients of variation		Variance ratios		
	Manual technique (M)	Work-simplified technique (WS)	M/WS	Senior/student (M)	Senior/student (WS)
Senior	2.00	2.15	1.12	4.02[b]	1.16
Student	4.01	1.96	4.12[b]		

[a] Mitchell *et al.* (M10).
[b] Variance ratio significant at the 5% level.

partate aminotransferase, and Table 5 shows the improvement in CV for assay of plasma cholesterol by the AutoAnalyzer when the initial extraction of the plasma is carried out by an automatic diluter; the amount of plasma required is also considerably reduced.

In a similar way, Fig. 13 shows the effect on the AutoAnalyzer record for the assay of blood glucose of carrying out an initial dilution of the sample in three different ways. In the first procedure, both the blood sample and the diluting fluid were measured by conventional pipets, but in the second, the diluting fluid was added from an automatic dispenser. In the third, sampling and dilution were carried out completely automatically, using the Filamatic diluter.

TABLE 5
REPRODUCIBILITY OF RESULTS FOR THE ASSAY OF PLASMA CHOLESTEROL BY AUTOANALYZER[a]

Technician	Initial plasma extraction by		Variance ratios	
	Manual pipeting (M) (0.5 ml of plasma, 4.5 ml of isopropanol) (CV)	Automatic dilution (A) (0.2 ml of plasma, 1.8 ml of isopropanol) (CV)	M/A	Senior/student (M)
Senior	3.5	2.7	1.7	2.01[c]
Student	5.0	2.7	3.5[b]	

[a] Mitchell *et al.* (M10).
[b] Variance ratio significant at the 1% level.
[c] Variance ratio significant at the 5% level.

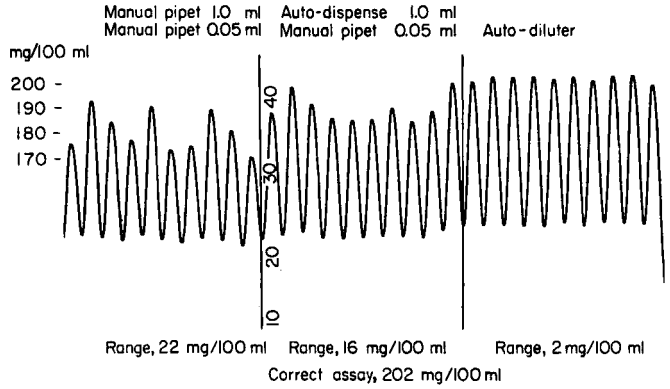


FIG. 13. AutoAnalyzer trace for blood glucose assayed by a glucose oxidase technique, showing the effect of three different methods (described in text) of making the initial dilution of the blood sample; the assumed true value for the glucose content of the blood was 202 mg/100 ml [Mitchell *et al.* (M10)].

6.2. THE ADVANTAGES AND RESULTS OF INTRODUCING AUTOMATIC ANALYSIS

Automatic analysis may be considered an extension of the techniques of work simplification discussed (Sections 5.4.2 and 6.1), and as such possesses the same advantages to a greater degree. Data will be presented for AutoAnalyzer performance only, since corresponding information for alternative systems is not yet available.

Table 6 shows the SD for several determinations using manual methods, and compares these with two sets of data for AutoAnalyzer operation, (a) during the first month after introduction of the automated method, and (b) 6 months after the changeover; progressive improvement in the SD has occurred in several cases. This is not always to be found, however, and Table 7 shows the results for long-term quality control assessment of five other analyses carried out on the AutoAnalyzer; in all cases the SD shows some deterioration, and this serves to emphasize the need to calculate the SD at regular intervals (Section 3.3.3). This deterioration during long-term operation of a method is probably the result of undetected relaxation of laboratory discipline (e.g., decrease in the care taken when reading AutoAnalyzer charts in a particularly busy sector of the laboratory).

The error obtained with serum calcium assays by the Clark and Collip (C6) method requires special comment (Table 6). This figure was recorded at a time when the technicians were grossly overworked; intro-

TABLE 6
QUALITY CONTROL RESULTS USING MANUAL AND AUTOMATED METHODS[a]

Determination	Reference value	Manual techniques		Automated techniques	
		Result (SD)	Method ref.	Result[b] (SD)	Result[c] (SD)
Alkaline phosphatase (K.A. units/100 ml)	13	1.8	(K1)	1.5	1.0
Bilirubin (mg/100 ml)	1.2	0.25	(V2)	0.15	0.13
Cholesterol (mg/100 ml)	175	14	(J4)	8[d]	8[d]
Uric acid (mg/100 ml)	4.8	0.8	(E1)	—	0.45
Creatinine (mg/100 ml)	0.79	—		0.14	0.06
Phosphate (mg/100 ml)	3.4	0.6	(H1a)	0.2	0.2
PBI (μg/100 ml)	4.9	—		0.3[e]	0.2[f]
Iron (μg/100 ml)	130	19[g]	(R2)	4.1	5.6
		7.7[g]	(C2)		
Calcium (mg/100 ml)	10.0	1.4[g]	(C6)	0.4	0.23
		0.6[g]	(T5)		

[a] Mitchell (M9).
[b] The SD of results obtained during the first month, using the AutoAnalyzer.
[c] The SD of results obtained during the sixth month, using the AutoAnalyzer.
[d] Preliminary extraction done, using an automatic diluter (Section 5.4.2.2).
[e] Colorimetric part of technique done by AutoAnalyzer.
[f] Complete technique done by AutoAnalyzer.
[g] Bench standard sera only used.

TABLE 7
QUALITY CONTROL RESULTS USING AUTOMATED METHODS[a]

Determination	Results at commencement of control		Results after 6 months	
	Reference value	Result (SD)	Reference value	Result (SD)
Urea (mg/100 ml)	18	1.06 (5.9%)	52	1.9 (3.6%)
	120	1.9 (1.6%)	262	6.7 (2.6%)
	170	3.4 (2.0%)		
	219	3.3 (1.5%)		
Sodium (mEq/liter)	135	1.5	142	1.8
Potassium (mEq/liter)	4.6	0.10	5.1	0.12
Chloride (mEq/liter)	100	1.5	107	2.1
Total protein (g/100 ml)	6.0	0.15	6.0	0.20

[a] Mitchell (M9).

duction of a simpler manual technique (T5) significantly reduced the error, but more acceptable results were obtained only by introduction of an AutoAnalyzer method. Both manual methods are widely accepted as satisfactory when carried out under favorable conditions, but this example emphasizes the fact that even competent staff when overworked can obtain results that are essentially valueless and, predictably, the more complicated the technique the greater chance for error from this source. Data have been presented elsewhere (H5) indicating the adverse effects of increasing workloads on serum calcium determinations. It is interesting to compare the reproducibility of these calcium methods with results reported when an atomic absorption spectrophotometer is used (S11); with the standard Jarrell-Ash instrument, these authors obtained a CV of 3.8% (i.e., an SD of ±0.38 mg/100 ml) and this figure narrowed to 1.1% (an SD of ±0.11 mg/100 ml) after the installation of a Zeiss burner.

A similar but less striking example is illustrated by the results for serum iron determinations (Table 6). The Caraway method (C2) has a smaller number of manipulations and gives better reproducibility than Ramsay's method (R2), but automation improves upon both of them.

6.3. Preliminary Results of the Application of Computer Control

The particular value of mechanical data-processing equipment and of computers in the rapid performance of statistical calculations based on laboratory data has been discussed (Section 3.3.4), as has their contribution to easing the burden of collecting data from measuring instruments, the performing of repetitive calculations, and the preparation of reports for laboratory work (Section 5.5). This section will consider the few examples that at present provide objective evidence for improvements in the reliability of laboratory work attributable to the introduction of modern data-processing equipment.

McKay *et al.* (M7), using a modified double-beam photometer, compared the results for a series of hemoglobin determinations calculated by conventional means with the corresponding results obtained by both an analog and two different digital computer programs. They showed that the data obtained by all three of the computer systems compared well with the manually obtained results, and concluded that a relatively inexpensive analog computer could be designed for the performance of photometric calculations with much increased speed and with the elimination of human sources of error in this type of calculation.

Lotito *et al.* (L7) later described the application of a similar data-acquisition system for accepting signals obtained during the scanning of electrophoresis strips; the data were stored as individual integration

voltages, and these stored voltages were subsequently used (in separate stages of the program) to compute the percentage contributed by the individual protein fractions, and to present these graphically for reporting purposes. The repeatability of the results proved very satisfactory, and the savings in time and labor were again emphasized.

In the ALA system described by Flynn *et al.* (F5), the accuracy of the readings obtained by visual assessment of the chart records was compared with the values punched on the paper tape. Only minor differences were reported, but these comparisons of accuracy were less valid (since the observer records the height of the peak, whereas the ALA system punches the reading of the pen after it has fallen away by a predetermined amount from the peak position) than the earlier set of comparisons (F3), which recorded a close degree of agreement between manually produced and computer-produced results. For long series of results, the computer-prepared data had clear advantages, since the program allowed the regular application of correction factors which become tedious to apply manually, and the overall time required for the actual computation of results and preparation of reports was greatly reduced. In our experience, however, these advantages are to be set against the major shortcoming of this particular off-line system of data processing that derives from the fact that none of the data can be computed until the end of a run of analyses. It sometimes happens, therefore, that a whole series of results have to be calculated manually as a matter of extreme urgency if, at the end of the run (which with plasma urea and electrolytes also means near the end of the working day), some fault in the operation of the AutoAnalyzers or in the working of the computer or its peripheral equipment is discovered. In addition, results requiring urgent treatment during the day have to be reported by conventional means.

On-line computer systems have also been evaluated in a limited number of clinical chemistry laboratories, and these systems have great potential in process control (e.g., for validation of peaks and rejection of artifacts), as well as in data processing (Section 5.5). It is stated that the results produced by them compare acceptably with those obtained by conventional means (B4, C3), but data substantiating these claims have yet to be published.

7. Conclusions

Medicine is becoming increasingly an objective and quantitative science, and like other sciences demands a realistic appraisal of the value of the numerical data upon which its conclusions are based. No longer is it permissible to regard a result of a blood glucose estimation, for instance, as an absolute value beyond question. Instead, such a figure must

be seen as an approximation, made with a stated probability of its correctness, to the biochemical state of the patient at the moment when the specimen was collected. The clinical chemist can contribute to this attitude by his ability to define the accuracy and precision which should be attributed to his analytical techniques. For his part, the clinician must learn to appreciate that changes in the chemical composition of samples from patients are not to be regarded as significant unless they exceed the range of variation inherent in the analytical method, or in normal fluctuations of the individual's biochemical makeup; in addition he must take into account the distribution of values found within the normal population.

In deciding on the confidence limits appropriate for his analyses, the clinical chemist will take into account several factors. The first and most important of these is the effect on the clinical value of the result of variations in each method's degree of reliability; if, because of a narrow normal range for a particular blood constituent, small variations from normal are of considerable diagnostic significance (as is the case for serum calcium), he must endeavor to ensure that the total error of his estimations is such that these small fluctuations can be detected. In general, however, other factors, such as expense in terms of staff and equipment and the reduction in clinical usefulness of chemical results unduly delayed, may dictate a compromise between the speed of a technique and its reported ability to provide results to a greater number of significant figures. It must again be emphasized that urgency is never an excuse for failing to meet the standards of accuracy and precision demanded by the clinical situation.

We would also draw attention once more to the growing dependence on chemical investigations for the early recognition of disease or of a tendency to develop disease. The emphasis in the various surveys which form the basis for this statement (e.g., C7, J3) has been on quantitative chemical measurements, the object in some cases (e.g., regular insurance checks) being to detect changes or trends in individuals attending for follow-up assessment. If the hypothesis is true that for many diseases there is a presymptomatic stage showing biochemical abnormalities, the ability to detect this will clearly depend on laboratory work that conforms to high standards, particularly with respect to reproducibility; this will necessarily place great emphasis on a searching program of quality control. Acceptance of van Peenen and Lindberg's (V1) contention that the clinical state of the patient is at present the most effective means of quality control would mean rejection of the concept of possible presymptomatic recognition of many diseases by means of chemical tests, and would serve only to delay developments in the field of quantitative, and especially preventative, medicine.

References

A1. Alcock, N., MacIntyre, I., and Radde, I., The determination of magnesium in biological fluids and tissues by flame spectrophotometry. *J. Clin. Pathol.* **13,** 506–510 (1960).

B1. Belk, W. P., and Sunderman, F. W., A survey of the accuracy of chemical analyses in clinical laboratories. *Am. J. Clin. Pathol.* **17,** 853–861 (1947).

B1a. Benenson, A. S., Thompson, H. L., and Klugerman, M. R., Application of laboratory controls in clinical chemistry. *Am. J. Clin. Pathol.* **25,** 575–584 (1955).

B2. Berry, R. E., A comparative study of laboratory performance using frozen serum unknowns. *Am. J. Clin. Pathol.* **46,** 193–197 (1966).

B2a. Berry, R. E., An analysis of some components of variation in clinical chemistry. *Am. J. Clin. Pathol.* **47,** 337–343 (1967).

B3. Birchall, K., Cathro, D. M., Forsyth, C. C., and Mitchell, F. L., A method for the separation and estimation of neutral steroids in the urine of newborn infants. *J. Endocrinol.* **27,** 31–51 (1963).

B4. Blaivas, M. A., Application of a process control computer in the automated clinical chemistry laboratory. *In* "Automation in Analytical Chemistry" (L. T. Skeggs, Jr., ed.), pp. 452–454. Mediad, New York, 1966.

B5. Borth, R., Steroids in human blood. *Vitamins Hormones* **15,** 259–290 (1957).

B6. Broughton, P. M. G., and Gowenlock, A. H., "Control Solutions for Clinical Biochemistry," Tech. Bull. No. 4. Assoc. Clin. Biochem. Sci. Comm., 1965.

B7. Brown, J. B., Bulbrook, R. D., and Greenwood, F. C., An evaluation of a chemical method for the estimation of oestriol, oestrone and oestradiol-17β in human urine. *J. Endocrinol.* **16,** 41–48 (1957).

B7a. Burgess, A. M., Fejfar, Z., and Kagan, A., Arterial hypertension and ischaemic heart disease: comparison in epidemiological studies, pp. 10–11. World Health Organization, Geneva, 1963.

B8. Bywaters, E. G. L., and Holloway, V. P., Measurement of serum uric acid in Great Britain in 1963. *Ann. Rheumatic Diseases* **23,** 236–239 (1964).

C1. Campbell, D. G., and Annan, W., Precision of plasma urea and electrolytes estimated by the AutoAnalyzer. *J. Clin. Pathol.* **19,** 513–516 (1966).

C2. Caraway, W. T., Macro and micro methods for the determination of serum iron and iron-binding capacity. *Clin. Chem.* **9,** 188–199 (1963).

C3. Cheek, C. S., Simultaneous multiple automated chemical analyses with data acquisition, storage, and retrieval. *In* "Automation in Analytical Chemistry" (L. T. Skeggs, Jr., ed.), pp. 458–462. Mediad, New York, 1966.

C4. Chiaraviglio, E. del C., Wolf, A. V., and Prentiss, P. G., Total protein/protein nitrogen ratio of human serum. *Am. J. Clin. Pathol.* **39,** 42–45 (1963).

C5. Clancey, V. J., Statistical methods in chemical analyses. *Nature* **159,** 339–340 (1947).

C6. Clark, E. P., and Collip, J. B., A study of the Tisdall method for the determination of blood serum calcium with a suggested modification. *J. Biol. Chem.* **63,** 461–464 (1925).

C7. Collen, M. F., Periodic health examinations using an automated multitest laboratory. *J. Am. Med. Assoc.* **195,** 830–833 (1966).

C8. Cotlove, E., Determination of chloride in biological materials. *Methods Biochem. Anal.* **12,** 277–391 (1964).

C9. Craig, A., Griffiths, B., and Higgins, G., A comparison of transaminase determinations by various 'kit' outfits. *Proc. Assoc. Clin. Biochem.* **4,** 109–112 (1966).

C10. Cramér, K., Cramér, H., and Selander, S., A comparative analysis between

variation in 24-hour urinary creatinine output and 24-hour urinary volume. *Clin. Chim. Acta* **15**, 331–335 (1967).

D1. Desmond, F. B., A clinical chemistry proficiency survey. *New Zealand Med. J.* **63**, 716–720 (1964).

E1. Eichhorn, F., Zelmanowski, S., Lew, E., Rutenberg, A., and Fanias, B., Improvement of the uric acid determination by the carbonate method for serum and urine. *J. Clin. Pathol.* **14**, 450–452 (1961).

F1. Fawcett, J. K., and Wynn, V., Effects of posture on plasma volume and some blood constituents. *J. Clin. Pathol.* **13**, 304–310 (1960).

F2. Flokstra, J. H., Varley, A. B., and Hagans, J. A., Reproducibility and accuracy of clinical laboratory determinations. *Am. J. Med. Sci.* **251**, 646–655 (1966).

F3. Flynn, F. V., Computer-assisted processing of biochemical test data. *In* "Progress in Medical Computing" (H. Atkins, chairman), pp. 46–51. Elliott Med. Automation, London, 1965.

F4. Flynn, F. V., Use of a computer by a clinical chemistry service. *Proc. Roy. Soc. Med.* **59**, 779–782 (1966).

F5. Flynn, F. V., Piper, K. A., and Roberts, P. K., Equipment for linking the Auto-Analyzer to an off-line computer. *J. Clin. Pathol.* **19**, 633–639 (1966).

F6. Flynn, F. V., and Vernon, J., Cumulative reporting of chemical pathology. *J. Clin. Pathol.* **18**, 678–683 (1965).

G1. Gaddie, R. The contribution of biochemistry to clinical practice. *J. Roy. Coll. Physicians (London)* **1**, 148–156 (1966).

G2. Gerbrandy, J., van Leeuwen, A. M., Hellendoorn, H. B. A., and de Vries, L. A., The binding between electrolytes and serum proteins calculated from an *in vivo* filtration method. *Clin. Sci.* **19**, 181–193 (1960).

G3. Gooszen, J. A. H., The use of control charts in the clinical laboratory. *Clin. Chim. Acta* **5**, 431–438 (1960).

G3a. Gould, H. E., and Brooks, A., Laboratory-Based Administrative Computer Monitoring System. *In* "Automation in Analytical Chemistry," Technicon Symposium 1966, Vol. I, pp. 378–382. Mediad, New York, 1967.

G4. Gowenlock, A. H., Preliminary communication. *6th Intern. Congr. Clin. Pathol., Rome, 1966.*

G5. Grant, G. H., "Control of Accuracy in Chemical Pathology," Broadsheet No. 30 [N. S.]. Assoc. Clin. Pathol., 1961.

H1. Halpin, J. F., "Zero Defects." McGraw-Hill, New York, 1967.

H1a. Harrison, G. A., "Chemical Methods in Clinical Medicine," 4th ed., p. 394. Churchill, London, 1957.

H2. Hendry, P. I. A., Proficiency survey in clinical chemistry, 1961. Report of Adelaide Meeting, pp. 8–15. College of Pathologists of Australia, 1961.

H3. Hendry, P. I. A., Proficiency survey in clinical chemistry, 1962. Report of Sydney Meeting, pp. 7–18. College of Pathologists of Australia, 1962.

H4. Hendry, P. I. A., Proficiency survey in clinical chemistry, 1963. Report of Melbourne Meeting, pp. 5–14. College of Pathologists of Australia, 1963.

H5. Hendry, P. I. A., Proficiency survey in clinical chemistry, 1964. Report of Sydney Meeting, pp. 17–25. College of Pathologists of Australia, 1964.

H6. Henry, R. J., Use of the control chart in clinical chemistry. *Clin. Chem.* **5**, 309–319 (1959).

H7. Henry, R. J., "Clinical Chemistry, Principles and Technics." Harper (Hoeber), New York, 1964.

H7a. Henry, R. J., and Segalove, M., The running of standards in clinical chemistry and the use of the control chart. *J. Clin. Pathol.* **5**, 305–311 (1952).

H8. Hersey, M. Q., Hartwell, K., and Doe, R. P., Use of a lyophilized serum enzyme preparation in quality control. *Clin. Chem.* **9**, 557–565 (1963).

H9. Hoffmann, R. G., and Waid, M. E., The number plus method of quality control of laboratory accuracy. *Am. J. Clin. Pathol.* **40**, 263–269 (1963).

H10. Hoffmann, R. G., and Waid, M. E., The "Average of Normals" method of quality control. *Am. J. Clin. Pathol.* **43**, 134–141 (1965).

H11. Hughes, H. K., Suggested nomenclature in applied spectroscopy. *Anal. Chem.* **24**, 1349–1354 (1952).

J1. Jenkins, D. J., Quality control. *J. Med. Lab. Technol.* **15**, 262–269 (1958).

J2. Jorgensen, K., Automatic processing of data in clinical chemistry and haematology. *Proc. Assoc. Clin. Biochem.* **3**, 246 (1965).

J3. Jungner, G., Chemical health screening. *In* "Surveillance and Early Diagnosis in General Practice" (G. Teeling-Smith, ed.), pp. 14–16, 48–52. Office of Health Economics, London, 1966.

J4. Jurand, J., and Albert-Recht, F., The estimation of serum cholesterol. *Clin. Chim. Acta* **7**, 522–528 (1962).

K1. King, E. J., and Wootton, I. D. P., "Micro-Analysis in Medical Biochemistry," 3rd ed., p. 83. Churchill, London, 1956.

K2. Kramer, B., and Tisdall, F. F., A simple technique for the determination of calcium and magnesium in small amounts of serum. *J. Biol. Chem.* **47**, 475–481 (1921).

L1. Lathe, G. H., and Mitchell, F. L., Staffing of clinical biochemistry laboratories serving the National Health Service. *Lancet* **I**, 1413–1415 (1966).

L2. Leading Article, The AutoAnalyzer—practical automation in wet chemistry. *Sci. Res.* **1**, No. 9, 48–50 (1966).

L2a. Lee, S., and Schoen, I. Quality control aspects of the chemistry laboratory form. *Am. J. Clin. Pathol.* **47**, 329–336 (1967).

L3. Levey, S., and Jennings, E. R., The use of control charts in the clinical laboratory. *Am. J. Clin. Pathol.* **20**, 1059–1066 (1950).

L4. Lindberg, D. A. B., van Peenen, H. J., and Couch, R. D., Patterns in clinical chemistry. *Am. J. Clin. Pathol.* **44**, 315–321 (1965).

L5. Loraine, J. A., and Bell, E. T., Hormone excretion during the normal menstrual cycle. *Lancet* **I**, 1340–1342 (1963).

L6. Loraine, J. A., and Bell, E. T., "Hormone Assays and their Clinical Application," 2nd ed. Livingstone, Edinburgh and London, 1966.

L7. Lotito, L. A., McKay, D. K., and Seligson, D., Analog computation of electrophoresis patterns. *Clin. Chem.* **11**, 386–394 (1965).

M1. MacIntyre, I., The flame-spectrophotometric determination of calcium in biological fluids and an isotopic analysis of the errors in the Kramer-Tisdall procedure. *Biochem. J.* **67**, 164–172 (1957).

M2. Mandel, J., "The Statistical Analysis of Experimental Data." Wiley (Interscience), New York, 1964.

M3. Marsh, W. H., Automation. *Advan. Clin. Chem.* **2**, 301–362 (1959).

M4. Marsh, W. H., Fingerhut, B., and Kirsch, E., Determination of urea nitrogen with the diacetyl method and an automatic dialyzing apparatus. *Am. J. Clin. Pathol.* **28**, 681–688 (1957).

M5. Mason, W. B., Some alternative paths of simplification of clinical chemistry. *Ann. N. Y. Acad. Sci.* **102**, 171–177 (1962).

M6. McClellan, E. K., Nakamura, R. M., Haas, W., Moyer, D. L., and Kunitake, G. M., Effect of pneumatic tube transport system on the validity of determinations in blood chemistry. *Am. J. Clin. Pathol.* **42**, 152–155 (1964).

M7. McKay, D. K., Lotito, L. A., Seligson, D., and Wogrin, C. A., Adaptation of a photoelectric colorimeter to a computer program for calculating and storing data in a clinical chemistry laboratory. *Clin. Chem.* **10,** 291–297 (1964).
M8. Ministry of Health, "Pneumatic Tube Communication Systems," Hosp. Tech. Mem. No. 9. H. M. Stationery Office, London, 1963.
M9. Mitchell, F. L. Quality control in a large laboratory. *Proc. Assoc. Clin. Biochem.* **4,** 38–41 (1966).
M10. Mitchell, F. L., Proffitt, J., and Annan, W., The effect of the introduction of auto-dilution on precision in a service laboratory. *Proc. Assoc. Clin. Biochem.* **4,** 71–74 (1966).
M11. Moroney, M. J., "Facts from Figures." Penguin Books, Harmondsworth, Middlesex, England, 1951.
M12. Moss, D. W., Automation in clinical biochemistry—a survey. *Med. Electron. Biol. Eng.* **3,** 227–237 (1965).
N1. Nabarro, J. D. N., The physician and the biochemist. *J. Roy. Coll. Physicians (London)* **1,** 156–163 (1966).
N2. Northam, B. E., "Variability Between AutoAnalyzer Modules," Tech. Bull. No. 7. Assoc. Clin. Biochem., Sci. Comm., 1965.
N3. Northam, B. E., Discontinuous discrete systems of analysis. *Proc. Assoc. Clin. Biochem.* **4,** 62–65 (1966).
O1. Owen, J. A., Iggo, B., Scandrett, F. J., and Stewart, C. P., The determination of creatinine in plasma or serum, and in urine; a critical examination. *Biochem. J.* **58,** 426–437 (1954).
P1. Peacock, A. C., Bunting, S. L., Brewer, D., Cotlove, E., and Williams, G. Z., Data processing in clinical chemistry. *Clin. Chem.* **11,** 595–611 (1965).
R1. Radin, N., What is a standard? *Clin. Chem.* **43,** 55–76 (1967).
R2. Ramsay, W. N. M., The determination of iron in blood plasma or serum. *Clin. Chim. Acta* **2,** 214–220 (1957).
R3. Report of a study carried out by the Division for Architectural Studies of the Nuffield Foundation, "The Design of Research Laboratories" (L. Farrer-Brown, chairman), Oxford Univ. Press, London and New York, 1961.
R4. Report of a Symposium on the design of hospital laboratories with special reference to biochemistry. *Proc. Assoc. Clin. Biochem.* **2,** 29–39 (1962).
R5. "Report of the Government Chemist 1965." H. M. Stationery Office, London, 1966.
R6. Robinson, R., The four o'clock phenomenon. *Lancet* **II,** 744–745 (1966).
R6a. Robinson, R., Tedium and the law of diminishing returns in clinical biochemistry laboratories. *Lancet* **I,** 433–434 (1967).
R7. Rootwelt, K., and Nesvik, B., Er analysesvarene fra Norske klinisk-kjemiske laboratorier pålitelige? *Tidsskr. Norske Laegeforen.* **86,** 1085–1090 (1966).
S1. Seligson, D., An automatic pipetting device and its application in the clinical laboratory. *Am. J. Clin. Pathol.* **28,** 200–207 (1957).
S2. Siggaard-Anderson, O., "The Acid-Base Status of the Blood." Munksgaard, Copenhagen, 1963.
S3. Simon, L. E., "Engineers' Manual of Statistical Methods." Wiley, New York, 1941.
S4. Skeggs, L. T., Jr., An automatic method for colorimetric analysis. *Am. J. Clin. Pathol.* **28,** 311–322 (1957).
S5. Skeggs, L. T., Jr., and Hochstrasser, H., Multiple automatic sequential analysis. *Clin. Chem.* **10,** 918–936 (1964).

S6. Snedecor, G. W., Queries. *Biometrics* **8,** 85–86 (1952).
S7. Snodgrass, P. J., Fuwa, K., and Hviid, K., Validation of automatic flame photometric analysis of sodium and potassium. *J. Lab. Clin. Med.* **60,** 983–990 (1962).
S8. Sparapani, A., and Berry, R. E., Pools of frozen serum in the quality control in clinical chemistry. *Am. J. Clin. Pathol.* **42,** 129–132 (1964).
S9. Sparapani, A., and Berry, R. E., An evaluation of standard deviations in clinical chemistry. *Am. J. Clin. Pathol.* **43,** 591–593 (1965).
S10. Straumfjord, J. V., Jr., and Copeland, B. E., Clinical chemistry quality control values in thirty-three university medical school hospitals. *Am. J. Clin. Pathol.* **44,** 252–258 (1965).
S11. Sunderman, F. W., Jr., and Carroll, J. E., Measurements of serum calcium and magnesium by atomic absorption spectrometry. *Am. J. Clin. Pathol.* **43,** 302–310 (1965).
S12. Sunderman, F. W., Jr., Sunderman, F. W., Falvo, E. A., and Kallick, C. J., Studies of the serum proteins. II. The nitrogen content of purified serum proteins separated by continuous flow electrophoresis. *Am. J. Clin. Pathol.* **30,** 112–119 (1958).
T1. Thiers, R. E., and Cole, R. R., A chart for concentrational calculations in quality control. *Clin. Chem.* **11,** 1036–1039 (1965).
T2. Thiers, R. E., and Oglesby, K. M., The precision, accuracy, and inherent errors of automatic continuous flow methods. *Clin. Chem.* **10,** 246–257 (1964).
T3. Thin, C. G., and Thomson, P. A., The estimation of calcium and magnesium in serum and urine by atomic absorption spectrophotometry. *J. Clin. Pathol.* **20,** 280–282 (1967).
T4. Tonks, D. B., A study of the accuracy and precision of clinical chemistry determinations in 170 Canadian laboratories. *Clin. Chem.* **9,** 217–233 (1963).
T5. Trinder, P., Colorimetric micro-determination of calcium in serum. *Analyst* **85,** 889–894 (1960).
V1. van Peenen, H. J., and Lindberg, D. A. B., The limitations of laboratory quality control with reference to the "Number plus" method. *Am. J. Clin. Pathol.* **44,** 322–330 (1965).
V2. Varley, H., "Practical Clinical Biochemistry," 3rd ed., p. 282. Heinemann, London, 1962.
V3. Vogel, A. I., "A Text-Book of Quantitative Inorganic Analysis." 3rd ed. (corrected). Longmans, Green, New York, 1962.
W1. Wallenius, G., and Hjelm, M., Automation and data processing in a clinical laboratory—a pilot study. *Proc. Assoc. Clin. Biochem.* **3,** 246–247 (1965).
W2. Whitby, L. G., Clinical chemistry: time for investment. *Lancet* **II,** 1239–1243 (1963).
W3. Whitby, L. G., Automation in clinical chemistry, with special reference to the AutoAnalyzer. *Brit. Med. J.* **II,** 895–899 (1964).
W4. Whitby, L. G., and Owen, J. A., Serial laboratory reports and records for chemical analyses. *J. Clin. Pathol.* **18,** 668–677 (1965).
W5. Whitehead, E. C., Sequential multiple analysis. *In* "Automation in Analytical Chemistry" (L. T. Skeggs, Jr., ed.), pp. 437–451. Mediad, New York, 1966.
W6. Whitehead, T. P., Computer-assisted statistical uses of laboratory data. *In* "Progress in Medical Computing" (H. Atkins, chairman), pp. 52–56. Elliott Med. Automation, London, 1965.
W7. Whitehead, T. P., Computers for laboratory services. *Hosp. Management Planning & Equipment* **29,** 708–711 (1966).

W7a. Whitehead, T. P., Personal communication (1966).
W8. Willis, J. B., The determination of metals in blood serum by atomic absorption spectroscopy. I. Calcium. *Spectrochim. Acta* **16**, 259–272 (1960).
W9. Woodward, R. H., and Goldsmith, P. L., "Cumulative Sum Techniques." Oliver & Boyd, Edinburgh and London, 1964.
W10. Wootton, I. D. P., International biochemical trial 1954. *Clin. Chem.* **2**, 296–301 (1956).
W11. Wootton, I. D. P., Standardization in clinical chemistry. *Clin. Chem.* **3**, 401–405 (1957).
W12. Wootton, I. D. P., Normal variations in blood constituents. *Proc. Nutr. Soc. (Engl. Scot.)* **21**, 129–135 (1962).
W13. Wootton, I. D. P., "Micro-Analysis in Medical Biochemistry," 4th ed., p. 8. Churchill, London, 1964.
W14. Wootton, I. D. P., and Haslam, R. M., The use of pooled sera for laboratory control. *Proc. Assoc. Clin. Biochem.* **3**, 247 (1965).
W15. Wootton, I. D. P., and King, E. J., Normal values for blood constituents: Inter-hospital differences. *Lancet* **I**, 470–471 (1953).
Z1. Zilva, J. F., and Patston, V. J., Variations in serum-iron in healthy women. *Lancet* **I**, 459–462 (1966).

METABOLISM OF OXYPURINES IN MAN

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1. Introduction

The physiological role of the oxypurines is not completely understood, but certain facts have been amply confirmed. There are many pathological or physiologically abnormal conditions which can lead to concomitant alterations in the metabolism of these compounds. Under certain condi-

tions abnormalities in oxypurine metabolism can exist with no known related malfunction, and, finally, it is possible for abnormalities in oxypurine metabolism to lead to pathological conditions. For these reasons the study of the production, distribution, and fate of the oxypurines is of great importance.

Purines are integral components of various fundamental biological entities. Their production and function are vital to all life. One of the most disturbing aspects of studies of oxypurines in man, however, is the fact that one can find little evolutionary advantage to modern man in the production of these compounds. The products are inherently dangerous because of their low solubility. Their synthesis does not seem to be a necessary by-product of a vital biochemical reaction, and their production results in the ultimate loss from the body of purine moieties that were produced at great biochemical expense. Nevertheless, they are very much a fact of life, the study of which is of fundamental significance.

Current research has begun to make it possible to discuss physiological conditions causing or resulting from disorders in oxypurine metabolism in terms of their biochemical bases. The action of drugs that influence the synthesis and interconversion of these compounds can be considered in light of the intricate balance and control systems regulating purine metabolism. The purpose of this review is to cover the synthesis of purine nucleotides and the transformation of these compounds leading to oxypurines. The control of the production is exerted ultimately in terms of enzyme level and function, and by various transport systems in the kidney at least. Superimposed on this are the genetic composition of the host and influence of his environment.

2. Purine Anabolism

The early experimental work that determined the mechanism by which purines are synthesized was carried out on a variety of laboratory animals and microorganisms. None of the data so obtained indicates a difference in the reactions utilized by the various species, nor is there evidence to indicate that man is unique in this regard.

2.1. Synthesis *de Novo* of Purines

All animals are able to synthesize purines. In 1874 Miescher (M13) noted the ability of migrating salmon to synthesize large amounts of nucleic acid, apparently from other body constituents. The direct demonstration of the ability of cells to synthesize purines was first recorded in 1885 by Tichomiroff (T3), who noted production of purines in developing silkworm ova. A year later, Kossel (K14) reported that, although he could not demonstrate purines in fresh eggs, after incubation of fertilized

eggs for several days considerable amounts of purines could be isolated. The ability of mammals to synthesize purines was demonstrated in 1891 by Socin (S24), who showed that mice were able to grow and hence synthesize purines on a purine-free diet. There were early demonstrations that amino acid nitrogen (K11) or ammonia (S7) was converted to uric acid by fowl, but the direct demonstration came from the work of Barnes and Schoenheimer (B10), who showed by use of heavy nitrogen that ammonia is a precursor of nucleic acid purines in the rat.

The classical work on the details of the synthesis of purines was carried out by Buchanan and his co-workers (B35, B36, S25, S26) and Greenberg and his associates (G8, G9, G14, G15, G16). Each of these groups used the pigeon liver system that had long been recognized to have the advantage of lacking xanthine oxidase. These investigations

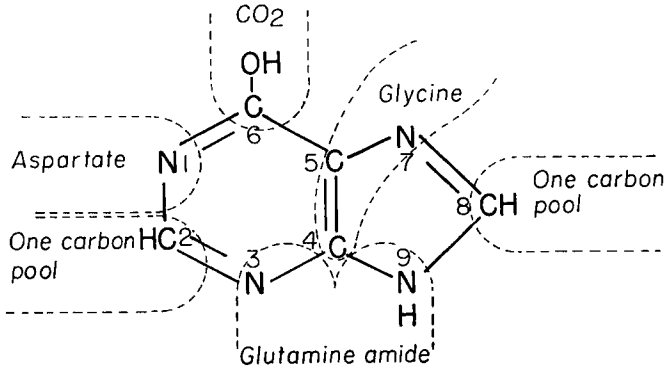


FIG. 1. Source of the atoms of the purine ring.

showed the carbons of the purine ring to be derived as follows: carbon dioxide is the source of carbon atom 6, the carbon atom of formate is the precursor of carbon atoms 2 and 8, the carboxyl carbon of glycine is the source of carbon atom 4, and the methylene carbon of glycine is the precursor of carbon atom 5 (Fig. 1). In 1947 Shemin and Rittenberg (S15) administered glycine containing ^{15}N to an adult human subject, isolated uric acid from the urine, and determined by chemical degradation that most of the label was located in position 7. This, in conjunction with the carbon studies, led to the conclusion that glycine supplies atoms 4, 5, and 7 of the purine ring. Sonne *et al.* (S27) were able to show that nitrogen atoms 3 and 9 were derived from glutamine, and that nitrogen atom 1 came from the amino group of either glutamic or aspartic acid. The ambiguity regarding the source of nitrogen atom 1 was later resolved by Levenberg *et al.* (L12), who showed unequivocally that it was the

nitrogen of aspartic acid that was the source of nitrogen atom 1 of the purine ring. It was largely through these amino acids that the exogenous ammonia given by Barnes and Schoenheimer (B10) was incorporated into purines.

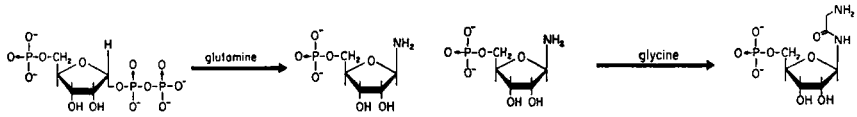
Greenberg, by comparing the rate of labeling of inosinate and hypoxanthine, was able to deduce that a ribosylpurine is synthesized prior to the free purine (G15). This established the then surprising fact that the first purine-containing entity was a ribonucleotide. As a result of the investigations carried out in the laboratories of Buchanan and Greenberg, the specific reactions involved in the synthesis of purine nucleotides have been delineated,[1] as summarized in Fig. 2.

2.2. Interconversion of Purines

Just as most living cells are able to synthesize inosinate from smaller precursors, so most cells are able to convert inosinate into the other purines that are physiologically important, and here, too, all cells appear to use the same sequence of reactions shown in Fig. 3. The role of the intermediate adenylosuccinate was detected by Carter and Cohen (C1). Inosinate reacts with aspartate in the presence of guanosine triphosphate to form the intermediate adenylosuccinate, cleavage of which results in adenylate and fumarate. This reaction is catalyzed by the enzyme adenylosuccinate AMP-lyase and is reversible. Inosinate with the mediation of NAD can also be converted to xanthylate, which can be aminated to guanylate. In most mammalian species, this amino nitrogen is donated by glutamine (A3, L1, L2, L3, M2, M17). These reactions are all catalyzed by specific enzymes. However, it appears that the enzyme adenylosuccinate AMP-lyase is the same enzyme responsible for the cleavage of the succinoimidazole ribonucleotide derivative that is an intermediate in the synthesis of purines (Fig. 2). Purification of the enzyme over 200-fold did not change the ratio of activities toward the two substrates (M14). Furthermore, in a certain class of mutants, the loss of activity for one substrate has always been accompanied by an equal loss of activity toward the other (G11). Similarly, in those tumors shown to be susceptible to 6-mercaptopurine (6-MP) because of a deficiency in adenylosuccinase, examination has shown that the tumors also have a reduced level of the activity toward the imidazole substrate (B2).

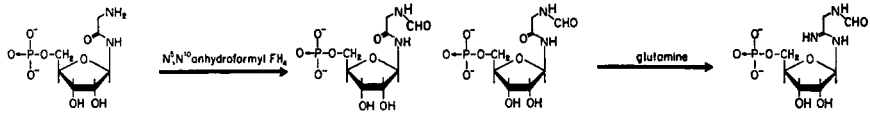
As was mentioned above, most species are able to convert inosinate into adenylate and guanylate to varying degrees. Many microorganisms, however, are unable to carry out one or both of these conversions, and mutant strains have been isolated that differ from their parent in that they have lost the enzymes necessary for catalyzing various reactions

[1] These reactions are discussed in detail in reviews by Buchanan (B34) and Davidson (D4).



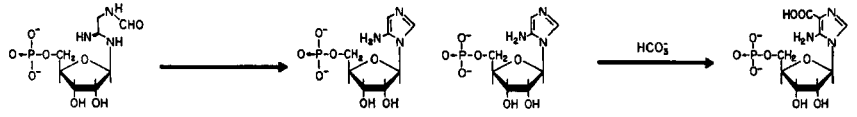
Ribosylamine-5-phosphate: pyrophosphate phosphoribosyltransferase (glutamate-amidating)

Ribosylamine-5-phosphate: glycine ligase (ADP)



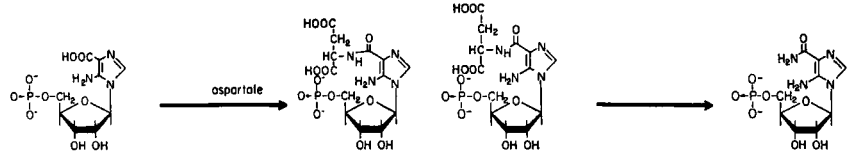
5'-Phosphoribosyl-N-formylglycineamide: tetrahydrofolate 5, 10-formyltransferase

5'-Phosphoribosyl-formylglycineamide: L-glutamine amido-ligase (ADP)



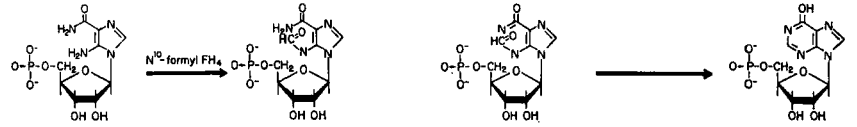
5'-Phosphoribosyl-formylglycineamidine cycloligase (ADP)

5'-Phosphoribosyl-5-amino-4 imidazole-carboxylate carboxy-lyase



5'-Phosphoribosyl-4-carboxy-5-aminoimidazole: L-asparate ligase (ADP)

Adenylosuccinate AMP-lyase



5'-Phosphoribosyl-5-formamido-4-imidazolecarboxamide: tetrahydrofolate 10-formyltransferase

IMP 1,2 Hydrolase (decyclizing)

FIG. 2. Pathways of purine synthesis.

(M1). Although no clear-cut case of a mammalian tissue being unable to carry out these conversions has been reported, it is theoretically possible for such mutations to occur.

2.3. UTILIZATION OF PREFORMED PURINE DERIVATIVES

The first unequivocal demonstration that an exogenously synthesized purine could enter into the normal purine anabolic pathway was the

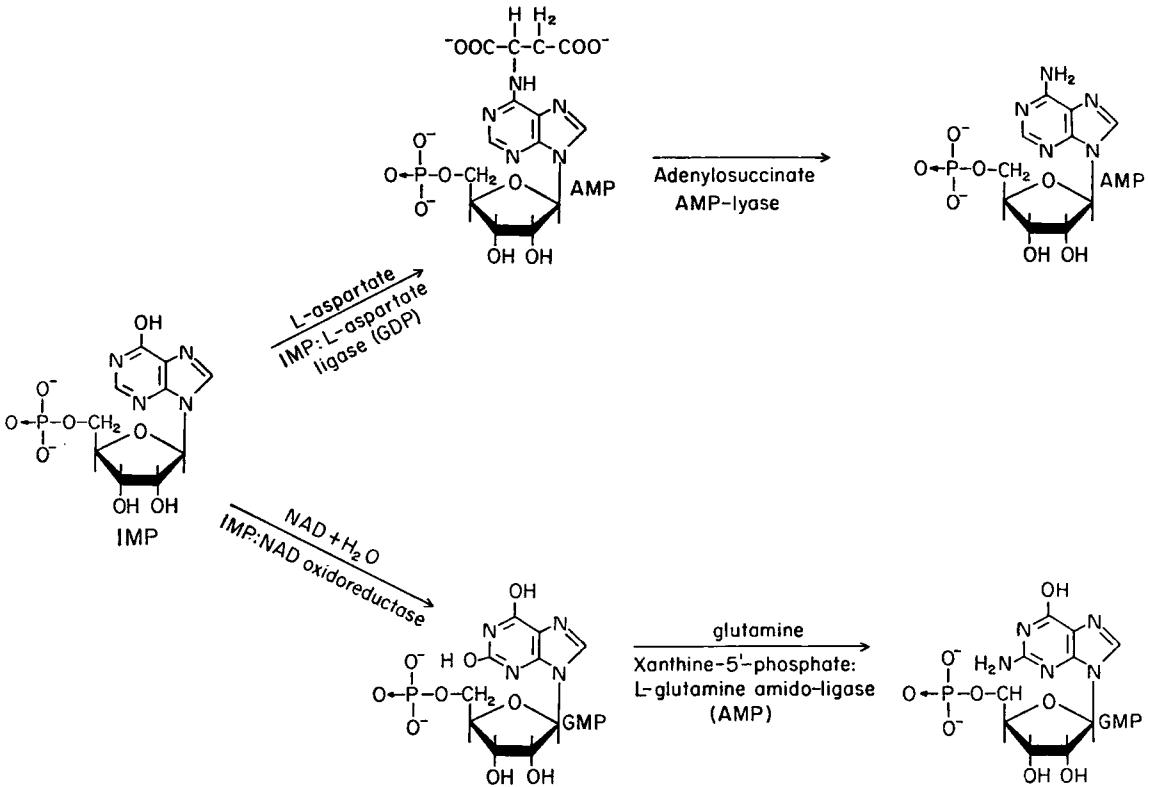


FIG. 3. Interconversion of purine ribonucleotides.

finding (B29, B31) that, in the rat, dietary adenine is incorporated into tissue nucleic acids as adenine. In addition, these workers showed that exogenously supplied adenine could also serve as a precursor of nucleic acid guanine. Following the feeding of 0.2 mmole of adenine/kg of body weight for 3 days, more than 5% of the nucleic acid adenine was derived from this exogenously supplied material. When higher levels were fed, almost 14% of the nucleic acid adenine was found to be derived from this exogenous source after 5 days. These results were all the more striking because in 1944 Plentl and Schoenheimer (P5) had reported that ^{15}N-labeled dietary guanine was not used per se in the synthesis of nucleic acid, and these results had been confirmed by Brown and his associates. Later, with a more sensitive tracer, ^{14}C, it was possible to show that guanine was incorporated into nucleic acid guanine in the rat; about 0.1% of guanine was derived from the exogenous source (B4). Similar results were found by Abrams (A2). Kidder and his associates had suggested that tumors were better able to utilize exogenous guanine for nucleic acid synthesis than were normal tissues (K7). This postulate was substantiated when, after massive doses of labeled guanine were administered to tumor-bearing mice, the tumor did in fact incorporate guanine into its nucleic acid (B30). Later work with smaller amounts of highly labeled guanine did not bear out these initial conclusions (B5, B6, M5). An investigation of the degree of incorporation and the level of administration of exogenous guanine revealed that, in fact, the utilization of guanine by tumors was much greater than by host tissues if the level of precursor were high, and much lower if the level were low (B1). Presumably these utilizations were related to the known presence of the enzyme guanase, which degrades exogenous guanine (see Section 3.3). The simultaneous administration of a guanase inhibitor regularly increased the utilization of guanine, so apparently the inability or ability of tissues to utilize exogenous adenine and guanine is related to the level of degradative enzymes, which might destroy these potential substrates before they could be anabolized.

Adenosine was shown to be incorporated into nucleic acid purines, but to only about half the extent of adenine itself (L2). The incorporation of guanosine was also studied and here again the guanine derivative was a poorer precursor than the adenine derivative, but the utilization of guanosine was considerably greater than that of the aglycone (L22). The administration of the adenine nucleotides revealed that these compounds, whether as the 3′ isomer or the 5′ isomer, were poorer precursors than the free base adenine (W1). With all nucleotides studied, the incorporation of the 5′ isomer was less extensive than of the 3′ isomer (R9). Roll *et al.* have shown that guanine nucleotides are considerably

better precursors than guanine *per se* (R10, R12). These findings were all the more interesting because the phosphorus of the guanylic acid was not incorporated preferentially into the nucleic acid of the cells (R11). A similar pattern obtains with microorganisms, in which it was shown that the phosphorus of neither adenine nor guanine nucleotides was incorporated into the nucleic acid while the purine moieties were (B3).

Evaluation of the potential role of hypoxanthine and xanthine as precursors of nucleic acid purines led to preliminary conclusions that the purine moieties of these two compounds could not be incorporated as such into nucleic acids, but instead were rapidly eliminated as allantoin in the rat (G3). It had been believed that this was a result of the absence of enzymes necessary for the conversion of the exogenous hypoxanthine and xanthine to the intermediate inosinate and xanthylate, which are known to be interconverted (Fig. 3). An alternative explanation, namely, that actually hypoxanthine and xanthine are excellent precursors, but are too rapidly degraded to be incorporated, is in fact correct. The demonstration of this was made possible by the availability of allopurinol, an inhibitor of the degrading enzymes to be discussed later. Concurrent administration of this inhibitor with hypoxanthine or xanthine as potential precursors led to relatively extensive incorporation of these purines into nucleic acid purines (P6, P7). Thus, all the purines whose nucleotides are involved in the interconversion shown in Fig. 3 are, in fact, capable of serving as precursors of nucleic acid purines and entering into this scheme in mammals. The extent to which this utilization occurs is a function not only of the ability of the animal to utilize these compounds, but also, inversely, of the presence of enzymes which can degrade the purine into a form not available for anabolism. As a corollary of this fact, purines of cells breaking down are not readily available to other cells in the animal for anabolic purposes. The nucleic acids of fetuses growing in mothers whose purines contained ^{14}C were not labeled, although most of the maternal purines had been replaced during the period of the experiment. Tumors implanted into animals with previously labeled nucleic acids did not utilize these purines in the course of their growth, nor did the tissues of animals that were parabiosed with animals whose nucleic acid purines were highly labeled (D2). Therefore it appears that, in normal animals, purines that leak out of cells are rapidly degraded and removed, and do not reach the available metabolic pools.

3. Purine Catabolism

Oxypurines are end products of purine metabolism in man. They do not serve as a general end product of nitrogen, as is the case with some

other species. The enzymes that produce them are varied in location and nature. Their properties have been studied in detail, but extensive description is beyond the scope of this review, which should be more concerned with their role in catalyzing a large variety of reactions.

3.1. Degradation of Ribonucleic Acids

Jones showed that nucleic acids could be degraded by the pancreas (J4) to yield a mixture of products less complex than nucleotides. Several years later, it was shown (J5) that boiled extracts of the pancreas are capable of degrading ribonucleic acid to water-soluble products without the liberation of inorganic phosphates or purines or pyrimidines. This was the first demonstration of the existence of the enzyme now referred to as pancreatic ribonuclease or ribonuclease I, capable of cleaving the internucleotide linkage. Crystallized by Kunitz (K25), it has received much attention (S4) and been shown to cleave the bonds between the 3′-pyrimidine nucleoside phosphate group and the 5′-hydroxyl group of adjacent purine or pyrimidine nucleotides (L21, S6). Even after exhaustive incubation with ribonuclease, the nucleic acid is not degraded entirely to mononucleotides, although all four mononucleotides are present. In the course of hydrolysis of the internucleotide bond, the intermediate cyclic 2′,3′-nucleoside phosphate diesters are formed (B27, M7), which may be further hydrolyzed to the 3′-nucleotides, the final product of the enzymatic reactions. The cyclic pyrimidine nucleotides which are intermediates in this reaction are, of course, of themselves substrates for this enzyme. Apparently the acidic polysaccharide, heparin, is a competitive inhibitor of ribonuclease (Z2), which is also inhibited by the sulfhydryl binding reagent *p*-chloromercuribenzoate. It has been suggested that this latter substance forms a complex with sulfur-containing groups of the enzyme that is inhibitory, and also that it combines with polar groups of the ribonucleic acid *per se* and so inhibits the overall reaction (L6). The presence of copper also inhibits the reaction, although no mechanism has been defined (Z1).

Many other ribonucleases have been described. They differ with respect to specificity toward various internucleotide bonds, in heat stabilities, and in pH optima. In 1949 Maver and Greco (M8) reported the presence in extracts of spleen of a heat-labile nuclease that had a pH optimum of approximately 5.2 in the presence of magnesium, and 6.6 in the absence of magnesium. That this was a different enzyme entirely from pancreatic RNase was shown most dramatically by Hilmoe and Heppel (H7), who found that this enzyme was able to hydrolyze the limit polynucleotide obtained by exhaustive treatment of RNA with ribonuclease I. The ubiquity of ribonucleases can perhaps best be demonstrated by noting

that there is a ribonuclease on human skin that can become a serious problem in laboratory work, since glassware, dialysis tubing, and other common laboratory tools can be contaminated merely by being handled without kid (polyethylene) gloves (H10).

There may be many proteins that inhibit ribonucleases. Clear evidence has been presented that there is at least one such substance in rat liver that is a powerful and specific inhibitor of the pancreatic enzyme, but is inactive against RNases obtained from plants or bacteriophage (R14, S17).

Variations in RNase levels may occur in several diseases (Section 5.2). These changes may be of a fundamental nature; for example, Daoust and Amano (D3) have postulated that the loss of ribonucleases is a common characteristic of tumors in general. In view of the several RNA's that are known and the several RNases that have been described, a variety of relationships are possible. Without more information it is impossible to evaluate this hypothesis.

Another enzyme of possible significance in this metabolic area is polynucleotide phosphorylase, which was first viewed as primarily a catalyst for RNA synthesis. The reaction involved is the reversible polymerization of ribonucleotide 5′-diphosphates with the concomitant liberation of inorganic phosphate (G17). It is quite possible that the biological role of this enzyme is the reverse reaction, i.e., the production of nucleoside diphosphates from RNA.

3.2. Degradation of Deoxyribonucleic Acid

There are two main types of mammalian DNase; both have been very well identified. These are endonucleases, that is, they cleave the polymer in the interior rather than at the terminal nucleotides. These two DNases differ in pH requirements and in the products formed. The first enzyme is that initially characterized (A1, S5) from the pancreas, and cleaves DNA to yield some monomers with a 5′ terminal group. The other type of DNase, referred to as DNase II, is found largely in spleen and thymus. DNase I yields oligonucleotides of an average chain length of 4, with a free hydroxyl group in the terminal 3′-position and a phosphate group on the terminal 5′-position. It is activated by magnesium, operates in the alkaline range slightly above pH 7, and has long been known to be inhibited by ions such as citrate, borate, and fluoride; this inhibition is undoubtedly caused by the chelating properties of these ions, which bind the magnesium ions necessary for activation. On the other hand, inhibitors such as sulfides and thioglycolate appear to act by reacting chemically with a functional group of the enzyme (G6).

The destruction of DNA is obviously of intense biological interest *per se*. The reports that the action of DNA as a primer for DNA replication

is increased by mild treatment with DNase have created considerable interest in the mechanisms that control the action of this enzyme or these enzymes. Inhibitors of the enzyme have been shown to exist. A protein inhibitor of deoxyribonuclease I has been demonstrated in several tissues, and one such protein has been partially purified (D1). These conclusions regarding the varying amounts of inhibitor may explain the observation that, in nonmalignant growing tissues, placentas or regenerating rat liver, for example, there are measurably higher levels of deoxyribonuclease activity, although in malignant tumors there is no pronounced increase in the apparent level of this enzyme (B24, B25, B26).

The second DNase referred to as DNase II differs from DNase I in that it completely hydrolyzes DNA to small fragments and will digest the cores not hydrolyzed by DNase I. DNase II is an acid enzyme with a pH optimum of approximately 5. It is not susceptible to the specific protein inhibitor that is active against DNase I, and does not have a requirement for magnesium. As a matter of fact, higher concentrations of magnesium can inhibit it (L3). There are, of course, many nucleases in bacteria and other microorganisms, but they are not relevant to the present discussion (see K6). The assay of these enzymes is made extremely complicated by the fact that inhibitors for each have been described, but the levels and stabilities of these inhibitors have not been accurately determined. Thus, it is difficult to distinguish between changes in enzyme level and changes in inhibitor activity, and this latter may be an artifact of the assay itself.

DNase inhibitors from a variety of sources have been investigated, as, for example, those from plasma (D12) and spleen (L17). The latter inhibitor has been extensively purified and studies have been carried out on the mechanism of action, but no specific conclusions have been reached.

The existence of inhibitors in various tissues probably explains the discrepancies in the literature regarding the level of DNase in serum. Red blood cells contain a large amount of inhibitor of DNase that is easily released, and it has been postulated that the presence of this inhibitor in serum samples has led to the variations between published analytical results (G18). It is quite natural to speculate on the possible significance of the levels of these nucleic acid-destroying enzymes to the production of purine breakdown products from nucleic acids.

3.3. Nonspecific Phosphoesterases

As phosphodiesters, both RNA and DNA are subject to hydrolysis by nonspecific phosphodiesterases. Historically, the enzymes of greatest interest in this area are snake venom phosphatases. Certain venoms con-

tain an enzyme or enzymes capable of hydrolyzing all polydeoxyribonucleotides and polyribonucleotides that do not contain a phosphate group on the terminal 3′-position. Crude snake venom usually contains, in addition to the phosphodiesterases, phosphomonoesterases capable of cleaving the nucleotide products further. These phosphodiesterases are not specific for nucleic acids. As a matter of fact, the assay of activity is usually done by treating esters such as bis-*p*-nitrophenyl phosphate; the liberated nitrophenol can be determined directly. A phosphodiesterase obtained from intestinal mucosa and from duodenal juice acts not only on nucleic acids, but is a more general phosphatase. It is without effect upon high molecular weight nucleic acids. Highly polymerized DNA is resistant to it (S2), but digests obtained by partial hydrolysis of DNA with pancreatic DNase are susceptible to its hydrolytic action (S5). These nonspecific diesterases are exonucleases, that attack from the end and remove nucleotides one at a time.

3.4. Degradation of Nucleotides and Nucleosides

Following the breakdown of nucleic acids by specific nucleases or nonspecific phosphodiesterases, a number of possible fates can await the nucleotides. Intracellularly they could mix with the normal pool of nucleotides to increase its size and be further anabolized, or they might leak out or be excreted from the cell. Normally, purine-containing breakdown products do not find themselves incorporated to an appreciable extent into the nucleic acids of other cells (D2). As was mentioned, many enzymes are able to degrade the derivatives until the breakdown products are biologically useless. There are many additional nonspecific phosphatases such as those found in the intestines, bone, and prostatic tissue. Reis (R3, R4, R5) in a series of studies established the existence in many tissues, including human, of specific enzymes capable of splitting the phosphate ester from 5′-nucleotides. This enzyme is present in rather high concentrations in bull semen; it has been highly purified and its properties studied by Heppel and Hilmoe (H5). More recently Bodansky and his associates (B22, L14) have shown that this enzyme has the unusual property of possessing two pH maxima in the presence of magnesium, and that the activity is altered by the presence of certain amino acids. This enzyme can cleave the phosphate from all the purine 5′-nucleotides. A second nucleotide phosphatase was described by Shuster and Kaplan (S19), who demonstrated its presence in germinating rye grass and certain bacteria, and its specific cleavage of 3′-nucleotides without effect upon 2′ and 5′ isomers. Its action is probably responsible for the poor utilization in microorganisms of the 2′-adenylic acid as a purine source when contrasted with that of the 3′ isomer (B3).

Purine nucleosides are cleaved by the action of purine nucleoside phosphorylase with the liberation of ribose 1-phosphate (K1, P1). The enzyme is apparently specific for purines. The material from erythrocytes catalyzes the phosphorolysis of purine but not pyrimidine nucleosides (T6.) Purine phosphorylase activity is found widespread in nature and in many animal tissues (F10). Friedkin and Kalckar investigated an enzyme capable of cleaving purine deoxynucleosides to the aglycone and deoxyribose 1-phosphate. They concluded that the enzyme was identical to that which splits purine ribonucleosides (F8, F9). This enzyme is capable of degrading inosine, xanthosine, and guanosine to forms readily attacked by other enzymes. In so doing, it permits living cells to retain the ribose and deoxyribose moieties.

3.5. Hydrolysis of Amino Purines

The amino groups of purines can be hydrolyzed by a variety of enzymes. Adenine deaminases are present in bacteria, but the tissues of higher animals do not seem to contain an enzyme capable of hydrolyzing the amino group of free adenine. Schmidt (S3) has commented, "Statements to the contrary in the literature are based upon indirect evidence such as a decoloration of methylene blue by adenine in the presence of xanthine dehydrogenase." Adenine is slowly oxidized at the 2- and 8-positions by xanthine oxidase (B23, K9). Adenosine deaminase, on the other hand, is widely distributed and found in most tissues of higher animals (C7). This enzyme has been of great interest because of its role in limiting the physiological effects of adenosine. The enzyme not only deaminates adenosine, but also acts upon synthetic compounds which could otherwise be of greater interest pharmacologically, such as arabinosyl- or xylosyladenine and 2,6-diaminopurine ribonucleoside (C4, L10). The presence of adenosine deaminase in rodents correlates with the less extensive incorporation of adenosine than adenine into nucleic acid purines (L22). The finding that adenylic acids were incorporated to about the same extent as adenosine is consistent with the abundance of both nucleotidases and nonspecific phosphatases.

There is a widely distributed enzyme capable of hydrolyzing guanine to xanthine; it is absent in pigs and spiders. So nearly ubiquitous is this enzyme in higher animals that, in practically all species studied, guanine is not a precursor of nucleic acid purines to an appreciable extent (B28) (see Section 2.3). It is not possible to say with certainty whether guanosine is deaminated to xanthosine, which is then hydrolyzed to xanthine, or whether guanosine is first hydrolyzed to guanine, which is then acted upon by guanase. It is well known that nucleotides do not pass freely in and out of cells. Therefore it would appear that cleavage to either

nucleoside or aglycone must occur within the cell before purine derivatives escape from it. The hydrolysis of these derivatives proceeds, as outlined in Fig. 4, to hypoxanthine or xanthine. From the studies of Ayvazian and Skupp (A7) with labeled precursors, in which they demonstrated that, after the administration of labeled adenine, the urinary adenine and hypoxanthine had the same specific activity, it can be concluded that hypoxanthine, which is found in the blood and consequently in the urine, is derived not from inosinate but from adenosine, so that the normal pathways of breakdown of nucleic acid derivatives would be those indicated by the solid heavy lines in the figure. It is not possible to say whether the primary source of xanthine is via xanthylic acid to xanthosine and thence to xanthine, or via guanosine and guanine, with possibly some xanthosine arising from guanosine.

3.6. Oxidation to Uric Acid

Xanthine oxidase, which is capable of catalyzing the conversion of hypoxanthine and xanthine to uric acid, was first detected in 1882 by Horbaczewski (H11), who noted that extracts of various tissues could catalyze the conversion of xanthine to uric acid. A similar enzyme was detected in milk (M15). These enzymes contain a flavin–adenine dinucleotide prosthetic group (C9). As a result of the essential nature of the flavin–adenine dinucleotide portion of the enzyme, a striking parallelism was seen between the riboflavin content of the diet and the xanthine oxidase concentration in tissues of growing rats (D10). The enzyme contains molybdenum. That the molybdenum is contained in a functionally important component has been demonstrated by several workers (G13, T5). Totter and his associates injected labeled molybdate into a cow, and then isolated the enzyme from the milk to show that the proportion between the molybdenum and flavin remained constant at a value of 0.5. Corran *et al.* (C9) postulated that the xanthine oxidase of milk is identical with the xanthine oxidase of liver, but the protein portions of the enzyme appear to differ.

Xanthine oxidase is a rather nonspecific enzyme; it not only catalyzes the oxidation of hypoxanthine and xanthine, but also the conversion of adenine to 2,8-dihydroxyadenine (B23, K9) as well as the oxidation of many unusual purines, such as 2-azaadenine (S14). It also acts on xanthopterin (K4), and catalyzes the oxidation of a variety of aldehydes and NADH. Several pterins, notably 2-amino-4-hydroxy-6-formyl- (K3), 2-amino-4-hydroxy-6-carboxy-, 2-amino-4-hydroxy-, and 6-hydroxymethyl- (P3) pterins, inhibit xanthine oxidase. A variety of purines are both substrate and inhibitor of the enzyme. Antabuse (tetraethylthiuram disulfide) has a considerable inhibitory effect on xanthine oxidase in rat

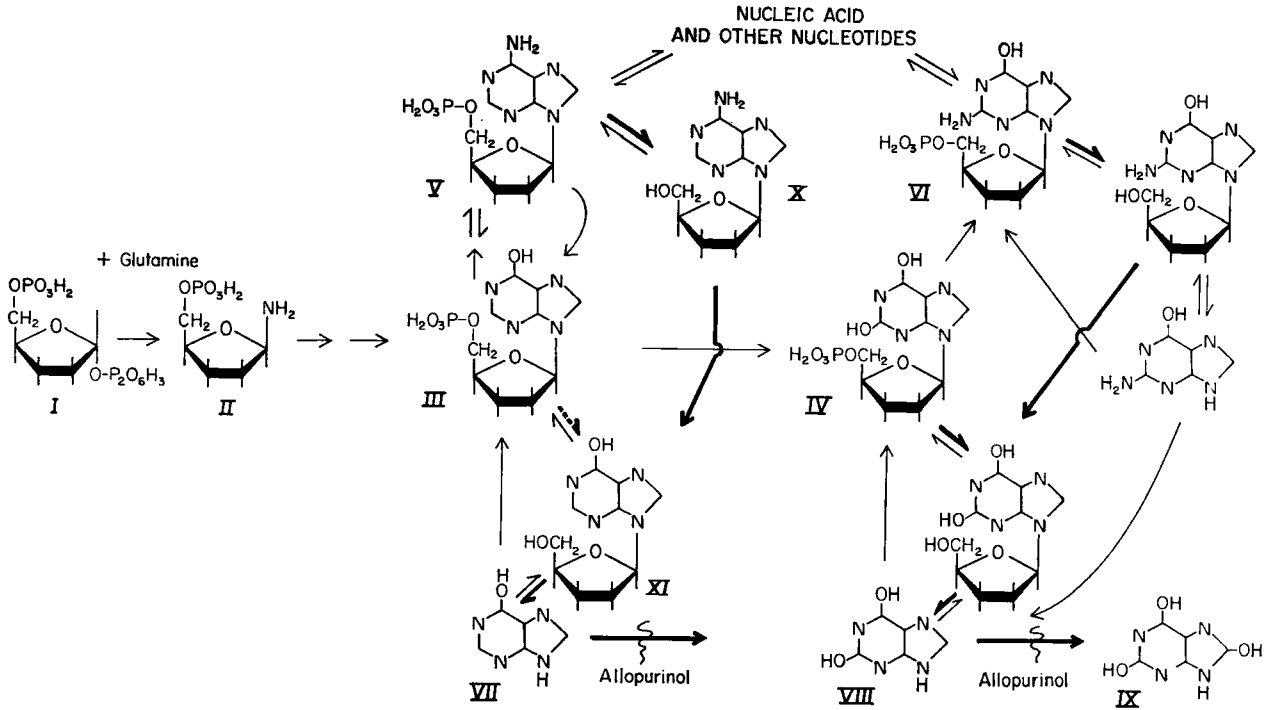


FIG. 4. Possible pathways of purine nucleotide anabolism and catabolism. The *heavy arrows* indicate the normal routes of degradation in man. I = phosphoribosylpyrophosphate, II = phosphoribosylamine, III = inosinic acid, IV = xanthylic acid, V = adenylic acid, VI = guanylic acid; VII = hypoxanthine, VIII = xanthine, IX = uric acid, and X = adenosine.

liver, but does not inhibit that from milk. Even small concentrations of copper have been shown to inactivate xanthine oxidase (S38). The differences in response to inhibitors support the concept that the enzyme prepared from milk is not identical with that from liver, and suggest that the protein carrier is different. More recently some purine analogs have been shown to be very potent inhibitors of xanthine oxidase (see Section 6.3).

3.7. Degradation in Other Species

The degradation of purines varies with the species. Actually uric acid is excreted as the principal end product of purine metabolism by very few mammals, of which man is unfortunately one. Most nonuricotelic animals possess the enzyme uricase, which converts uric acid to the much more soluble end product allantoin. Man and certain of the higher apes, as well as fowl and reptiles, do not possess this enzyme, so that they must excrete uric acid as the end product of purine metabolism. Despite the fact that it possesses uricase, as do other dogs, the Dalmatian coach hound is peculiar in that it excretes uric acid. This anomaly results from the absence of tubular reabsorption of uric acid in the kidney (F11).

Man, of course, excretes a relatively small proportion of nitrogen as uric acid, and there probably is little significance in the uric acid excretion in terms of nitrogen elimination (N3). Needham postulated that the method of elimination of excess nitrogen can be correlated with the mode of reproduction. Ureotelic metabolism, that is, elimination of nitrogen waste as urea, is associated with viviparity. On the other hand, uricotelic metabolism, uric acid as the principal nitrogenous end product, is associated with development in a cleidoic egg. His reasoning is essentially the following. Cleidoic eggs develop under a strict limitation of the water supply. The developing embryo metabolizes carbohydrate and protein in the course of its development and must produce ammonia. The toxic ammonia is not easily removed. Converted to urea its toxicity would be overcome, but by the end of development the amount of urea produced could be so considerable as to disturb the osmotic balance. The animal has a survival advantage if it can convert the ammonia into something nontoxic and insoluble; uric acid fulfills these requirements.

In man and other animals that produce uric acid, the product is not normally toxic but may be upon occasion. The animals that produce allantoin seem to have an advantage over man, since they are not in danger of producing a harmfully insoluble end product. Most fish cleave allantoin through the action of the enzyme allantoinase to allantoic acid. Some fish and amphibia split the allantoic acid further to urea and

glyoxylic acid by the action of a second enzyme, allantoicase (F3). The temptation to generalize is restrained by such oddities as the fact that the spider eliminates its nitrogenous waste as guanine!

4. Fate of Oxypurines

In view of the obvious relationship between high uric acid levels and gout there has been a great deal of interest in hyperuricemia, and relatively little consideration has been given to a possible significance of hypouricemia. Studies have revealed, however, that in addition to the greatly discussed hyperuricemias there exist a certain small but real number of individuals whose uric acid levels run from a third to a sixth of the normal value (E7, F6).

4.1. Uric Acid Pool

The determination of pool size has been one of the most important studies in the investigation of the metabolism of uric acid. The initial work was done by Benedict *et al.* (B14); a similar series of experiments was done shortly afterward by Geren *et al.* (G2). The rationale behind these experiments was that, if labeled uric acid were injected into individuals and if this uric acid were able to mix quickly with the endogenous uric acid and specific radioactivity of the resultant uric acid could be determined, then, by simple calculations of the "isotope dilution" method, the size of the endogenous pool could be determined. The samples of uric acid could be obtained most readily from the urine. If a series of urine collections were made over a period of days and the activity of the urinary uric acid determined, these values should decrease logarithmically as a straight line function. Extrapolation of this line back to zero time gives the value of the pool uric acid at the time of injection. From this it is possible to calculate the size of the endogenous miscible pool. The rationale was probably correct, as is evidenced by the fact that the activity of the uric acid did decrease as a logarithmic function (Fig. 5). Extrapolation of this line back to zero time permitted the calculation of pool sizes, which were of the order of 1 g for a normal adult. About 50% of this was replaced per day, as shown by the slope of the curve. With normal subjects, approximately 80% of the daily turnover was found as urinary uric acid.

The serum uric acid pool and the amount of uric acid excreted are dependent upon many physiological factors. One of the more obvious of these is kidney function. About 60 years ago, Leathes (L5) noted that during work there was a decrease in urinary uric acid excretion coupled with an apparent increase in excretion of other purine bases. He felt that

this increased excretion resulted from differential retention of uric acid by various tissues. Shortly afterward Kennaway (K5) made similar observations in subjects who had exercised vigorously. Unfortunately the determination of other bases at that time involved a laborious and not very quantitative silver precipitation step. Kennaway interpreted his observation in terms of the decreased oxidation of purines. These observations have been repeated and confirmed with better techniques by Quick (Q2). Attempts were recently made to understand the nature of the altered balance in purine excretion, and the suggestion was made that there were specific increases in hypoxanthine and perhaps xanthine elimination following exercise (N1). The methods of differential spectroscopy that were used are not, however, extremely accurate, nor can they distinguish between hypoxanthine and xanthine.

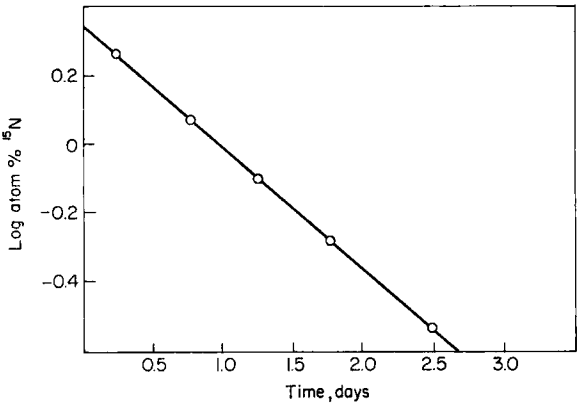


FIG. 5. Decrease in label of urinary uric acid with time. The pool size can be calculated from the y intercept and the daily turnover (k) calculated from the equation $k = l/t \ln A/(A\text{-}X)$, in which A and X are isotope excess at t_0 and t, respectively. [From (G2).]

Diminished uric acid excretion has been attributed to the action of organic acids produced during exercise. The effects can be mimicked by administration of benzoic (Q1) and lactic (G4, M12, Q2) acids. Similarly, the changes in serum uric acid levels associated with the toxemia of pregnancy (H2, S32) and glycogen storage disease (J1) have been related to lactic acid production. Traditionally, alcoholic beverages have been considered a precipitating agent in gout, and there is reason to believe that this may be true. Administration of alcohol does alter uric acid levels. Since ethanol can be converted to lactate enzymatically, Lieber

et al. (L16) proposed that the hyperuricemia associated with alcohol results from a similar mechanism of reduced urinary urate output.

4.2. Uricolysis

The great bulk of the oxypurines released as a result of the action of hydrolytic enzymes normally is ultimately oxidized to uric acid. Not all the uric acid, however, is eliminated as such. This was first demonstrated by Benedict *et al.* (B14), who showed that as much as 20% of the injected uric acid was broken down and eliminated as forms other than urate. These findings have been confirmed by several investigators (B37, B38, W11). Sorensen (S28) investigated the possible role of intestinal bacteria in uricolysis. He found that in a particular patient 69% of intravenously administered uric acid-2-^{14}C was eliminated in 10 days as urinary urate, while 22.5% of the injected radioactivity was recovered as degradation products: half as expired CO_2 and one-third as fecal products. Following the establishment of effective bacteriostasis through the concurrent administration of sulfonamide, streptomycin, and neomycin, 56% of an equivalent dose of urate was recovered as urinary uric acid after 5 days. Only 3% of the radioactivity was found as breakdown products, and only 0.5% of the injected activity was expired as $^{14}CO_2$. In the treated patient 30% of the ^{14}C was found in fecal urate, but in the control experiment there was no fecal urate. As Sorenson pointed out, there is uric acid in sweat but the quantity is so small as to be of no significance in this context.

One of the more interesting observations in this regard was the finding that in patients with renal insufficiency there was a far greater extrarenal elimination of injected urate (S30). In three such patients, 42, 33, and 61% of the uric acid radioactivity was recovered in expired CO_2, and each had approximately 10% in fecal products. At the other end of the spectrum is the observation that patients with hepatolenticular degeneration excreted approximately 90% of an administered dose of uric acid as urinary uric acid; here this is attributable to the abnormality in kidney function that favors nonreabsorption of urate. In view of the observations of Lesch and Nyhan (L11), that their pediatric patients (Section 5.4) with excessive uric acid production eliminated a relatively small amount of their daily turnover of uric acid in urine, and that several gouty patients excreted relatively large amounts of uric acid extrarenally (B39), there is a temptation to attribute the concurrence of such extrarenal elimination to excessive production of urate. On the other hand, it may well be that extrarenal elimination of urate can in some cases be attributed to undetected abnormal kidney function.

4.3. Xanthinuria

There have been reports in the literature of hypouricemia coincident with specific inborn metabolic errors, but many of these cases are attributable to defects in the kidney leading to failure of renal tubular reabsorption. It was mentioned above that the excretion of uric acid by the Dalmatian coach hound can be attributed to such a mechanism (F11). Similarly, the hypouricemia found in the Fanconi syndrome (L4) and Wilson's disease (B12) can be attributed to kidney malfunction. These are not true examples of underproduction of oxypurines, including uric acid, since the daily output of uric acid is normal. The large number of healthy people who have extremely low serum urate values, however, may indicate that there are individuals who underproduce oxypurines but suffer no ill effects because of this. The one well-documented inborn error that results in underproduction of uric acid is xanthinuria. It has been reported in relatively few cases, probably because individuals with this metabolic abnormality who suffer no ill effects would not come to the attention of a physician.

Although the existence of xanthine was first discovered in a stone (M6), it is only in the past 30-odd years that xanthine stones have been widely recorded. The precipitation of xanthine in cases studied in the past was accompanied by normal or even somewhat high levels of serum uric acid (J6, P2, R2, T2). The nature of this abnormality has not been determined; presumably it results from an overproduction of xanthine *per se*. A second group of patients, who also have high levels of xanthine, are characterized as a distinct entity by the fact that they have extremely low values of uric acid. The uric acid levels are in fact so low that the small amount determined can be attributed to dietary sources or perhaps to the action of intestinal bacteria. The first reported case of xanthinuria was presented by Dent and Philpot (D9). They found only traces of uric acid in the blood and urine of a 4-year-old girl; the urinary purine was primarily xanthine, and this was quantitatively comparable to a normal uric acid excretion. At that time Dent and Philpot felt that the defect was attributable to either a deficiency of liver xanthine oxidase or a renal tubular defect that resulted in greatly increased clearance of xanthine. This girl passed a urinary stone that was almost pure xanthine. She was seen clinically because of hematuria and urinary frequency. Five years later Dickinson and Smellie (D11) examined the same patient in more detail. Through enzymatic determinations and more refined chromatographic procedures, these investigators demonstrated that 10–40% of the oxypurines in the urine of the patient was hypoxanthine. They essentially agreed with Dent and Philpot's interpretation of the disorder,

except that they felt it to be not a question of either/or, but of both abnormalities being present. However, it has since been shown that clearance of xanthine in such patients is normal (K10). Other patients have since been reported and studied in considerable detail. One such patient, a 47-year-old man, also had hemochromatosis (A5, A7); another was suffering from pheochromocytoma (E6). In these patients the absence of xanthine oxidase was demonstrated in the liver and intestinal mucosa, two places where the enzyme is presently reported in normal human subjects. It has thus been demonstrated that the fundamental biochemical alteration leading to this abnormality is the absence of xanthine oxidase.

Administration of labeled xanthine resulted in the production of a small amount of labeled uric acid, but this could be explained in terms of the action of intestinal bacteria. Several labeled purines were administered to these patients, and the fate of the labeled compounds was consistent with the possible mechanisms of purine metabolism developed through studies of laboratory animals and microorganisms (see Section 2). Thus there was greater utilization of hypoxanthine, xanthine, and guanine than has been seen in individuals who possessed xanthine oxidase (W13), as determined by the delayed release of labeled purines in urine (A7).

The bulk of the oxypurines are eliminated as xanthine, which is consistent with the presence of a large amount of enzymes capable of degrading guanine, and relatively small amounts of enzymes capable of degrading adenine. This is consistent with the fact that guanine is a poor precursor of nucleic acid purines in most animals, although adenine is an excellent one (B28). On the other hand, guanine is an excellent precursor of nucleic acid purines *in vitro* (B1). Following the administration of labeled adenine to a xanthinuric, urinary hypoxanthine and urinary adenine had essentially the same specific activity (A7). Similar findings have been made with normal subjects (W13). Since it is not possible for urinary adenine to have been derived from the hypoxanthine that was being eliminated and since the two had the same specific activity, it must follow that the hypoxanthine was derived from an adenine derivative. Thus studies of these patients would indicate that the normal routes to urinary oxypurines are the degradations of guanylic or xanthylic acid and of adenylic acid. These two purine nucleotides are ultimately hydrolyzed to hypoxanthine and xanthine, which are then oxidized to uric acid if the enzyme xanthine oxidase is functioning.

The patients with xanthinuria who have been studied came to the attention of clinicians because of secondary ailments or because of the fact that relatives had the disease. Since the absence of xanthine oxidase

does not produce overt symptoms, many individuals with this defect might exist who escape detection.

5. Overproduction

The most common defect in oxypurine metabolism is that manifested by hyperuricemia and hyperuricosuria. The many epidemiological investigations of uric acid levels have shown a distribution of serum uric acid values ranging from approximately 0.5 to 9.5 mg/100 ml of serum (E7, F6, N2). Many of the individuals with extreme levels are perfectly healthy, although there is a correlation between secondary complications and high uric acid values.[2]

5.1. Gout

Hyperuricemia is found not only in gout, and not all gouty individuals are hyperuricemic, but there is a very distinct correlation between hyperuricemia and gout when populations are studied. Seegmiller *et al.* measured the uric acid levels of 940 normal individuals and 60 gouty individuals. There was a mean serum value among the gouty of greater than 9 mg/100 ml, and of approximately 5 mg/100 ml for the normal. This was true despite appreciable overlap (S10).

Just as there is no clear line of differentiation between hyperuricemic gout and normo-uricemic nongout, so there is no clear indication of the mechanism of the overproduction of uric acid. Many investigators have shown, by the administration of various labeled precursors, that in hyperuricemic and hyperuricosuric individuals there is a greater incorporation of labeled precursors into uric acid than there is in normal controls. Actually these studies merely confirm the fact that more uric acid is made by these "overexcretors." If more uric acid is made, then more of the precursor must be incorporated, and we should therefore be most surprised to find that there is no increase in the labeled precursors.

[2] Interestingly, several studies in the United States have indicated an apparent correlation between mean serum uric acid level and social or academic achievement. For example, urate values were shown to have some correlation with scores of inductees in the Army aptitude test (S37). Another survey indicated that executives have an average higher uric acid level than do craftsmen in several Pittsburgh companies. It was also shown that Ph.D. scientists have a higher value than supervisors, and supervisors in turn higher uric acid levels than craftsmen, at the Oak Ridge National Laboratory (D13). On the other hand, similar elevations in the level of uric acid have been noted in the serum of mongoloids (C5, F12). Since, in addition, there are differences in urate levels of various races (L9, O1, S39) and since urate concentration seems to vary geographically (P8), it is difficult to draw firm conclusions regarding the interrelationship between uric acid levels and intelligence or aptitude. Nevertheless, hyperuricemia apparently predisposes to certain mental aberrations, and is a result of certain other infirmities.

These studies seem to indicate that the uric acid production found in cases of hyperuricemia and gout occurs by the usual biosynthetic pathways.

In microorganisms, it has been shown that *de novo* synthesis of purines can be prevented by the addition of relatively large amounts of the "normal" purines to the growth medium (B9). Examination of these and similar data led to the postulate that purines were able to repress the synthesis of purine nucleotides at an early step (G10). The postulates of Gots have been confirmed, and it has been shown that the actual mechanism is interference with the functioning of phosphoribosylpyrophosphate amidotransferase by nucleoside phosphates (M11, N4). The control of the synthesis of many natural metabolites by internal feedback control mechanisms has been demonstrated to be an exquisitely efficient mechanism for maintaining the proper balance of cell constituents, and it is tempting to postulate that destruction of the normal system is what has led to the metabolic error resulting in hyperuricemia. However, no one has been able to gather information that would clearly delineate this as the mechanism of action in hyperuricemia.

The first step in purine synthesis involves the condensation of phosphoribosylpyrophosphate (PRPP) with glutamine. The PRPP also reacts with orotic acid in the normal synthesis of pyrimidines (K12). An attempt to deplete the PRPP supply was the basis for the attempts to use orotic acid as an inhibitor of uric acid production in the treatment of gout (see Section 6.2) (D8). Another reaction of PRPP is the condensation with imidazoleacetic acid to form a ribonucleotide. A study of the synthesis of this ribonucleotide was used as a method to investigate whether or not the turnover of PRPP was normal in gouty subjects. Imidazoleacetic acid was administered to subjects also given labeled glucose as an ultimate source of labeled ribose (H6). The results of the study indicated an increased turnover of the ribose moiety in overproducers of uric acid, but no difference in ribosyl synthesis between gouty and nongouty subjects who produced approximately the same amount of uric acid (J3). Thus there is no support for the postulate that altered metabolism of this initial precursor of purines is involved either in gout or in the overproduction of uric acid.

On the basis of the relative distribution of ^{15}N among the nitrogens of urinary uric acid of control and gouty individuals given labeled glycine, particularly the nitrogens N3 and N9, Gutman and Yü (G19) postulated that an abnormality in glutamine metabolism was responsible for the overproduction of purines. They believed that there was a defect or a deficiency in glutaminase in gouty individuals. This would result in a diversion of glutamine from the hydrolytic pathway to purine synthesis;

the increased amount of substrate would cause an increased reaction with PRPP, and an increased production of inosinate and the ultimate purine products. Although the interpretation of their data was consistent with the facts, many other explanations of their observations are possible (W10). No direct experimental verification or support of this suggestion has been obtained, but it is a plausible postulate and one that only further research can evaluate.

The most fundamental observation regarding the biochemical nature of gout was in all probability that of Benedict *et al.* (B14), who showed that the uric acid pool size in gouty overproducers was considerably larger than that of normal individuals. In those cases where the urinary output was not greater than normal, there were greater extrarenal elimination and degradation of the uric acid. This was shown both by the fact that the slope of the activity curve (Section 4.1) indicated a replacement of much more uric acid than was actually found in the urine, and by the fact that only a small fraction of the injected uric acid was recovered as urate. The fraction of the injected uric acid recovered from the urine was equal to the fraction of the daily turnover of uric acid which appeared as urinary uric acid.

All that is really known about the hyperuricemia of gout is the fact that there is a greater synthesis of uric acid in gouty than in normal individuals, and in some cases this excessive production is disposed of extrarenally and in some cases it is not. The amount of uric acid produced leads to the creation of a pool much greater than that found in normal individuals, and in some cases the pool is so great that it exceeds the solubility of urate under physiological conditions. The values of pool size determined experimentally may be minimal, since part of the pool may be slowly miscible or immiscible and not exchange with the injected labeled uric acid (B14, B15, B19). There is then precipitation in sensitive sites, which is one step toward the ultimate development of gouty arthritis.

The fact that the uric acid content of blood is increased in gout was first reported by Garrod (G1) and has been confirmed countless times. Given the fact that most gouty individuals are hyperuricemic does not in any way indicate the nature of the initiating reaction that causes some hyperuricemics to become gouty or furthermore, why some individuals have attacks of arthritis that disappear and then return again under certain other conditions. It would appear that there are two requirements for gout: one is hyperuricemia, and the other, an initiation of crystallization in the sensitive sites. Some years ago Hellman (H4) showed that precipitation of acute attacks of gouty arthritis could be induced by adrenocorticotropic hormone. Hellman was led to carry out his studies

because of the observations of Shipley *et al.* (S16) that nonspecific stresses such as trauma, infection, X-irradiation, or chilling produced increased adrenocortical activity, and the fact that these same stresses have been known to precipitate acute gouty arthritis. Ayvazian and Ayvazian (A6), studying the changes in serum and urinary uric acid in patients who had developed acute attacks of gout, noted an increased excretion of 17-hydroxycorticosteroids during the development of gouty symptoms. They were unable to postulate whether these were cause or effect, but, in relationship to the observations of Hellman, one would tend to believe that there is presumptive evidence that the changes in corticosteroid production may be a link in the chain initiating the events of acute symptomatic gout.

A second aspect of the precipitation of acute attacks of gout is related to the inflammatory reaction produced by the injection of sodium urate crystals. The historical background for believing that such materials are related to the occurrence of acute attacks is reviewed by McCarty (M10). Since synovial fluid from patients with acute attacks contains microcrystalline sodium urate, it appeared reasonable to believe that the presence of these crystals would cause gouty attacks. A number of investigators showed that administration of a microcrystalline sodium urate resulted in attacks in normal human subjects, in dogs, and in gouty patients in a quiescent phase (F1, H12, M4).

A condition known as pseudogout has been investigated by Kohn *et al.* (K12). Clinically, their patients appeared to have gouty arthritis. However, the serum urate levels were normal. When fluid from these patients was removed, it was found to contain crystals that were not sodium urate but calcium pyrophosphate. The mechanistic parallels to gout are obvious.

Not only does sodium urate precipitate acute attacks, but other substances with similar crystal structures, such as sodium orotate, can also cause symptoms of acute gout (S9). Since solutions of urate do not have the same effect (M3), it would be consistent with these data to suppose that precipitation, induced by seed crystals in the presence of high uric acid concentrations, is another link in the chain leading to acute gouty symptoms. The gouty symptoms precipitated by injection of crystals have another factor in common with normal gout, in that they are relieved by treatment with colchicine (H12, M4, S9). Seegmiller *et al.* had postulated that the mechanism of the gouty paroxysm required a crystal either of urate or something isomorphic in the target area and the presence of high concentrations of the substance in solution so that precipitation of additional crystals could occur. This would then be followed by leukocytosis and phagocytosis of these crystals, producing a

resultant inflammatory response. The response to stress and adrenocorticotropic hormones would also have to involve precipitation of uric crystals in the target area. These data and their interpretation would lead to the postulate that gout is a combination of at least two defects. The first leads to high concentrations of uric acid and, more than simply high serum levels, high miscible pools so that uric concentrations are present throughout the host, presumably leading to high concentration in certain target areas. A second defect leads to a more ready precipitation of the acute phase, either by increased sensitivity to the effects of stress and/or hormones or by another defect leading to the precipitation of crystals in these same target areas. The fact that any agent that lowers the level of urate production and decreases the size of the urate pool can prevent the symptoms from developing is consistent with this interpretation.

5.2. Psoriasis

This disease is of considerable interest in relation to oxypurine metabolism, although a wide variety of potential metabolic abnormalities have been said to be associated with it. In a review in 1961 Tickner (T4) asserted that there was evidence to support the postulate that psoriasis was associated with alterations in lipid, protein, carbohydrate, and mineral metabolism as well as in serum protein level. Investigators have since supported a myriad of hypotheses. Note has been taken of variations in carbohydrate metabolism (R16), and of changes in the synthesis of hyaluronic acid with resultant alterations in transport mechanisms (C10). Changes in fatty acid levels and metabolism (C8), alterations of aldolase activity (C6), increased proteolytic activity (S33), and alterations in the composition of proteins in psoriatic scales (L20) have been suggested. Changes in serum copper content in psoriatic patients have been observed (L18).

There were early reports that in patients with psoriasis there was a greater output of urinary uric acid (B11) and increases in serum uric acid levels (E2). Eisen and Weissman showed that the increase in uric acid excreted was proportional to the amount of skin involved in the disease. This increase in uric acid may be partially the result of increased turnover of the diseased cells and release of their nucleic acid to the purine catabolic pool. There are other alterations in nucleic acids related to cellular metabolism in psoriasis. The ribonucleic acid concentration of psoriatic tissues is approximately 3 times that of normal skin, and the DNA can be as much as 13–14 times greater than in normal tissues (H8, W5). A fundamental change in the nature of psoriatic deoxyribonucleic acid was suggested by Steigleder *et al.* (S36), who isolated DNA's from psoriatic skin and from normal skin and

compared their sensitivity to DNase. They found that the psoriatic DNA was relatively resistant to the action of this depolymerase. In view of the constancy of the DNA content of cells (V1), these excessive amounts of DNA could represent either enormous polyploidy and/or the presence of unusual, perhaps nonfunctional, nucleic acids. If so, differential susceptibility to DNase would not be unexpected.

The DNase levels of psoriatic tissue vary depending upon the fraction of the tissue examined (S34). That it is lower in psoriatic scales (S35) must be considered together with the fact that DNase is high in normal skin (T1). On the other hand, the RNase level was found to be much higher in affected skin, approximately 3 times the normal level (L19, S35). Examination of the enzyme indicated that it behaved like pancreatic RNase (D5, S34).

The levels of acid phosphatases were also much greater in psoriatic tissue and higher in the surrounding normal tissue as well (D6, W7). The high level of acid phosphatase found in adjacent normal tissues led to the postulate that the basic defect of psoriasis is an inborn error leading to greater levels of acid phosphatase (or lowered amounts of an inhibitor), so that there would be a degradation of nucleotides resulting in a hyperuricemia and abnormal metabolism in the cells *per se*. That this explanation might be too simple is indicated by the observation that not only is there a large amount of destruction of the nucleotides, but also a high level of nucleotides in psoriatic tissue (M9).

Eisen and Seegmiller (E1) attempted to investigate the cause of the large amount of uric acid produced by psoriasis patients by administration of labeled precursors, followed by study of the rate and mechanism of uric acid synthesis. They also administered labeled uric acid and determined the pool size and the turnover rate. Half of their patients were hyperuricemic, and the extent of the elevation in the serum uric was roughly proportional to the amount of skin involvement. These patients incorporated more label from a dose of glycine-^{14}C into urinary uric than did normal individuals, but the increase was not so great as that seen with gouty patients (W9). There was nothing unusual in any other aspect of the utilization of the precursor. The increase was commensurate with that to be expected from the larger amount of uric acid produced and excreted, and the larger pool. No abnormal metabolic pathway appeared to be involved, and the only unusual biochemical feature of the uric acid metabolism of these patients seemed to be its rapidity. Several of the patients studied by Eisen and Seegmiller had quite elevated uric acid levels, but none developed the symptoms of gout. One with both psoriasis and gout did not have a very high serum uric acid level. These results emphasize the fact that hyperuricemia alone

does not always produce gout, but a secondary effect, apparently unrelated to that found in psoriasis, is necessary to precipitate the arthritic symptoms.

5.3. Myeloproliferative Diseases

These diseases are characterized by, among other features, the presence of a large number of cells undergoing increased proliferation and death. As a result, in the host there is a greatly increased nucleic acid degradation that produces an excessive amount of nucleic acid breakdown products and ultimately uric acid. This in turn may bring the serum and urinary urate concentrations to a level high enough to precipitate symptomatic gout. The incidence of gout in patients with polycythemia vera and myeloid metaplasia is too high (5–9%) to be a coincidence (Y1). In Yü's studies, a positive family history of gout was found in about 30% of the primary gout patients, but in only 3 of 42 patients with gout secondary to the myeloproliferative disease. Thus he concludes that this secondary gout is a separate entity from primary gout.

Excessive uric acid production is consistently seen in untreated chronic granulocytic leukemia patients, but not in those with chronic lymphocytic leukemia (K15, K24, S1). That the increased production of uric acid is a symptom of the disease is shown in a series of patients studied before and after initiation of therapy. When the disease was under control, there was a marked decrease in both serum and urinary uric acid (Table 1). The unusual nature of the high uric acid production and its relationship

TABLE 1
Uric Acid Excretion in Untreated and Controlled Chronic Granulocytic Leukemia[a]

	Untreated			Controlled		
Patient	Urine uric acid (mg/day, mean)	Serum uric acid (mg/100 ml, mean)	WBC ($\times 10^3$, mean)	Urine uric acid (mg/day, mean)	Serum uric acid (mg/100 ml, mean)	WBC ($\times 10^3$, mean)
F.S.	2044	9.9	116	504	7.4	39
M.P.	1055	10.4	263	511	7.1	13
M.W.	1217	5.7	212	717	4.0	5
M.Y.	810	6.6	198	500	5.8	16
T.Y.	891	5.6	228	433	3.7	15
P.K.	618	8.0	73	341	5.7	10
Average	1106	7.7	177	507	5.6	16

[a] These values represent the mean of 8–12 consecutive days of observation during each phase in each patient (K19).

to the renewal of granulocytes were shown in an experiment in which the administration of labeled formate to patients with chronic granulocytic leukemia resulted in a large incorporation of this precursor into the uric acid, with a maximum not only at the end of 2–3 days, but also a second maximum that appears 12 days after administration of the isotope (Fig. 6). Presumably the first peak of excretion results from the direct syn-

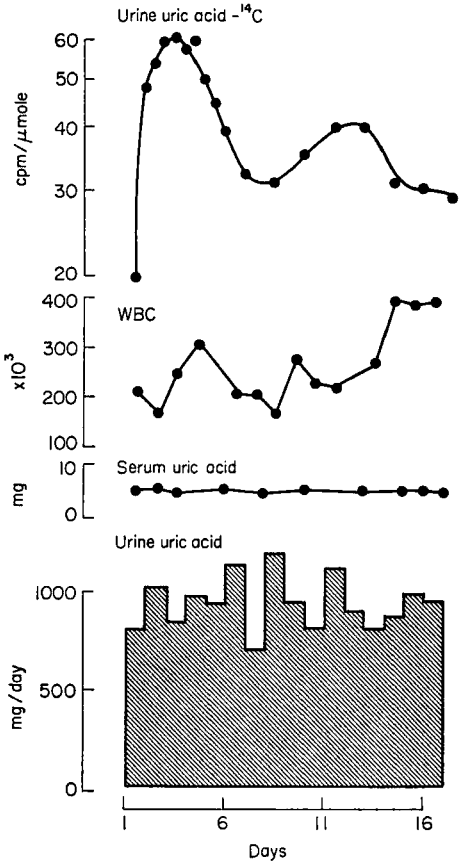


FIG. 6. Incorporation of formate-^{14}C into urinary uric acid of a patient with chronic granulocytic leukemia. [From (K24).]

thesis of labeled uric acid through the breakdown of purine nucleotides in the soluble cell pool, and the second is attributable to the destruction of granulocytes. Mathematical analysis and interpretation of the isotope data indicate that approximately five-eighths of the area under the curve, and therefore of the radioactivity obtained, is attributable to the turnover

of granulocytes, and is consistent with the magnitude of the overproduction of uric acid seen in such patients. Labeled formate incorporation curves from patients with chronic lymphocytic leukemia had a single maximum like those from nonleukemic patients (K19). These findings, consistent with the data of Hamilton (H1), indicate the differences in life span of human granulocytes and lymphocytes. Some attempts, made by Adams *et al.* (A4) to analyze the oxypurines other than uric acid in the urine of leukemic subjects, were inconclusive. No significance was attached to the findings by these investigators, who also recognized their data to be semiquantitative, like those with many of the earlier methods. Their work was important in that it established the existence of the oxypurines in urine, but, because of the difficulty in determining small amounts, the data could not be considered adequate for complete evaluation of the significance of these compounds (W3).

5.4. Congenital Juvenile Hyperuricogenesis

There have been reports of young children with elevated serum uric acid levels, some of whom appeared to have concurrent mental aberrations (C2, R7). In at least one case there were no symptoms other than those normally associated with gout (R13).

In 1965 Lesch and Nyhan (L11) described the third and fourth cases diagnosed until then of elevated serum uric acid accompanied by mental retardation. The patients were hyperuricosuric as well. The most striking characteristic was the self-mutilation of the patients (L11). They reported studies of two brothers, listed the clinical manifestations of the disease, and followed the rate of uric acid synthesis as well as the turnover of the uric acid pool. As in gout, incorporation of the labeled precursors resulted in the rapid extensive labeling of urinary waste. Examinations of the rate of excretion of injected labeled urate indicated the pool sizes in the two children to be 530 and 787 mg, or values similar to those seen in patients with nontophaceous gout (B14). However, the rate of replacement of the pool was higher than that of most gouty patients (S11); one of the brothers replaced 2.06 pools per day, and the other 1.61. Only a relatively small fraction of the uric acid was eliminated in the urine by these infants, and the turnover was so rapid that after a few days the amount of label was too small to be detected. On the basis of the first 5 days, Lesch and Nyhan concluded that none of the deviation from linearity to be expected with slow equilibration with tophi existed. Because of the rapid turnover in these patients, however, the decay curve was so short that curvature might well have been missed. The concentration of uric acid/kg of body weight was comparable to that of gouty patients (B15, S29), for whom it has been calculated

that the average concentration of uric in the total body water is about half that of the serum. The uric acid/body water ratio obviously increases in patients with tophaceous gout. It may well be that, in these two children, the concentration would increase in time as the kidneys failed to handle the massive amounts of uric acid presented to them.

The most striking thing is not so much the size of the pool but the rapidity of its replacement, i.e., amount of uric acid synthesized daily. The adults with primary gout, secondary gout, and hyperuricemia studied by Seegmiller *et al.* (S11) eliminated at most 1.7 g/day compared to the greater than 1 g/day put out by these 14-kg children. Benedict *et al.* (B14) reported one case of tophaceous gout that produced even more uric acid than these children, but the daily turnover was still only about half the pool of uric acid. It is therefore quite possible that the high figures reported in Benedict's case were misleading because of temporary mobilization of the uric acid tophi. Certainly nontophaceous gouty individuals do not produce amounts of uric acid of the same order of magnitude as did these children. Thus, the disease is characterized more by hyperuricogenesis than by hyperuricosuria or hyperuricemia *alone*.

Nyhan later described another patient who was a hyperexcretor and displayed the associated clinical symptoms. This child synthesized uric acid from labeled glycine 200 times as rapidly as did normal controls (N5). Another aspect of Nyhan's studies was the rapid decrease of labeled uric acid from patients given glycine-^{14}C. The fact that, a week after administration of the isotope, the radioactivity of uric acid in the control and the patients under study approached approximately the same value would seem to indicate that the initial large amount of labeled uric acid was formed by the synthesis of purine nucleotides, which were rapidly broken down and did not involve an increased turnover of nucleic acid *per se*. It reflects a labile nucleotide pool, not a highly labeled one.

That children can have gout and excessive uric acid production and/or excretion without suffering the cerebral damage of Nyhan's patients would seem to indicate a fundamental difference in the mechanism of uric acid production in these children and in gouty patients. The postulate that the control enzymes were deficient could explain symptoms similar to those seen in gout; such a mechanism had already been suggested for gout. An alternative explanation is that the purine intermediate that is overproduced or that leaks from the cell is different from that found in gout. The secondary effects could be attributed to the perfusion of the patient by higher than normal levels of some particular purine derivative. Ultimately the compound would be broken down to uric acid, by which time the damage may have been done. The dissimilar symptomatology

of the two diseases suggests that overproduction of uric acid is not the cause of both syndromes.

That two patients are brothers indicates the possibility that there is a familial trait involved. Examination of the pedigree of patients suggests that the disease is carried by an X-linked recessive gene. The disease has been seen only in males but, interestingly, female carriers of the gene appear to be hyperexcretors of uric acid, although they do not develop the mental and neurological symptoms (H9, S13). The uric acid metabolism of relatives of gouty patients and of hyperuricogenic children is quite different (Section 7), again indicating that the two diseases are fundamentally different.

Recently it has been shown that patients with this disease excrete more hypoxanthine than xanthine in their urine. All other subjects studied excrete more xanthine than hypoxanthine (B7). Furthermore, although the administration of allopurinol reduces total oxypurine output in other patients, it does not do so in these children (B7, O2). These observations have been clarified by the demonstration that patients with juvenile hyperuricemia lack inosinate pyrophosphorylase, the enzyme that catalyses the conversion of hypoxanthine (and guanine) to inosinic acid (and guanylic acid) (S10a). They, thus, excrete any hypoxanthine formed rather than reutilize it. This may identify the fundamental difference between this entity and gout, but offers no explanation of the neurological damage.

5.5. Other Hyperuricemias

Hyperuricemia has been found associated with myxedema, where it is accompanied by normal or low urinary urate excretion. On the other hand, hyperthyroidism is usually found with slightly increased urinary excretion (L8, M16). These variations have been interpreted in terms of changes in pool size and the relative role of extrarenal excretion (L7).

It has been known for some time that rapid destruction of tumor or leukemic tissue can result in excretion of large amounts of uric acid. The mechanisms by which various agents can cause this have been studied by Krakoff (K15). Agents capable of reducing leukocyte mass may do so either by destroying cells (e.g., X-rays, alkylating agents, and steroids) or by inhibiting additional cellular proliferation (e.g., antimetabolites). Substances of the first class cause an increase in uric acid production. The nucleic acid released by the dead cells is degraded by the enzymes referred to (in Section 3) (K24). Normally purine breakdown products of nucleic acids are not reutilized (D2). There is no reason to assume that only neoplastic tissue releases purines upon destruction, although these are the most common cases where massive quantities of tissues are known to be destroyed.

In general, hyperuricemia would result from a condition that causes the sudden destruction of tissue; in these cases, hyperuricosuria would also result. Interference with renal clearance (referred to in Section 4.1) could also result in hyperuricemia, but in this case there would be a decrease in urinary output. Most reported cases of hyperuricemia, aside from those involving hormonal abnormalities, are most likely related to these changes in renal clearance, possibly resulting from changes in the level of various organic acids (Section 4.1).

6. Drugs Affecting Purine Metabolism

Compounds active in the regulation of oxypurine metabolism can influence the production of purines *de novo* and the interconversion or

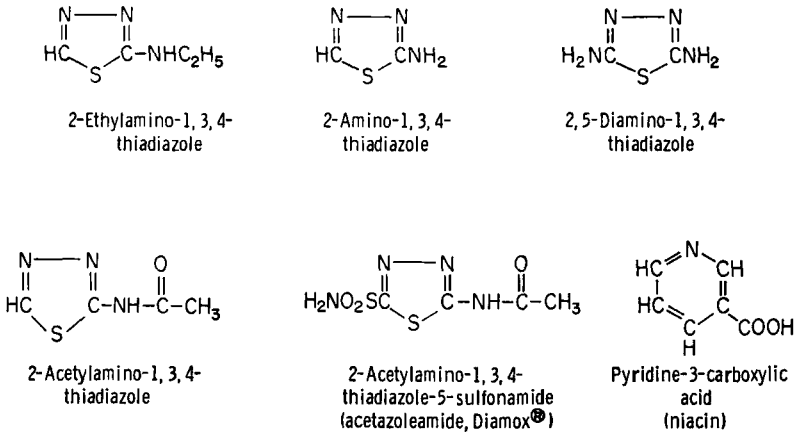


FIG. 7. Structural formulas of some thiadiazoles and niacin.

excretion of the purine moieties. The latter compounds are of considerable clinical importance, but are not active in the metabolism of the oxypurines *per se*. A recent review by Krakoff (K17) deals with the clinical aspects of compounds active in this area. Suffice it to say that drugs are known which can alter the secretion and/or the reabsorption of urates in the kidney. Such actions lead to altered concentrations of urate in blood and urine without necessarily changing the production or destruction of any of the oxypurines.

6.1. Compounds Stimulating Oxypurine Production

A number of 2-substituted thiadiazoles (Fig. 7) are unique in that they, among all known drugs, cause an increase in the production of uric acid. The original observation came as a result of the evaluation of 2-ethylamino-1,3,4-thiadiazole (EATDA) as a potential antitumor

agent (K21). Patients receiving the compound had elevated serum uric acid as well as elevated urinary output. The several derivatives shown in Fig. 7 were tested. The three compounds mono-substituted in the 2-position were active, and the compounds with substituents in both the 2- and 5-positions were inactive (K18). The uricogenic effect of the thiadiazoles was accompanied by a characteristic oral toxicity.

The effects of thiadiazole were blocked by simultaneous administration of nicotinamide (K18) or nicotinic acid (S8). Concurrent administration of 6-diazo-5-oxo-L-norleucine (DON), an antagonist of glutamine that is also an inhibitor of purine biosynthesis (L13), abolished the uricogenic effect of the thiadiazoles although the toxicity remained. Azaserine, a compound structurally related to DON which has a similar activity (L13), is also capable of preventing the uricogenic effect of EATDA. The antimetabolite, 6-mercaptopurine, can also inhibit the increased production of urate without preventing the oral toxicity (K23). On the other hand, 6-thioguanine, a purine antagonist that resembles 6-mercaptopurine in structure and in some of its biochemical actions, was incapable of inhibiting any of the effects of thiadiazole even at toxic doses (K16). Allopurinol, an inhibitor of xanthine oxidase, also prevents the uricogenic effect of thiadiazole without affecting the oral toxicity (K20).

Not only are the effects of thiadiazoles blocked in patients, but the antileukemic action in experimental animals is also reversed by nicotinamide (C3). No direct evidence of interference with NAD synthesis or function has been obtained, but these reversal studies obviously suggest this as a possible explanation of the biochemical actions of these compounds. Ciotti and associates (C3) demonstrated an exchange reaction with NAD *in vitro* yielding an analog of NAD, but no evidence was obtained that this occurs *in vivo*.

Other investigations into the action of these compounds revealed that patients receiving EATDA incorporated more sodium formate-^{14}C into uric acid than did controls. The increase was similar to that seen with gouty overexcretions of uric acid (K18). The increase in incorporation was rapid, which suggested that the action was attributable to increased *de novo* synthesis. This hypothesis was confirmed by an experiment in which a patient was given adenine-^{14}C to label the nucleic acid purines and, some time later when the label in urinary uric became stable, the patient was given thiadiazole and ammonia-^{15}N. The latter compound was used as a measure of *de novo* synthesis. Administration of ^{15}N was repeated after the effects of thiadiazole were gone. The thiadiazole treatment caused an increase in serum and urinary uric acid. It also resulted in a drop of the specific activity of the urinary uric acid, indicating a

dilution of the nucleic acid source (Fig. 8). The incorporation of the ^{15}N, when the patient was receiving EATDA, was approximately 3-fold that seen during the control period, which indicates a 3-fold greater *de novo*

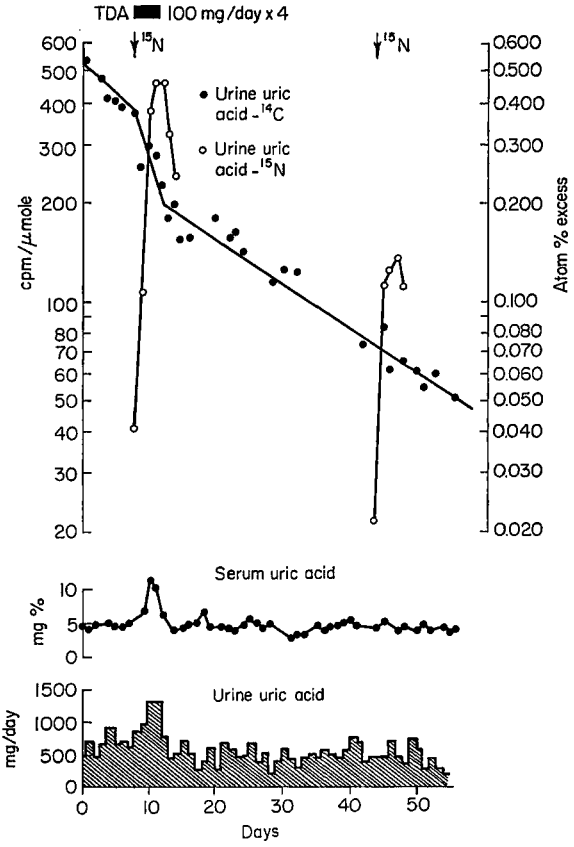


FIG. 8. Study of a patient (with spindle cell sarcoma) given adenine-8-^{14}C (200 μc) 3 weeks prior to 2-ethylamino-1,3,4-thiadiazole (TDA). The thiadiazole was given at a time when the decay curve of urine uric acid-^{14}C was a straight line. Note the sharp drop in ^{14}C concentration in urine uric acid produced by the thiadiazole. Ammonium chloride-^{15}N was incorporated to a much greater extent when given during thiadiazole administration than during the control study. The second ^{15}N curve has been corrected here for the extrapolated value of the first curve. [From (K18).]

synthesis of purines. A somewhat similar observation was made in experimental animals by Shuster and Goldin (S18), who demonstrated that thiadiazole results in enhanced incorporation of carbon precursors into soluble adenine derivatives in the liver of mice.

Examination of the urine of subjects receiving EATDA showed that several, if not all, of the urinary purines were excreted in increased amounts. There was, furthermore, no change in the mode of excretion of urates or in the proportion disposed of extrarenally (S12). The suggestion has been made (K18) that the fundamental mechanism of action of these compounds is the blocking of the incorporation of newly synthesized adenine into polynucleotides and/or coenzymes, with the production of an undefined deficiency state. This block could stimulate a compensatory increase in purine biosynthesis. The excess purines would not be utilizable and, therefore, would be excreted from the cells, and the normal degradation by enzymes would convert this material to uric acid.

6.2. Inhibitors of Purine Synthesis

Two compounds referred to in Section 6.1, azaserine and DON, are known to inhibit purine synthesis. These two compounds inhibit the conversion of formylglycineamide ribonucleotide to formylglycineamidine ribonucleotide (Section 2.1), presumably by virtue of their action as glutamine antagonists (L13). Both of these compounds inhibit the biosynthesis of uric acid in man. This has been demonstrated by the fact that the amount of glycine-^{14}C incorporated into urinary uric acid is decreased (G12, Z3) by the glutamine antagonist. The actual reduction in the quantity of urinary uric acid brought about by these compounds is too small to be detected in view of the daily variations in output. Both of these compounds are toxic to bone marrow and the gastrointestinal tract. Presumably this action is a result of their interference with purine synthesis *de novo*. Although these compounds do not normally cause a measurable decrease in the uric acid production, it is interesting, as was mentioned (Section 6.1), that they are able to prevent the excessive production of uric acid stimulated by the thiadiazoles. In view of the action of these compounds as antagonists of glutamine, it is not surprising that in some systems large doses lead to inhibition of conversion of xanthylate to guanylate (A3). Although this might potentially lead to an imbalance of purine interconversion, the levels required are so high as to be extremely toxic.

One of the first studies that indicated the possible interference of amethopterin and aminopterin with purine synthesis was the demonstration by Skipper *et al.* (S21) that administration of these compounds to mice inhibited the incorporation of labeled formate into nucleic acid purines. Amethopterin (4-amino-N^{10}-methyl-pteroylglutamic acid) has produced some inhibition of uric acid synthesis during the use of this compound for treatment of leukemia (K15, K24). Another inhibitor of

purine metabolism, 6-mercaptopurine, which acts either by pseudofeedback inhibition of purine synthesis (M11) or as an inhibitor of the interconversion of purines (B8), does not normally cause detectable reductions in purine synthesis in man. However, as was mentioned (Section 6.1), when given in conjunction with EATDA, it too can reduce the overproduction of the uric acid stimulated by this compound.

Orotic acid reacts with phosphoribosylpyrophosphate to form orotidine 5′-phosphate (K13). Since PRPP is also a reactant in *de novo* synthesis of purines (Section 2.1), the suggestion was made that competition could exist between the orotic acid and glutamine for the limited supply of PRPP, and thus the presence of large amounts of orotate would prevent purine synthesis. The feeding of large amounts of orotic acid caused fatty livers. An analysis of this situation and its reversal by the addition of adenine to the diet led Handschumacher *et al.* to postulate that *in vivo* there was in fact an inhibition of purine synthesis because of the depletion of PRPP by orotic acid (E8, H3). Despite the logic, data to the contrary indicated that orotic acid stimulated hepatic purine synthesis (W6). The therapy of gout with orotic acid was undertaken and apparently was somewhat successful (D8). The success, however, is probably attributable to the fact that orotic acid is a uricosuric agent (S31).

Although many compounds pharmacologically able to inhibit *de novo* purine synthesis are known, none has been useful in the treatment of overproduction of uric acid because of their general toxicity, since they are not specific for the overproduction *per se*.

6.3. Inhibitors of Xanthine Oxidase

A variety of pyrazolo(3,4-d)pyrimidines were shown to be both substrates and inhibitors of xanthine oxidase (F2). The 4-hydroxy compound was later shown to be the most potent of these derivatives (E4). This inhibition also occurs *in vivo*. In view of this fact it was suggested that the compound might prevent the destruction by xanthine oxidase of 6-mercaptopurine (6-MP) and thus permit the use of smaller amounts of this antileukemic drug. The hypothesis was tested in animals and in patients and the hoped for results were obtained (E3, R15). These investigators also noted that in patients with hyperuricemia treated with 4-hydroxypyrazolo(3,4-d)pyrimidine (allopurinol) there was a reduction in the uric acid level, a reduction not seen when 6-MP alone was administered. They then used the drug in the therapy of gout and were successful in reducing the uric acid level and the symptoms of gout in the patients under study. Their results have been confirmed in a number of other studies (K10, W12, Y2). As might have been predicted,

since the xanthine oxidase inhibitor prevents the conversion of oxypurines to uric acid, they are excreted as hypoxanthine and xanthine. Serum levels of hypoxanthine and xanthine usually approach 0.3–0.4 mg% (about 4 times normal) (R15). They do not reach the concentration that uric acid does, since the renal clearance of these compounds is at least 10 times greater than that of uric acid (G7, K10). The amount of oxypurine excreted per day does not usually equal the decrease in uric acid output (Y2). As is seen with patients with xanthinuria, the output of xanthine in these patients usually exceeds that of hypoxanthine (K20). The drug is useful not only in treatment of primary gout but also in alleviating the hyperuricemia of leukemia (K22). The availability of this compound has permitted the demonstration that hypoxanthine and xanthine are excellent precursors of nucleic acid purines, if they are not degraded first (P6, P7). When allopurinol was used to inhibit xanthine oxidase in experimental animals, both of these compounds were well utilized.

Allopurinol is a substrate as well as an inhibitor of xanthine oxidase. The product, 4,6-dihydroxypyrazolo(3,4-d)pyrimidine (alloxanthine), is an analog of xanthine (E5). It is also an inhibitor of xanthine oxidase, although less potent than allopurinol. The dihydroxy compound is cleared much more slowly by the kidney than the mono compound, however (E5); therefore in practice it is the active inhibitor *in vivo*.

A wide variety of compounds have been shown to inhibit xanthine oxidase: guanidines and some triazines (F7), purine derivatives such as purine 6-aldehyde (G5), 6-mercaptopurine (S20), 2,6-diaminopurine (W8), flavonoids (B13), and Antabuse (R6). Although these compounds and the inhibitions they produce are of interest in understanding the nature of the action of xanthine oxidase, none has been useful clinically in limiting urate production. Antabuse, as a result of its action, produces other effects that (fortunately?) have not been found with allopurinol.

7. Genetic Controls

The investigators of gout have been lucky in that a reasonable number of the old wives' tales proved to be useful leads. The influence of alcohol, although not necessarily red wine, has been confirmed, and the belief that gout is hereditary has a good deal of basis in fact. As Blumberg (B21) has pointed out, most investigators started with the assumption that gout, or at least hyperuricemia, is hereditary, and have set out to study the mode of heredity, assuming that tradition has taken care of the first problem. Examination of a variety of data has long since led to statistical confirmation of these beliefs. For example, asymptomatic hyperuricemia is often found in relatives of patients with gout (S23). On the basis of determinations of serum urate levels, Smyth *et al.* (S22) concluded that

hyperuricemia is apparently the result of a single autosomal dominant gene. On the basis of uric acid excretion studies and clinical diagnoses of 19 gouty families, Rakic and associates (R1) concluded that not only hyperuricemia but gout, too, is hereditary. This may mean merely that there is an increased predisposition toward gout in hyperuricemics, of course. No one has yet been able to demonstrate clear evidence of hyperuricemia free of gout in any family group. Nevertheless the postulate that there is more than one gene involved in gout persists, and it certainly has the acceptance of the great majority of investigators in this area. Further evidence for the genetic nature of hyperuricemia in gout was obtained by Decker *et al.* (D7), who found the occurrence of hyperuricemia and of gout higher among Filippinos, and the distribution of high uric acid values seemed to indicate that there were two populations, one hyperuricemic and a larger normo-uricemic one.

The juvenile hyperuricogenesis described by Nyhan also appears to be a genetically controlled disease, as suggested by the fact that the first patients reported by Lesch and Nyhan (L11) were brothers. Examination of the pedigree of patients with this disease led to the conclusion (H9, S13) that the disease is hereditary, and that it is X-linked and inherited in a recessive mode. The female carriers appeared to be hyperexcretors of uric acid; the males who did not develop this specific syndrome did not seem unusually prone to gout and were not hyperexcretors of uric acid. This is radically different from the picture seen with gout, and is at variance with the fact that in general males excrete more uric acid than do females. The genetic disorder of juvenile hyperuricogenesis probably acts at a locus quite different from that involved in gout.

8. Assays of Oxypurines

The oxypurines can be determined by their ultraviolet absorption, changes in absorption following reaction with enzymes, colorimetric reactions, or combinations of these methods. The assay of large quantities of reasonably pure oxypurines is quite simple; practical problems, however, arise in the determination of small quantities in the presence of unknown impurities. No completely satisfactory methods for the determination of these compounds have been developed. One of the difficulties in interpreting some of the results in the literature is to decide whether the cerebrations of the investigators exceed the limitations of their methods.

8.1. Uric Acid

A colorimetric method for determination of uric acid was introduced by Folin and Denis (F5); this initial method, which used phosphotungstate, was modified by a series of investigators (B16, B32, F4). Brown

(B33) summed up the objections when he showed that the color was not linear in response to the amount of uric acid, that recoveries of added uric acid were not complete, and that the reaction was not completely specific. Improvements on this have been suggested, based upon prior purification of the uric acid. One method, for example, not designed to be used with the colorimetric procedure but with an enzymatic determination, is to pass serum through a molecular exclusion resin to remove interfering large molecules (B18). Another suggested approach is to purify the uric acid by precipitation; it could then be redissolved and assayed free of many interfering substances. A procedure that uses acid mercuric acetate has been developed (B17, W4); unfortunately, the precipitation of the urate may not be quantitative under these conditions. Another way of avoiding the blank color by the use of uricase was suggested some time ago (B20, B37); uricase converts uric acid to allantoin, which does not react with phosphotungstate or have an absorption at 290 mμ. Uricase is added to one sample followed by the phosphotungstate reagent; a duplicate sample is reacted with the reagent without enzyme. The color produced in the uricase-treated sample serves as a blank. The objections to this method are that it involves twice as many determinations, and, like all methods depending upon differences, that the errors of the determinations are additive. Kalckar (K2) suggested the differential spectrophotometric method, where the amount of uric acid is calculated from the decrease in optical density at 290 mμ following the addition of uricase; this method was developed further by Praetorius (P9) and Praetorius and Poulsen (P10). A very extensive analysis of the method, its limitations, and its advantages was carried out by Liddle *et al.* (L15). The uricase procedure is the method of choice for routine laboratory analyses, and many investigators have found it to be the most reliable method within the limits of convenience. The uricase method is not perfect; for example, there may be inhibitors of uricase present, the serum absorption itself may change even without the addition of uricase, and no perfect method of blanks has been developed. This problem has been investigated and no simple solution is available (K8). Chromatographic methods have been developed (A4, A7) and there is the isotope dilution technique (R8), but these are practical only in limited research projects and are too time-consuming for normal routine determinations.

The recent introduction of automatic analyzers with their standard and reproducible conditions has made the colorimetric method consistent and accurate, although the problem of the nonspecific blank still remains. This method is undoubtedly the quickest, easiest, and most reliable for routine analyses of serum uric acid (B40, O3).

8.2. Hypoxanthine and Xanthine

The problems outlined for uric acid also exist for xanthine and hypoxanthine assays; however, they are amplified by the fact that the levels of xanthine and hypoxanthine are considerably lower. The method of Kalckar (K2) is excellent if sufficient material is present. Attempts to use this method in urine meet with the difficulty that urea is an inhibitor of xanthine oxidase, and the color change to be expected is so low that spontaneous changes are often as great as those seen with the enzyme. A method was developed to overcome these difficulties by Jørgensen and Poulsen (J7). More recently the method has been improved (P4) so that, through the use of differential spectrophotometry, xanthine and hypoxanthine can be individually determined and the sum confirmed through the use of uricase to hydrolyze the uric acid formed by the action of xanthine oxidase. However, because of the low optical densities observed and the extreme sensitivity of the optical density of hypoxanthine and xanthine to changes in pH, the results often do not agree with theory. Improvements have been noted with urinary oxypurines by first absorbing them on Dowex ion-exchange resin, according to the method of Weissmann *et al.* (W2), and determining hypoxanthine and xanthine in the material eluted from the resin. However, even here the impurities present are of sufficient magnitude that the individual oxypurines hypoxanthine and xanthine cannot be determined, although the sum can be easily measured by the increase of optical density at 290 mμ, which is lost upon the addition of uricase. Probably the only truly quantitative method available for determining the small amounts of hypoxanthine and xanthine present in normal blood and urine is the ion-exchange or isotope dilution method (A4, A7). The enzymatic methods, however, are sufficiently accurate for determination of these substances in xanthinuric patients or patients receiving allopurinol in whom the levels are considerably increased.

Methods have been devised for the determination of these compounds in the presence of uric acid through the use of uricase. The uricase is destroyed and the xanthine and hypoxanthine determined enzymatically. In the absence of uric acid (e.g., patients with xanthinuria), addition of xanthine oxidase converts the hypoxanthine and xanthine to uric acid and any method for uric acid determination could then be employed. The determination of hypoxanthine and xanthine by any method is complicated if the patient has received allopurinol, since allopurinol does not separate well from xanthine on ion-exchange resins and both allopurinol and alloxanthine are inhibitors of xanthine oxidase. Carefully

controlled chromatography will permit separation, and excess xanthine oxidase overcomes the inhibition by the pyrazolo compounds.

References

A1. Abderholden, E., and Schittenhelm, A., Der Ab und Aufbau Nucleinsäuren in Tierischen Organismus. *Z. Physiol. Chem.* **47,** 452–457 (1906).

A2. Abrams, R., Some factors influencing nucleic acid purine renewal in the rat. *Arch. Biochem. Biophys.* **33,** 436–447 (1951).

A3. Abrams, R., and Bentley, M., Biosynthesis of nucleic acid purines. III. Guanosine 5′-phosphate formation from xanthosine 5′-phosphate and L-glutamine. *Arch. Biochem. Biophys.* **79,** 91–110 (1959).

A4. Adams, W. S., Davis, F., and Nakatani, B. S., Purine and pyrimidine excretion in normal and leukemic subjects. *Am. J. Med.* **28,** 726–734 (1960).

A5. Ayvazian, J. H., Xanthinuria and hemochromatosis. *New Engl. J. Med.* **270,** 18–22 (1964).

A6. Ayvazian, J. H., and Ayvazian, L. F., Changes in serum and urinary uric acid with the development of symptomatic gout. *J. Clin. Invest.* **42,** 1835–1839 (1963).

A7. Ayvazian, J. H., and Skupp, S. J., The study of purine utilization and excretion in a xanthinuric man. *J. Clin. Invest.* **44,** 1248–1260 (1965).

B1. Balis, M. E., Studies on the metabolism of human tumors. IV. Certain aspects of guanine metabolism. *Cancer Res.* **19,** 297–302 (1959).

B2. Balis, M. E., and Salser, J., Interconversion of purines by tumors and the action of anti-purines. *Acta, Unio Intern. Contra Cancrum* **20,** 115–117 (1964).

B3. Balis, M. E., Lark, C. T., and Luzzati, D., Nucleotide utilization by *Escherichia coli. J. Biol. Chem.* **212,** 641–645 (1955).

B4. Balis, M. E., Marrian, D. H., and Brown, G. B., On the utilization of guanine by the rat. *J. Am. Chem. Soc.* **73,** 3319–3320 (1951).

B5. Balis, M. E., Van Praag, D., and Aezen, F., Studies on the metabolism of human tumors. II. Pentosenucleic acid synthesis in tumor-bearing rats. *Cancer Res.* **16,** 628–631 (1956).

B6. Balis, M. E., Van Praag, D., and Brown, G. B., Studies on the metabolism of human tumors. I. Pentosenucleic acid synthesis in tumor-bearing hamsters. *Cancer Res.* **15,** 673–678 (1955).

B7. Balis, M. E., Krakoff, I. H., Berman, P. H., and Dancis, J., Urinary metabolites in congenital hyperuricosuria. *Science,* **156,** 1122–1123 (1967).

B8. Balis, M. E., Levin, D. H., Brown, G. B., Elion, G. B., Nathan, H., and Hitchings, G. H., Effects of 6-mercaptopurine on *Lactobacillus casei. Arch. Biochem. Biophys.* **71,** 358–366 (1957).

B9. Balis, M. E., Levin, D. H., Brown, G. B., Elion, G. B., Vanderwerff, H., and Hitchings, G. H., The incorporation of exogenous purines into pentose nucleic acid by *Lactobacillus casei.* I. *J. Biol. Chem.* **196,** 729–747 (1952).

B10. Barnes, F. W., and Schoenheimer, R., Biological synthesis of purines and pyrimidines. *J. Biol. Chem.* **151,** 123–139 (1943).

B11. Baumann, R. R., and Jillson, O. F., Hyperuricemia and psoriasis. *J. Invest. Dermatol.* **36,** 105–107 (1961).

B12. Bearn, A. G., Wilson's disease. *In* "The Metabolic Basis of Inherited Disease" (J. B. Stanbury, J. B. Wyngaarden, and D. S. Fredrickson, eds.), pp. 761–779. McGraw-Hill (Blakinston), New York, 1966.

B13. Beiler, J. M., and Martin, G. J., The inhibition of xanthine oxidase by flavonoids and related compounds. *J. Biol. Chem.* **192,** 831–834 (1951).

B14. Benedict, J. D., Forsham, P. H., and Stetten, D., Jr., The metabolism of uric acid in the normal and gouty human studied with the aid of isotopic uric acid. *J. Biol. Chem.* **181,** 183–193 (1949).

B15. Benedict, J. D., Forsham, P. H., Rocke, M., Soloway, S., and Stetten, D., Jr., The effect of salicylates and adrenocorticotropic hormone upon the miscible pool of uric acid in gout. *J. Clin. Invest.* **29,** 1104–1111 (1950).

B16. Benedict, S. R., The determination of uric acid in blood. *J. Biol. Chem.* **51,** 187–207 (1922).

B17. Bergmann, F., and Dikstein, S., Studies on uric acid and related compounds. I. Quantitative determination of uric acid in biological fluids. *J. Biol. Chem.* **211,** 149–153 (1954).

B18. Bergmann, F., and Kalina, M., Determination of uric acid and its thio analogs in biological fluids. *Anal. Biochem.* **10,** 73–85 (1965).

B19. Bishop, C., Garner, W., and Talbott, J. H., Pool size, turnover rate, and rapidity of equilibration of injected isotopic uric acid in normal and pathological subjects. *J. Clin. Invest.* **30,** 879–888 (1951).

B20. Blauch, M. B., and Koch, F. C., A new method for the determination of uric acid in blood with uricase. *J. Biol. Chem.* **130,** 443–454 (1939).

B21. Blumberg, B. S., Heredity of gout and hyperuricemia in gout and purine metabolism. *Arthritis Rheumat.* **8,** 627–633 (1965).

B22. Bodansky, O., and Schwartz, M. K., Comparative effects of L-histidine on the activities of 5'nucleotidase and alkaline phosphatase. *J. Biol. Chem.* **238,** 3420–3427 (1963).

B23. Booth, V. H., The specificity of xanthine oxidase. *Biochem. J.* **32,** 494–502 (1938).

B24. Brody, S., and Balis, M. E., Ribonuclease and deoxyribonuclease activities during normal and neoplastic growth. *Nature* **182,** 940–941 (1958).

B25. Brody, S., and Balis, M. E., Mechanism of growth. I. Interrelation between deoxyribonuclease and deoxyribonucleic acid synthesis in nonmalignant growth. *Cancer Res.* **19,** 538–543 (1959).

B26. Brody, S., and Thorell, B., Ribonuclease and desoxyribonuclease activities in normal and regenerating bone marrow homogenates. *Biochim. Biophys. Acta* **25,** 579–585 (1957).

B27. Brown, D. M., Dekker, C. A., and Todd, A. R., *J. Chem. Soc.* pp. 2715–2721 (1952).

B28. Brown, G. B., and Roll, P. M., Biosynthesis of nucleic acids. *In* "The Nucleic Acids" (E. Chargaff and J. N. Davidson, eds.), Vol. 2, pp. 341–392. Academic Press, New York, 1955.

B29. Brown, G. B., Roll, P. M., and Plentl, A. A., Studies on the metabolism of adenine. *Federation Proc.* **6,** 517–522 (1947).

B30. Brown, G. B., Bendich, A., Roll, P. M., and Sugiura, K., Utilization of guanine by the C57 black mouse bearing adenocarcinoma Eo771. *Proc. Soc. Exptl. Biol. Med.* **72,** 501–502 (1949).

B31. Brown, G. B., Roll, P. M., Plentl, A. A., and Cavalieri, L. F., The utilization of adenine for nucleic acid synthesis and as a precursor of guanine. *J. Biol. Chem.* **172,** 469–484 (1948).

B32. Brown, H., The determination of uric acid in blood. *J. Biol. Chem.* **68,** 123–133 (1926).

B33. Brown, H., The determination of uric acid in human blood. *J. Biol. Chem.* **158,** 601–608 (1945).

B34. Buchanan, J. M., Biosynthesis of purine nucleotides. *In* "The Nucleic Acids"

(E. Chargaff and J. N. Davidson, eds.), Vol. 3, pp. 303–322. Academic Press, New York, 1960.

B35. Buchanan, J. M., and Sonne, J. C., Utilization of formate in uric acid synthesis. *J. Biol. Chem.* **166,** 781 (1946).

B36. Buchanan, J. M., Sonne, J. C., and Delluva, A. M., Biological precursors of uric acid. II. The role of lactate, glycine, and carbon dioxide as precursors of the carbon chain and nitrogen atom 7 of uric acid. *J. Biol. Chem.* **173,** 81–98 (1948).

B37. Bulger, H. A., and Johns, H. E., The determination of plasma uric acid. *J. Biol. Chem.* **140,** 427–440 (1941).

B38. Buzard, J., Bishop, C., and Talbott, J. H., Recovery in humans of intravenously injected isotopic uric acid. *J. Biol. Chem.* **196,** 179–184 (1952).

B39. Buzard, J., Bishop, C., and Talbott, J. H., The fate of uric acid in the normal and gouty human being. *J. Chronic Diseases* **2,** 42–49 (1955).

B40. Bywaters, E. G. L., and Holloway, V. P., Measurement of serum uric acid in Great Britain in 1963. *Ann. Rheumatic Diseases* **23,** 236–239 (1964).

C1. Carter, C. E., and Cohen, L. H., The preparation and properties of adenylosuccinase and adenylosuccinic acid. *J. Biol. Chem.* **222,** 17–30 (1956).

C2. Catel, W., and Schmidt, J., Über familiäre Gichtische Diathese in Verbindung mit Zerebralen und Renalen Lymptomen bei einem Kleinkind. *Deut. Med. Wochschr.* **84,** 2145–2147 (1959).

C3. Ciotti, M. M., Humphreys, S. R., Venditti, J. M., Kaplan, N. O., and Goldin, A., Antileukemic action of two thiadiazole derivatives. *Cancer Res.* **20,** 1195–1201 (1960).

C4. Clarke, D. A., Davoll, J., Philips, F. S., and Brown, G. B., Enzymatic deamination and vasodepressor effects of adenosine analogs. *J. Pharmacol. Exptl. Therap.* **106,** 291–302 (1952).

C5. Coburn, S. P., Luce, M. W., and Mertz, E. T., Elevated levels of several nitrogenous non-protein metabolites in mongoloid blood. *Am. J. Mental Deficiency* **69,** 814–817 (1965).

C6. Comaisch, J. S., Epidermal aldolase levels in psoriasis and normal skin. *Brit. J. Dermatol.* **75,** 337–343 (1963).

C7. Conway, E. J., and Cook, R., Deaminases of adenosine and adenylic acid in blood and tissues. *Biochem. J.* **33,** 479–492 (1939).

C8. Coon, W. M., Wheatley, V. R., Herrmann, F., and Mandel, L., Free fatty acids of the skin surface and barrier zone in normal and abnormal keratinization. *J. Invest. Dermatol.* **41,** 259–264 (1963).

C9. Corran, H. S., Dewan, J. G., Gordon, A. H., and Green, D. E., Xanthine oxidase and milk flavoprotein. *Biochem. J.* **33,** 1694–1706 (1939).

C10. Cotton, D. W. K., and Mier, P. D., An hypothesis on the etiology of psoriasis. *Brit. J. Dermatol.* **76,** 519–528 (1964).

D1. Dabrowska, W., Cooper, E. J., and Laskowski, M., A specific inhibitor for desoxyribonuclease. *J. Biol. Chem.* **177,** 991–992 (1949).

D2. Dancis, J., and Balis, M. E., The reutilization of nucleic acid catabolites. *J. Biol. Chem.* **207,** 367–374 (1954).

D3. Daoust, R., and Amano, H., Ribonuclease and deoxyribonuclease activities in experimental and human tumors on the histochemical substrate film method. *Cancer Res.* **23,** 131–134 (1963).

D4. Davidson, J. N., "The Biochemistry of the Nucleic Acids." Methuen, London, 1952.

D5. de Bersaques, J., Activité de la ribonucléase cutanée. *Arch. Belges Dermatol. Syphiligraphie* **20,** 315–317 (1964).

D6. de Bersaques, J., and Fiers, W., Comparaison des activités phosphatasiques de l'épiderme et des squames psoriasigues. *Arch. Belges Dermatol. Syphiligraphie* **15,** 275–278 (1959).
D7. Decker, J. L., Lane, J. J., Jr., and Reynolds, W. E., Hyperuricemia in a Filipino population. *Arthritis Rheumat.* **5,** 144–155 (1962).
D8. Delbarre, F., and Auscher, C., Traitement de la goutte par l'acide uracil-6-carboxylique et ses dérivés. *Presse Med.* **71,** 1765–1768 (1963).
D9. Dent, C. E., and Philpot, G. R., Xanthinuria; inborn error (or deviation) of metabolism. *Lancet* **I,** 182–185 (1954).
D10. de Renzo, E. C., Kaleita, E., Heytler, P., Oleson, J. J., Hutchings, B. L., and Williams, J. H., The nature of the xanthine oxidase factor. *J. Am. Chem. Soc.* **75,** 753 (1953).
D11. Dickinson, C. J., and Smellie, J. M., Xanthinuria. *Brit. Med. J.* No. 5161, 1217–1221 (1959).
D12. Doctor, V. M., Studies on the purification and properties of human plasma deoxyribonuclease. *Arch. Biochem. Biophys.* **103,** 286–290 (1963).
D13. Dunn, J. P., Brooks, G. W., Mausner, J., Rodnan, G. P., and Cobb, S., Social class gradient of serum uric acid levels in man. *J. Am. Med. Assoc.* **185,** 431–436 (1963).
E1. Eisen, A. Z., and Seegmiller, J. E., Uric acid metabolism in psoriasis. *J. Clin. Invest.* **40,** 1486–1494 (1961).
E2. Eisen, A. Z., and Weissman, S., Pseudouridine excretion in psoriasis. *J. Invest. Dermatol.* **39,** 35–37 (1962).
E3. Elion, G. B., Callahan, S., Nathan, H., Bieber, S., Rundles, R. W., and Hitchings, G. H., Potentiation by inhibition of drug degradation, 6-substituted purines and xanthine oxidase. *Biochem. Pharmacol.* **12,** 85–93 (1963).
E4. Elion, G. B., Taylor, T. J., and Hitchings, G. H., Binding of substrates and inhibitor and xanthine oxidase from different species. *Proc. 6th Intern. Congr. Biochem., New York, 1964* Vol. IV, p. 42.
E5. Elion, G. B., Kovensky, A., Hitchings, G. H., Metz, E., and Rundles, R. W., Metabolic studies on allopurinol, an inhibitor of xanthine oxidase. *Biochem. Pharmacol.* **15,** 863–880 (1966).
E6. Engelman, K., Richard, W. E., Watts, J. R., Klinenberg, A. S., and Seegmiller, J. E., Clinical physiological and biochemical studies of a patient with xanthinuria and pheochromocytoma. *Am. J. Med.* **37,** 839–861 (1964).
E7. Epstein, F. H., An epidemiological study in a total community; the Tecumseh Project. *Univ. Mich. Med. Bull.* **26,** 307–314 (1960).
E8. von Euler, L. H., Rubin, R. J., and Handschumacher, R. E., Fatty livers induced by orotic acid. II. Changes in nucleotide metabolism. *J. Biol. Chem.* **238,** 2464–2469 (1963).
F1. Faires, J. S., and McCarty, D. P., Jr., Acute synovitis in normal joints of man and dog produced by injection of microcrystalline sodium urate, calcium oxalate and corticosteroid esters. *Arthritis Rheumat.* **5,** 295–296 (1962).
F2. Feigelson, P., Davidson, J. D., and Robins, R. K., Pyrazolopyrimidines as inhibitors and substrates of xanthine oxidase. *J. Biol. Chem.* **226,** 993–1000 (1957).
F3. Florkin, M., "Biochemical Evolution," p. 71. *Academic Press,* New York, 1949.
F4. Folin, O., A system of blood analysis. *J. Biol. Chem.* **54,** 153–170 (1922).
F5. Folin, O., and Denis, W., A new (colorimetric) method for the determination of uric acid in blood. *J. Biol. Chem.* **13,** 469–475 (1912).
F6. Francis, T., Jr., Aspects of a Tecumseh study. *Public Health Rept. (U.S.)* **76,** 963–965 (1961).

F7. Fridovich, I., A new class of xanthine oxidase inhibitors isolated from guanidinium salts. *Biochemistry* **4,** 1098–1101 (1965).
F8. Friedkin, M., Desoxyribose-1-phosphate. II. The isolation of crystalline desoxyribose-1-phosphate. *J. Biol. Chem.* **184,** 449–459 (1950).
F9. Friedkin, M., and Kalckar, H. M., Desoxyribose-1-phosphate. I. The phosphorolysis and resynthesis of purine desoxyribose nucleoside. *J. Biol. Chem.* **184,** 437–449 (1950).
F10. Friedkin, M., and Kalckar, H. M., Nucleoside phosphorylases. *In* "The Enzymes" (P. D. Boyer, H. Lardy, and K. Myrbäck, eds.), 2nd ed., Vol. 5, pp. 237–255. Academic Press, New York, 1961.
F11. Friedman, M., and Byers, S. O., Observations concerning the causes of the excess excretion of uric acid in the dalmatian dog. *J. Biol. Chem.* **175,** 727–735 (1948).
F12. Fuller, R. W., Luce, M. W., and Mertz, E. T., Serum uric acid in mongolism. *Science* **137,** 868–869 (1962).
G1. Garrod, A. B., Observations on certain pathological conditions of the blood and urine in gout. Rheumatism and Bright's Disease. *Med.-Chir. Trans.* **31,** 83 (1848) (as cited in Engelman *et al.*, E6).
G2. Geren, W., Bendich, A., Bodansky, O., and Brown, G. B., The fate of uric acid in man. *J. Biol. Chem.* **183,** 21–31 (1950).
G3. Getler, H., Roll, P. M., Tinker, J. F., and Brown, G. B., A study of the metabolism of dietary hypoxanthine and xanthine in the rat. *J. Biol. Chem.* **178,** 259–264 (1949).
G4. Gibson, H. V., and Edward, A. D., A note on the effect of some organic acids upon the uric acid excretion of man. *J. Biol. Chem.* **55,** 605–610 (1923).
G5. Gilbert, D. A., The chemistry of xanthine oxidase. The inhibition of the bovine enzyme by purine 6-aldehyde. *Biochem. J.* **93,** 214–219 (1964).
G6. Gilbert, L. M., Overend, W. G., and Nebb, M., The inhibition of pancreas deoxyribonuclease. *Exptl. Cell Res.* **2,** 349–365 (1951).
G7. Goldfinger, S. E., Klinenberg, J. R., and Seegmiller, J. E., The renal excretion of oxypurines. *J. Clin. Invest.* **44,** 623–628 (1965).
G8. Goldthwait, D. A., Peabody, R. A., and Greenberg, G. R., Glycine ribotide intermediates in the *de novo* synthesis of inosinic acid. *J. Am. Chem. Soc.* **76,** 5258–5259 (1954).
G9. Goldthwait, D. A., Peabody, R. A., and Greenberg, G. R., The biosynthesis of the purine ring. *In* "Amino Acid Metabolism" (W. D. McElroy and H. B. Glass, eds.), pp. 765–781. The Johns Hopkins Press, Baltimore, 1955.
G10. Gots, J. S., Purine metabolism in bacteria. V. Feed-back inhibition. *J. Biol. Chem.* **228,** 57–66 (1957).
G11. Gots, J. S., and Gollub, E. G., Sequential blockade in adenine biosynthesis by genetic loss of an apparent bifunctional deacylase. *Proc. Natl. Acad. Sci. U.S.* **43,** 826–834 (1957).
G12. Grayzel, A. I., Seegmiller, J. E., and Looe, E., Suppression of uric acid synthesis in the gouty human by the use of 6-diazo-5-oxo-L-norleuciue. *J. Clin. Invest.* **39,** 447–454 (1960).
G13. Green, D. E., and Beinert, H., Xanthine oxidase, a molybdo flavoprotein. *Biochim. Biophys. Acta* **11,** 599–600 (1953).
G14. Greenberg, G. R., Incorporation of carbon labeled formic acid and carbon dioxide with hypoxanthine in pigeon liver homogenates. *Arch. Biochem.* **19,** 337–339 (1948).
G15. Greenberg, G. R., *De novo* synthesis of hypoxanthine via inosine-5-phosphate and inosine. *J. Biol. Chem.* **190,** 611–631 (1951).

G16. Greenberg, G. R., and Jaenicke, L., On the activation of the one-carbon unit for the biosynthesis of purine nucleotides. *In* "The Chemistry and Biology of Purines" (G. E. Wolstenholme and C. M. O'Connor, eds.), pp. 204–232. Churchill, London, 1957.

G17. Grunberg-Manago, M., Ortiz, P. J., and Ochoa, S., Enzymic synthesis of polynucleotides. I. Polynucleotide phosphorylase of *Azotobacter vinelandii*. *Biochim. Biophys. Acta* **20,** 269–285 (1956).

G18. Gupta, S., and Herriott, R. M., Nucleases and their inhibitors in the cellular components of human blood. *Arch. Biochem. Biophys.* **101,** 88–95 (1963).

G19. Gutman, A. B., and Yü, T. F., An abnormality of glutamine metabolism in primary gout. *Am. J. Med.* **35,** 820–831 (1963).

H1. Hamilton, L. D., Metabolic stability of PNA and DNA Human leukemic leucocytes: The function of leucocytes. *In* "The Leukemias: Etiology, Pathophysiology and Treatment" (J. W. Rebuck, F. H. Bethell, and R. W. Monto, eds.), p. 381. Academic Press, New York, 1957.

H2. Handler, J. S., The role of lactic acid in the reduced excretion of uric acid in toxemia of pregnancy. *J. Clin. Invest.* **39,** 1526–1532 (1960).

H3. Handschumacher, R. E., Creasey, W. A., Jaffe, J. J., Pasternak, C. A., and Hankin, L., Biochemical and nutritional studies on the induction of fatty livers by dietary orotic acid. *Proc. Natl. Acad. Sci. U.S.* **46,** 178–186 (1960).

H4. Hellman, L., Production of acute gouty arthritis by adrenocorticotropin. *Science* **109,** 280–281 (1949).

H5. Heppel, L. A., and Hilmoe, R. J., Purification and properties of 5-nucleotidase. *J. Biol. Chem.* **188,** 665–676 (1951).

H6. Hiatt, H. H., Studies of ribose metabolism. II. A method for the study of ribose synthesis *in vivo*. *J. Biol. Chem.* **229,** 725–730 (1957).

H7. Hilmoe, R. J., and Heppel, L. A., Purification of nucleases from spleen and intestinal mucosa. *Federation Proc.* **12,** 217–218 (1953).

H8. Hodgson, C., Nucleic acids and their decomposition products in normal and pathological horny layers. *J. Invest. Dermatol.* **39,** 69–78 (1962).

H9. Hoefnagel, D., Andrew, E. D., Mireault, N. G., and Berndt, U. D., Hereditary choreoathetosis, self-mutilation and hyperuricemia in young males. *New Engl. J. Med.* **273,** 130–135 (1965).

H10. Holley, R. W., Apgar, J., and Merrill, S. H., Evidence for the liberation of a nuclease from human fingers. *J. Biol. Chem.* **236,** 42–43 (1961).

H11. Horbaczewski, J., Uber eine neue Synthese und die Constitution der Harnsäure. *Monatsch. Chem.* **12,** 221 (1882).

H12. Howell, R. R., and Seegmiller, J. E., A mechanism of action of colchicine. *Arthritis Rheumat.* **5,** 303 (1962).

J1. Jeaudet, J., and Lestradet, H., L'hyperlactacidémie cause probable de l'hyperuricémie dans la glycogénose hépatique. *Rev. Franc. Etudes Clin. Biol.* **6,** 71–72 (1961).

J2. Johnson, T. B., The chemistry of pyrimidines, purines, and nucleic acids. *In* "Organic Chemistry an Advanced Treatise" (H. Gilman, ed.), Vol. 11, pp. 948–1017. Wiley, New York, 1938.

J3. Jones, O. W., Jr., Ashton, D. M., and Wyngaarden, J. B., Accelerated turnover of phosphoribosylpyrophosphate—a purine nucleotide precursor in certain gouty subjects. *J. Clin. Invest.* **41,** 1805–1815 (1962).

J4. Jones, W., Uber das Enzyme der Thymusdrüse. *Z. Physiol. Chem.* **41,** 101–108 (1904).

J5. Jones, W., and Perkins, M. E., The nucleotides formed by the action of boiled pancreas on yeast nucleic acid. *J. Biol. Chem.* **55,** 557–565 (1923).

J6. Jordan, H., Bericht über einen Fall. *Deut. Z. Verdauungs- Stoffwechselkrankh.* **15,** 143–146 (1955).

J7. Jørgensen, S., and Poulsen, H. E., Enzymatic determination of hypoxanthine and xanthine in human plasma and urine. *Acta Pharmacol.* **11,** 223–245 (1955).

K1. Kalckar, H. M., Enzymatic synthesis of a nucleoside. *J. Biol. Chem.* **158,** 723–724 (1945).

K2. Kalckar, H. M., Differential spectrophotometry of purine compounds by means of specific enzymes. *J. Biol. Chem.* **167,** 429–443 (1947).

K3. Kalckar, H. M., Kjelgaard, N. O., and Klenow, H., Inhibition of xanthine oxidase and related enzymes by 6-pteridylaldehyde. *J. Biol. Chem.* **174,** 771–772 (1948).

K4. Kalckar, H. M., Kjelgaard, N. O., and Klenow, H., Xanthopterin oxidase. *Biochim. Biophys. Acta* **5,** 575–585 (1950).

K5. Kennaway, E. L., The effects of muscular work upon the excretion of endogenous purines. *J. Physiol. (London)* **38,** 1–27 (1909).

K6. Khorana, H. G., Phosphodiesterases. *In* "The Enzymes" (P. D. Boyer, H. Lardy, and K. Myrbäck, eds.), 2nd ed., Vol. 5, pp. 79–94. Academic Press, New York, 1961.

K7. Kidder, G. W., Dewey, V. C., Parks, R. E., Jr., and Woodside, G. L., Purine metabolism in Tetrahymena and its relation to malignant cells in mice. *Science* **109,** 511–514 (1949).

K8. Klein, F., and Lafeber, G. J. M., Improvements of the uricase-U.V. method for the determination of uric acid in serum and urine. *Clin. Chim. Acta* **14,** 708–710 (1966).

K9. Klenow, H., The enzymic oxidation and assay of adenine. *Biochem. J.* **50,** 404–407 (1952).

K10. Klinenberg, J. R., Goldfinger, S. E., and Seegmiller, J. E., The effectiveness of the xanthine oxidase inhibitor allopurinol in the treatment of gout. *Ann. Internal. Med.* [N.S.] **62,** 639 (1965).

K11. von Knieriem, W., Ueber das Verhalten der im Sägethierkörper als Vorstufen des Harnstoffs erkannten Verbindungen zum Organismus der Huhner. *Z. Biol.* **13,** 36 (1877).

K12. Kohn, N. N., Hughes, R. E., McCarty, D. J., Jr., and Faires, J. S., The significance of calcium phosphate crystals in the synovial fluid of arthritic patients: The "Pseudogout Syndrome." II. Identification of crystals. *Ann. Internal. Med.* [N.S.] **56,** 738–745 (1962).

K13. Kornberg, A., Lieberman, I., and Simms, E., Enzymatic synthesis and properties of 5-phosphoribosylpyrophosphate. *J. Biol. Chem.* **215,** 389–402 (1955).

K14. Kossel, A., Weitere Beiträge que Chemie des Zellkerns. *Z. Physiol. Chem.* **10,** 248–262 (1886).

K15. Krakoff, I. H., Urinary uric acid excretion in leukemia. *In* "The Leukemias: Etiology, Pathophysiology and Treatment" (J. W. Rebuck, F. H. Bethell, and R. W. Monto, eds.), pp. 401–416. Academic Press, New York, 1957.

K16. Krakoff, I. H., A difference between 6-mercaptopurine and 6-thioguanine in man. *Proc. Am. Assoc. Cancer Res.* **5,** 37 (1964).

K17. Krakoff, I. H., Clinical pharmacology of drugs which influence uric acid production and excretion. *Clin. Pharmacol. Therap.* **8,** 124–138 (1967).

K18. Krakoff, I. H., and Balis, M. E., Studies of the uricogenic effect of 2-substituted thiadiazoles in man. *J. Clin. Invest.* **38,** 907–915 (1959).

K19. Krakoff, I. H., and Balis, M. E., Abnormalities of purine metabolism in human leukemia. *Ann. N. Y. Acad. Sci.* **113,** 1043–1052 (1964).

K20. Krakoff, I. H., and Balis, M. E., Allopurinol in the prevention of hyperuricemia secondary to the treatment of neoplastic diseases with alkylating agents, adrenal steroids and radiation therapy. *Ann. Rheumatic Diseases* **25,** 651–654, 1966.

K21. Krakoff, I. H., and Magill, G. M., Effects of 2-ethylamino-1,3,4-thiadiazole hydrochloride on uric acid production in man. *Proc. Soc. Exptl. Biol. Med.* **91,** 470–472 (1956).

K22. Krakoff, I. H., and Meyer, R. L., Prevention of hyperuricemia in leukemia: Use of allopurinol, a xanthine oxidase inhibitor. *J. Am. Med. Assoc.* **193,** 1–6 (1965).

K23. Krakoff, I. H., Balis, M. E., and Karnofsky, D. A., Relation of 2-substituted thiadiazoles to purine and nicotinamide metabolism. *Ann. Internal. Med* [N.S.] **54,** 1045 (1961).

K24. Krakoff, I. H., Balis, M. E., Magill, J. N., and Nary, D., Studies of purine metabolism in neoplastic diseases. *Med. Clin. N. Am.* **45,** 521–535 (1961).

K25. Kunitz, M., Crystalline ribonuclease. *J. Gen. Physiol.* **24,** 15–32 (1940).

L1. Lagerkvist, U., Biosynthesis of guanosine 5′-phosphate. I. Xanthosine 5′-phosphate as an intermediate. *J. Biol. Chem.* **233,** 138–142 (1958).

L2. Lagerkvist, U., Biosynthesis of guanosine 5′-phosphate. II. Amination of xanthosine 5′-phosphate by purified enzyme from pigeon liver. *J. Biol Chem.* **233.** 143–149 (1958).

L3. Laskowski, M., Deoxyribonucleases. *In* "The Enzymes" (P. D. Boyer, H. Lardy, and K. Myrbäck, eds.), 2nd ed., Vol. 5, pp. 123–147. Academic Press, New York, 1961.

L4. Leaf, A., The syndrome of osteomalacia, renal glycosuria, aminoaciduria, and increased phosphorus clearance (The Fanconi syndrome). *In* "The Metabolic Basis of Inherited Disease" (J. B. Stanbury, J. B. Wyngaarden, and D. S. Fredrickson, eds.), pp. 1205–1220. McGraw-Hill (Blakinston), New York, 1966.

L5. Leathes, J. B., On diurnal and nocturnal variations in the excretion of uric acid. *J. Physiol. (London)* **35,** 125–130 (1906).

L6. Ledoux, L., Les groupes actifs de la Ribonucléase. I. Action du Parachloromercuribenzoate de sodium sur le systeme Arn-RNase. *Biochim. Biophys. Acta* **11,** 517–523 (1953).

L7. Leeper, R. D., Krakoff, I. H., Balis, M. E., and Rawson, R. W., The effect of thyroid hormones on uric acid metabolism in man. *Am. Thyroid Assoc.* Annual meeting, Chicago, May 9–11 (1963).

L8. Leeper, R. D., Benua, R. S., Brenner, J. L., and Rawson, R. W., Hyperuricemia in myxedema. *J. Clin. Endocrinol. Metab.* **20,** 1457–1466 (1960).

L9. Lennane, G. A. G., Rose, B. S., and Isdale, I. C., Gout in Maori. *Ann. Rheumatic Diseases* **19,** 120–125 (1960).

L10. LePage, G. A., and Junga, I. G., Metabolism of purine nucleoside analogs. *Cancer Res.* **25,** 46–52 (1965).

L11. Lesch, M., and Nyhan, W. L., A familial disorder of uric acid metabolism and central nervous system function. *Am. J. Med.* **36,** 561–570 (1964).

L12. Levenberg, B., Hartmann, S. O., and Buchanan, J. M., Biosynthesis of the purines. X. Further studies *in vitro* on the metabolic origin of nitrogen atoms 1 and 3 of the purine ring. *J. Biol. Chem.* **220,** 379–390 (1956).

L13. Levenberg, B., Melnick, I., and Buchanan, J. M., Biosynthesis of the purines. XV. The effect of aza-L-serine and 6-diazo-5-oxo-L-norleucine on inosinic acid biosynthesis *de novo*. *J. Biol. Chem.* **225,** 163–167 (1957).

L14. Levin, S. J., and Bodansky, O., The double pH optimum of 5'-nucleotidase of bull seminal plasma. *J. Biol. Chem.* **241,** 51–56 (1966).
L15. Liddle, L., Seegmiller, J. E., and Laster, L., The enzymatic spectrophotometric method for determination of uric acid. *J. Lab. Clin. Med.* **54,** 903–913 (1959).
L16. Lieber, C. S., Jones, D. P., Losowsky, M. S., and Davidson, C. S., Interrelation of uric acid and ethanol metabolism in man. *J. Clin. Invest.* **41,** 1863–1870 (1962).
L17. Lindberg, M. U., Crystallization from calf spleen of two inhibitors of deoxyribonuclease. *J. Biol. Chem.* **241,** 1246–1249 (1966).
L18. Lipkin, G., Herrmann, F., and Mandol, L., Studies of serum copper. I. The copper content of blood serum in patients with psoriasis. *J. Invest. Dermatol.* **39,** 543–546 (1962).
L19. Liss, M., and Lever, W. F., Purification and characterization of ribonuclease from psoriatic scales. *J. Invest. Dermatol.* **39,** 529–535 (1962).
L20. Liss, M., and Lever, W. F., The amino acid control and nucleotide composition of the proteins and of RNA psoriasis scales. *J. Invest. Dermatol.* **40,** 45–49 (1963).
L21. Loring, H. S., Carpenter, F. H., and Roll, P. M., The hydrolysis of yeast ribonucleic acid by ribonucleinase. I. The extent of hydrolysis and the preparation of ribonucleinase-resistant fractions after ribonucleinase treatment. *J. Biol. Chem.* **169,** 601–608 (1947).
L22. Lowy, B. A., Davoll, J., and Brown, G. B., The utilization of purine nucleosides for nucleic acid synthesis in the rat. *J. Biol. Chem.* **197,** 591–600 (1952).
M1. Magasanik, B., Nutrition of bacteria and fungi. *Ann. Rev. Microbiol.* **11,** 221–252 (1957).
M2. Magasanik, B., Moyed, H. S., and Gehring, L. B., Enzymes essential for the biosynthesis of nucleic acid guanine; inosine 5'-phosphate dehydrogenase of *Aerobacter aerogenes*. *J. Biol. Chem.* **226,** 339–350 (1957).
M3. Malawista, S. E., and Seegmiller, J. E., The effect of pretreatment with colchicine on the inflammatory response to microcrystalline urate. A model for gouty inflammation. *Ann. Internal. Med.* [N.S.] **62,** 648–657 (1965).
M4. Malawista, S. E., Howell, R. R., and Seegmiller, J. E., Factors modifying the inflammatory response to injected microcrystalline sodium urate. *Arthritis Rheumat.* **5,** 307 (1962).
M5. Mandel, H. G., and Carlo, P., The incorporation of guanine into nucleic acids of tumor-bearing mice. *J. Biol. Chem.* **201,** 335–341 (1953).
M6. Marcet, "An Essay on the Chemical History and Medical Treatment of Calcul Disorders." London, 1817 (as cited in Johnson, J2).
M7. Markham, R., and Smith, J. D., The structure of ribonucleic acids. I. Cyclic nucleotides produced by ribonuclease and by alkaline hydrolysis. *Biochem. J.* **52,** 552–557 (1952).
M8. Maver, M. E., and Greco, A. E., The nuclease activities of cathepsin preparations from calf spleen and thymus. *J. Biol. Chem.* **181,** 861–870 (1949).
M9. McCabe, M. G. P., and Mier, P. D., Fractionation of the acid-soluble phosphate esters in the lesions of eczema and psoriasis. *Brit. J. Dermantol.* **75,** 358–362 (1963).
M10. McCarty, D. J., Jr., The inflammatory reaction to microcrystalline sodium urate. *Arthritis Rheumat.* **8,** 726–735 (1965).
M11. McCollister, R. J., Gilbert, W. R., Jr., Ashton, D. M., and Wyngaarden, J. B., Pseudofeedback inhibition of purine synthesis by 6-mercaptopurine ribonucleotide and other purine analogues. *J. Biol. Chem.* **239,** 1560–1563 (1964).
M12. Michael, S. T., The relation of uric acid excretion to blood lactic acid in man. *Am. J. Physiol.* **141,** 71–74 (1944).

M13. Miescher, F., Ueber die chemische Zusammensetzung der Eiterzellen. *Hoppe-Seyler's Med.-Chem. Unters.* **6,** 138 (1874).
M14. Miller, R. W., Lukens, L. N., and Buchanan, J. M., The enzymatic cleavage of 5-amino-4-imidazole-N-succino-carboxamide ribotide. *J. Am. Chem. Soc.* **79,** 1513–1514 (1957).
M15. Morgan, E. J., Stewart, C. P., and Hopkins, F. G., Anaerobic and aerobic oxidation of xanthine and hypoxanthine by tissues and by milk. *Proc. Roy. Soc.* **B94,** 109–131 (1922).
M16. Moses, C., Sunder, J. H., Vester, J. W., and Donowski, T. S., Hydrocortisone and/or desiccated thyroid in physiological dosage. XI. Effects of thyroid hormone excesses on lipids and other blood and serum solutes. *Metab., Clin. Exptl.* **13,** 717–728 (1964).
M17. Moyed, H. S., and Magasanik, B., Enzymes essential for the biosynthesis of nucleic acid guanine; xanthosine 5'-phosphate aminase of *Aerobacter aerogenes*. *J. Biol. Chem.* **226,** 351–363 (1957).
N1. Nasrallah, S., and Khalidi, A., Nature of purines excreted in urine during muscular exercise. *J. Appl. Physiol.* **19,** 246–248 (1964).
N2. Neal, J. V., Rakic, M. T., Davidson, R. T., Valkenburg, H. A., and Mikklesen, W. N., Studies on hyperuricemia. II. A reconsideration of the distribution of serum uric acid values in the families of Smyth, Cotterman and Freyberg. *Am. J. Human Genet.* **17,** 14–22 (1965).
N3. Needham, J., "Biochemistry and Morphogenesis." Cambridge Univ. Press, London and New York, 1942.
N4. Nierlich, D. P., and Magasanik, B., Regulation of purine ribonucleotide synthesis by end product inhibition. *J. Biol. Chem.* **240,** 358–365 (1965).
N5. Nyhan, W. L., Oliver, W. J., and Lesch, M., A familial disorder of uric acid metabolism and central nervous system function. II. *J. Pediat.* **67,** 257–263 (1965).
O1. O'Brien, W., Burch, T. A., and Bunim, J. J., Genetics of hyperuricemia in Black Feet and Pima Indians. *Ann. Rheumatic Diseases* **25,** 117–119 (1966).
O2. Ogryzlo, M. A., Urowitz, M. B., Weber, H. M., and Haupt, J. B., The treatment of gout and disorders of uric acid metabolism with allopurinol. *Can. Med. Assoc. J.* **95,** 1120–1121 (1966).
O3. O'Sullivan, J. D., Francis, T., Jr., John, O. S., and Kantor, N., Comparison of a colorimetric (automated) with an enzymatic (manual) uric acid procedure. *Clin. Chem.* **11,** 427–435 (1965).
P1. Paege, L. M., and Schlenk, F., Pyrimidine riboside metabolism. *Arch. Biochem. Biophys.* **28,** 348–358 (1950).
P2. Pearlman, C. K., Xanthine urinary calculus. *J. Urol.* **64,** 799–800 (1950).
P3. Petering, H. G., and Schmitt, J. A., Studies in enzyme inhibition. I. Action of some pterines on xanthine oxidase. *J. Am. Chem. Soc.* **72,** 2995–2998 (1950).
P4. Petersen, B. B., Jørni, J., and Jørgensen, S., Separate enzymatic determination of hypoxanthine and xanthine in human urine. *Scand. J. Clin. Lab. Invest.* **17,** 454–459 (1965).
P5. Plentl, A. A., and Schoenheimer, R., Studies in metabolism of purines and pyrimidines by means of isotopic nitrogen. *J. Biol. Chem.* **153,** 203–217 (1944).
P6. Pomales, R., Elion, G. B., and Hitchings, G. H., Xanthine as a precursor of nucleic acid purines in the mouse. *Biochim. Biophys. Acta* **95,** 505–506 (1965).
P7. Pomales, R., Bieber, S., Friedman, R., and Hitchings, G. H., Augmentation of the incorporation of hypoxanthine into nucleic acids by the administration of an inhibitor of xanthine oxidase. *Biochim. Biophys. Acta* **72,** 119–120 (1963).

P8. Popert, A. J., and Hewitt, J. V., Gout and hyperuricemia in rural and urban populations. *Ann. Rheumatic Diseases* **20,** 154–163 (1962).
P9. Praetorius, E., An enzymatic method for the determination of uric acid by ultraviolet spectrophotometry. *Scand. J. Clin. Lab. Invest.* **1,** 222–230 (1949).
P10. Praetorius, E., and Poulsen, H., Enzymatic determination of uric acid by ultraviolet spectrophotometry. *Scand. J. Clin. Lab. Invest.* **5,** 273–280 (1953).
Q1. Quick, A. J., The relationship between chemical structure and physiological response. III. Factors influencing the excretion of uric acid. *J. Biol. Chem.* **98,** 157–169 (1932).
Q2. Quick, A. J., The effect of exercise on the excretion of uric acid. *J. Biol. Chem.* **110,** 107–112 (1935).
R1. Rakic, M. T., Valkenburg, H. A., Davidson, R. T., Engels, J. P., Mikkelsen, W. M., Neel, J. V., and Duff, I. F., Observation on the natural history of hyperuricemia and gout. I. An eighteen year follow-up of nineteen gouty families. *Am. J. Med.* **37,** 862–871 (1964).
R2. Ratner, M., and Strasberg, A., A case of xanthine calculosis. *Can. Med. Assoc. J.* **40,** 350–351 (1939).
R3. Reis, J., Über die Aktivitat der 5-Nukleotidase in den tierischen und menschlichen Geweben. *Enzymologia* **2,** 183–190 (1937).
R4. Reis, J., Über die Speziftät der Prostata-und Hodenphosphatase. *Enzymologia* **5,** 251–253 (1938).
R5. Reis, J., Sur la présence de la 5-nucléotidase dans les tissus animaux. *Bull. Soc. Chim. Biol.* **22,** 36–42 (1940).
R6. Richert, D. A., Vanderlinde, R., and Westerfeld, W. W., The composition of rat liver xanthine oxidase and its inhibition by antabuse. *J. Biol. Chem.* **186,** 261–274 (1950).
R7. Riley, I. D., Gout and cerebral palsy in a three year old boy. *Arch. Disease Childhood* **35,** 293–295 (1960).
R8. Rittenberg, D., and Foster, G. L., New procedure for quantitative analysis by isotope dilution with application to determination of amino acids and fatty acids. *J. Biol. Chem.* **133,** 737–744 (1940).
R9. Roll, P. M., The incorporation of guanosine 5′-phosphate into the ribonucleic acid of the rat. *J. Biol. Chem.* **231,** 183–186 (1958).
R10. Roll, P. M., and Weliky I., The utilization of nucleotides by the mammal. II. N^{15}-Labeled purine nucleotides. *J. Biol. Chem.* **213,** 509–522 (1955).
R11. Roll, P. M., Weinfeld, H., and Brown, G. B., The metabolism in the rat of guanylic acid labelled with ^{32}P. *Biochim. Biophys. Acta* **13,** 141–142 (1954).
R12. Roll, P. M., Weinfeld, H., Carroll, E., and Brown, G. B., The utilization of nucleotides by the mammal. IV. Triply labelled purine nucleotides. *J. Biol. Chem.* **220,** 439–454 (1956).
R13. Rosenthal, I. M., Gaballah, S., and Rofelson, M. E., Jr., Gout in infancy manifested by renal failure. *Pediatrics* **33,** 251–257 (1964).
R14. Roth, J. S., Comparative studies on tissue ribonucleases. *Ann. N.Y. Acad. Sci.* **81,** 611–617 (1959).
R15. Rundles, R. W., Wyngaarden, J. B., Hitchings, G. H., Elion, G. B., and Silberman, H. R., Effects of a xanthine oxidase inhibitor on thiopurine metabolism, hyperuricemia and gout. *Trans. Assoc. Am. Physicians* **76,** 126–140 (1963).
R16. Ruzicka, J., Biochemických Zneúach u Psoriazy. *Cesk. Dermatol.* **38,** 356–59 (1963) (as cited *Chem. Abs.* **61,** 1083b).
S1. Sandberg, A. A., Cartwright, G. E., and Wintrobe, M. M., Studies on leukemia: Uric acid excretion. *Blood* **11,** 154–166 (1956).

S2. Schmidt, G., The effect of enzymes on protein with prosthetic groups. I. The effect of nucleophosphatase on thymus nucleohistone. *Enzymologia* **1,** 135–141 (1936).

S3. Schmidt, G., Nucleases and enzymes attacking nucleic acid components. *In* "The Nucleic Acids" (E. Chargaff and J. N. Davidson, eds.), Vol. 1, pp. 555–626. Academic Press, New York, 1955.

S4. Schmidt, G., and Levene, P. A., Ribonucleodepolymerase (The Jones-Dubos Enzyme). *J. Biol. Chem.* **126,** 423–434 (1938).

S5. Schmidt, G., Pickels, E. G., and Levene, P. A., Enzymatic dephosphorylation of desoxyribonucleic acids of various degrees of polymerization. *J. Biol. Chem.* **127,** 251–259 (1939).

S6. Schmidt, G., Cubiles, R., Zöllner, N., Hecht, L., Strickler, N., Seraidarian, K., Seraidarian, M., and Thannhauser, S. J., On the action of ribonuclease. *J. Biol. Chem.* **192,** 715–726 (1951).

S7. Schröder, W., Über die Verwandhung des Ammoniaks in Harnsäure im Organismus des Huhns. *Z. Physiol. Chem.* **2,** 228–240 (1878–1879).

S8. Seegmiller, J. E., Grayzel, A. I., and Liddle, L., Excessive uric acid production in the human induced by 2-ethylamino-1,3,4-thiadiazoles. *Nature* **183,** 1463–1464 (1959).

S9. Seegmiller, J. E., Howell, R. R., and Malawista, S. E., The inflammatory reaction to sodium urate: Its possible relationship to genesis of acute gouty arthritis. *J. Am. Med. Assoc.* **180,** 469–475 (1962).

S10. Seegmiller, J. E., Laster, L., and Howell, R. R., Biochemistry of uric acid in relation to gout. *New Engl. J. Med.* **268,** 712–716 (1963).

S10a. Seegmiller, J. E., Rosenbloom, F. M., and Kelley, W. N., Enzyme defect associated with a sex-linked human neurological disorder and excessive purine synthesis. *Science* **155,** 1682–1684 (1967).

S11. Seegmiller, J. E., Grayzel, A. I., Laster, L., and Liddle, L., Uric acid production in gout. *J. Clin. Invest.* **40,** 1304–1314 (1961).

S12. Seegmiller, J. E., Grayzel, A. I., Liddle, L., and Wyngaarden, J. B., The effect of 2-ethylamino-1,3,4-thiadiazole on the incorporation of glycine into urinary purines and uric acid in man. *Metab., Clin. Exptl.* **12,** 507–515 (1963).

S13. Shapiro, S. L., Sheppard, G. L., Jr., Dreifuss, F. E., and Newcombe, D. S., X-linked recessive inheritance of a syndrome of mental retardation with hyperuricemia. *Proc. Soc. Exptl. Biol. Med.* **122,** 609–610 (1966).

S14. Shaw, E., and Woolley, D. W., Imidazo-1,2,3-triazines as substrates and inhibitors for xanthine oxidase. *J. Biol. Chem.* **194,** 641–654 (1952).

S15. Shemin, D., and Rittenberg, D., On the utilization of glycine for uric acid synthesis in man. *J. Biol. Chem.* **167,** 875–876 (1947).

S16. Shipley, R. A., Dorfman, R. I., Buchwald, E., and Ross, E., Effect of infection and trauma on excretion of urinary cortin. *J. Clin. Invest.* **25,** 673–678 (1946).

S17. Shortman, K., Studies of cellular inhibitors of ribonuclease. II. Some properties of the inhibitor from rat liver. *Biochim. Biophys. Acta* **55,** 88–96 (1962).

S18. Shuster, L., and Goldin, A., Some biochemical effects of 2-ethylamino-1,3,4-thiadiazole. *Biochem. Pharmacol.* **2,** 17–24 (1959).

S19. Shuster, L., and Kaplan, N. O., A specific nucleotidase. *J. Biol. Chem.* **201,** 535–546 (1953).

S20. Silberman, H. R., and Wyngaarden, J. B., 6-mercaptopurine as substrate and inhibitor of xanthine oxidase. *Biochim. Biophys. Acta* **47,** 178–180 (1961).

S21. Skipper, H. E., Mitchell, J. H., Jr., and Bennett, L. L., Jr., Inhibition of nucleic acid synthesis by folic acid antagonists. *Cancer Res.* **10,** 510–512 (1950).

S22. Smyth, C. J., Cotterman, C. W., and Freyberg, R. H., The genetics of gout and hyperuricemia—an analysis of nineteen families. *J. Clin. Invest.* **27,** 749–758 (1948).

S23. Smyth, C. J., Stecher, R. M., and Wolfson, W. Q., Genetic and endocrine determinants of the plasma urate level. *Science* **108,** 514–515 (1948).

S24. Socin, C. A., In Welchen Form Wird das Eisen Resor Birt. *Z. Physiol. Chem.* **15,** 93–139 (1891).

S25. Sonne, J. C., Buchanan, J. M., and Delluva, A. M., Biological precursors of uric acid carbon. *J. Biol. Chem.* **166,** 395–396 (1946).

S26. Sonne, J. C., Buchanan, J. M., and Delluva, A. M., Biological precursors of uric acid I. The role of lactate, acetate, and formate in the synthesis of the ureide groups of uric acid. *J. Biol. Chem.* **173,** 69–79 (1948).

S27. Sonne, J. C., Lin, I., and Buchanan, J. M., The role of N^{15}glycine, glutamine, aspartate and glutamate in hypoxanthine synthesis. *J. Am. Chem. Soc.* **75,** 1516–1517 (1953).

S28. Sorensen, L. B., Degradation of uric acid in man. *Metab. Clin. Exptl.* **8,** 687–703 (1959).

S29. Sorensen, L. B., The elimination of uric acid in man studied by means of C^{14}-labeled uric acid, uricolysis. *Scand. J. Clin. Lab. Invest.* **12,** 1–214 (1960).

S30. Sorensen, L. B., Role of the intestinal tract in the elimination of uric acid. *Arthritis Rheumat.* **8,** 694–706 (1965).

S31. Spanio, L., and De Battisti, C., Esperienze con orotato di potassio in soggetti iperuricemici. *Acta Vitaminol.* **19,** 215–218 (1965).

S32. Steenstrup, O. R., A note on hyperuricaemia during labour. *Scand. J. Clin. Lab. Invest.* **12,** 205–208 (1960).

S33. Steigleder, G. K., and Endres, E., Biochemistry of abnormal cornification especially in psoriasis. Proteolytic activity on the surfaces of pathologically altered skin. *Arch. Klin. Exptl. Dermatol.* **218,** 105–110 (1964).

S34. Steigleder, G. K., and Fissher, I., Localization of ribonuclease and deoxyribonuclease activity in normal skin, skin altered by inflammation and in skin tumors. *Arch. Klin. Exptl. Dermatol.* **217,** 553–562 (1963).

S35. Steigleder, G. K., and Raab, W. P., The localization of ribonuclease and deoxyribonuclease activities in normal and psoriatic epidermis. *J. Invest. Dermatol.* **38,** 209–214 (1962).

S36. Steigleder, G. K., Rust, S., and Koch, H., Digestibility of deoxyribonucleic acid of the nucleus by deoxyribonuclease in skin altered by psoriasis. *Arch. Klin. Exptl. Dermatol.* **221,** 203–206 (1965).

S37. Stetten, D., Jr., and Hearon, J. E., Intellectual level measured by army classification battery and serum uric acid concentration. *Science* **129,** 1737 (1959).

S38. Stetten, M. R., and Fox, C. L., An amine formed by bacteria during sulfonamide bacteriostasis. *J. Biol. Chem.* **161,** 333–349 (1945).

S39. Steurermann, N., and Farias, A. H., Hyperuricemia in Phillipinos. *Hawaii Med. J.* **21,** 151–153 (1960).

T1. Tabachnik, J., Studies on the biochemistry of epidermis. II. Some characteristics of deoxyribonucleases I and II of albino guinea pig epidermis and saline extracts of hair. *J. Invest. Dermatol.* **42,** 471–478 (1964).

T2. Taylor, W. N., and Taylor, J. N., Xanthine calculus: Case report. *J. Urol.* **68,** 659–660 (1952).

T3. Tichomiroff, A., Chemische Studien Über die Entwicklung der Insecteneir. *Z. Physiol. Chem.* **9,** 518–532 (1885).

T4. Tickner, A., Biochemistry of psoriasis. *Brit. J. Dermatol.* **73,** 87–98 (1961).

T5. Totter, J. R., Burnett, W. T., Jr., Monroe, R. A., Whitney, I. B., and Comar, C. L., Evidence that molybdenum is a nondialyzable component of xanthine oxidase. *Science* **118,** 555 (1953).

T6. Tsuboi, K. K., and Hudson, P. B., Enzymes of the human erythrocyte. I. Purine nucleoside phosphorylase: Isolation procedure. *J. Biol. Chem.* **224,** 879–887 (1957).

V1. Vendrely, R., The deoxyribonucleic acid content of the nucleus. *In* "The Nucleic Acids" (E. Chargaff and J. N. Davidson, eds.), Vol. 2, pp. 155–180. Academic Press, New York, 1955.

W1. Weinfeld, H., Roll, P. M., and Brown, G. B., The utilization of nucleotides by the mammal. III. Isomeric purine nucleotides labeled with C^{14}. *J. Biol. Chem.* **213,** 523–531 (1955).

W2. Weissmann, B., Bromberg, P. A., and Gutman, A. B., The purine basis in human urine. I. Separation and identification. *J. Biol. Chem.* **224,** 407–422 (1957).

W3. Weissmann, B., Bromberg, P. A., and Gutman, A. B., The purine basis in human urine. II. Semi quantitative estimation and isotope incorporation. *J. Biol. Chem.* **224,** 423–434 (1957).

W4. Wharton, M. L., and Wharton, R. A., The determination of uric acid in biological fluids. I. A modification of the method of Bergmann and Dikstein. *Anal. Biochem.* **1,** 213–217 (1960).

W5. Wheatley, V. R., and Farber, E. M., Studies on the chemical composition of psoriatic scales. *J. Invest. Dermatol.* **36,** 199–211 (1961).

W6. Windmueller, H. G., and Spaeth, A. E., Stimulation of hepatic purine biosynthesis by orotic acid. *J. Biol. Chem.* **240,** 4398–4405 (1965).

W7. Wohlrab, W., Histopography of the phosophomonoesterases in psoriasis foci and their environment. II. Acid phosophomonoesterasis. *Arch. Klin. Exptl. Dermatol.* **220,** 600 (1964).

W8. Wyngaarden, J. B., 2,6 diaminopurine as substrate and inhibitor of xanthine oxidase. *J. Biol. Chem.* **224,** 453–462 (1957).

W9. Wyngaarden, J. B., Normal glycine C^{14} incorporation into uric acid in primary gout. *Metab., Clin. Exptl.* **7,** 374–375 (1958).

W10. Wyngaarden, J. B., The overproduction of uric acid in primary gout. *Arthritis Rheumat.* **8,** 648–658 (1965).

W11. Wyngaarden, J. B., and Stetten, D., Jr., Uricolysis in normal man. *J. Biol. Chem.* **203,** 9–21 (1953).

W12. Wyngaarden, J. B., Rundles, R. W., and Metz, E. N., Allopurinol in the treatment of gout. *Ann. Internal. Med.* [N.S.] **62,** 842–847 (1965).

W13. Wyngaarden, J. B., Seegmiller, J. E., Laster, L., and Blair, A. E., Utilization of hypoxanthine, adenine and 4-amino-5-imidazolecarboxamide for uric acid synthesis in man. *Metab., Clin. Exptl.* **8,** 455–464 (1959).

Y1. Yü, T. F., Secondary gout associated with myeloproliferative diseases. *Arthritis Rheumat.* **8,** 765–771 (1965).

Y2. Yü, T. F., Fan, A. I., and Gutman, A. B., Effect of allopurinol (4-hydroxypyrazolo (3,4-d) pyrimidine) on serum and urinary uric acid in primary and secondary gout. *Am. J. Med.* **37,** 885–898 (1964).

Z1. Zittle, C. A., Ribonucleinase. II. Mononucleotides in commercial ribonucleic acids and their effect on ribonucleinase. *J. Biol. Chem.* **160,** 527–533 (1945).

Z2. Zöllner, N., and Fellig, J., Nature of inhibition of ribonuclease by heparin. *Am. J. Physiol.* **173,** 223–228 (1953).

Z3. Zuckerman, R., Drell, W., and Levin, M. H., Urinary purines in gout: Effect of azaserine. *Arthritis Rheumat.* **2,** 46–47 (1959).

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THE TECHNIQUE AND SIGNIFICANCE OF HYDROXYPROLINE MEASUREMENT IN MAN

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1. Introduction

Collagen is the only mammalian protein that contains appreciable amounts of hydroxyproline[1] (N4). Determination of this amino (or imino) acid can therefore be used to estimate collagen, and hydroxyproline found in the biological fluids, in the absence of dietary sources, is assumed to be derived from collagen and is useful as an index of collagen metabolism (S14). In this manner, observations of the state of collagen turnover can be obtained in the intact subject without introducing the variables of tissue sampling and the effects of tissue repair.

[1] Although all investigators do not agree, it is likely that elastin contains 1–2 residues of hydroxyproline per 100 residues. This is considered inconsequential for the purposes of this discussion.

It is the purpose of this review to outline critically the methods available for hydroxyproline assay, and to summarize the information obtained by hydroxyproline determinations in urine, plasma (or serum), and the tissues, with emphasis on understanding the physiological and pathological setting in which these measurements have been made. No attempt will be made to discuss recent developments in the chemistry (H3, H4) or the biosynthesis (U1) of collagen, since thorough reviews of these topics are available.

2. Analytical Methods

2.1. Historical Introduction

Emil Fischer isolated 4-hydroxyproline from acid hydrolyzates of gelatin in 1902 and identified it as a hydroxy derivative of proline that had been isolated previously (F7). The synthesis of the four stereoisomers was accomplished by Leuchs *et al.* shortly thereafter (L14–L16). The first method for the determination of hydroxyproline was developed in 1933 by Lang, who applied the earlier observation of Langheld that hydroxyproline could be oxidized to pyrrole or a pyrrole-like compound (L3). Lang's colorimetric data for the proline and hydroxyproline content of gelatin were lower than those obtained with isolation techniques by Dakin (D2) and by Bergmann (B10); this led McFarlane and Guest to introduce a modified method also based on the oxidation of hydroxyproline to pyrrole and the formation of a chromophore which could be quantified (M2). Isatin was used initially to form pyrrole blue, because the oxidation product of proline was believed to react with *p*-dimethylaminobenzaldehyde (*p*-DAB) and interfere with its use in the specific assay of hydroxyproline; the reaction with proline was later shown to result from the presence of hydroxyproline as an impurity (N4). Since the sensitivity of the pyrrole–*p*-DAB reaction was 4–10-fold that of the pyrrole–isatin reaction, Neuman and Logan introduced in 1950 a simple and sensitive procedure for hydroxyproline assay based on the formation of a pyrrole–*p*-DAB chromophore, which remains the prototype for comparison and the basis for a large number of subsequent modifications (N4). Table 1 lists the published procedures for hydroxyproline determination.[2]

2.2. Colorimetric Procedures

The first reproducible procedure for the determination of hydroxyproline, published by Neuman and Logan in 1950, placed the study of

[2] The small amounts of 3-hydroxyproline recently identified in bovine collagen do not influence the determination or significance of 4-hydroxyproline measurements (O2).

TABLE 1

PROCEDURES FOR HYDROXYPROLINE ASSAY

Colorimetric procedures

(A) *p*-DAB[a] chromophore of hydroxyproline oxidation product

(1) No separation prior to chromophore formation

(a) Oxidation with peroxide [Neuman and Logan, 1950 (N4); Martin and Axelrod, 1953 (M5); Baker *et al.*, 1953 (B1); Wierbicki and Deatherage, 1954 (W6); Miyada and Tappel, 1956 (M17); Grunbaum and Glick, 1956 (micromethod) (G9); Lollar, 1958 (L19); Fels, 1958 (F3); Bowes, 1959 (B18); Mitoma *et al.*, 1959 (M16); Leach, 1960 (L4); Onitsuka, 1960 (O4); Hutterer and Singer, 1960 (H14); Bekhor and Bavetta, 1961 (B4); Zaides *et al.*, 1964 (Z1); Dahl and Persson, 1963 (D1); Blomfield and Farrar, 1964 (B13); Ko, 1964 (K30); Filliat *et al.*, 1966 (F5)]

(b) Oxidation with Chloramine-T [Stegemann, 1958 (S22); Woessner, 1961 (W8); Hurych and Chvapil, 1962 (H12); Dahl and Persson, 1963 (D1); Goll *et al.*, 1963 (G4); Bergman and Loxley, 1963 (B9); Grant, 1964 (AutoAnalyzer) (G5); Koevoet, 1965 (K32); Firschein and Shill, 1966 (F6)]

(2) Distillation of hydroxyproline oxidation products [Lang, 1933 (L3); Waldschmidt-Leitz and Akabori, 1934 (W1); Wiss, 1949 (W7); Mitoma *et al.*, 1959 (M16); Serafini-Cessi and Cessi, 1964 (S10); Mühlbach *et al.*, 1966 (M23)]

(3) Extraction of hydroxyproline oxidation products [Prockop and Udenfriend, 1960 (for urine hydroxyproline) (P11); Goll *et al.*, 1963 (G4); Pil'v and Titaev, 1963 (P7)]

(4) Extraction of nonspecific chromogens [Prockop and Udenfriend, 1960 (P11); Hutterer and Singer, 1960 (H14); Woessner, 1961 (W8)]

(B) Isatin chromophore of hydroxyproline oxidation products [Lang, 1933 (L3); McFarlane and Guest, 1939 (M2)]

(C) Ninhydrin chromophore of hydroxyproline [Troll and Cannan, 1953 (T4)]

Chromatographic–colorimetric procedures
(Chromatographic separation prior to colorimetric determination)

[Rogers *et al.*, 1954 (ion-exchange, column) (R6); Piez *et al.*, 1956 (ion-exchange, column) (P6); Iachan *et al.*, 1959 (paper) (I1); Alexeenko and Orekhovich, 1960 (starch, column) (A1); Dunn and Murphy, 1961 (paper) (D10); Leach, 1961 (ion-exchange, column) (L5); Partridge and Elsden, 1961 (ion-exchange, column) (P1); Cucco, 1962 (ion-exchange, column) (C12); Myhill and Jackson, 1963 (thin layer) (M26); Nicholls *et al.*, 1963 (gas) (N7); Titaev, 1963 (paper) (T2); Gray *et al.*, 1964 (paper) (G8); LeRoy *et al.*, 1964 (ion-exchange, column) (for plasma protein hydroxyproline) (L9); Blau and Darbre, 1965 (gas) (B12); Marcucci and Mussini, 1965 (thin layer) (M4); Mussini and Marcucci, 1965 (gas) (M25); Oeriu and Tanase, 1965 (paper) (O1); Stalder, 1965 (ion-exchange, column) (S20); Firschein and Shill, 1966 (ion-exchange, column) (F6); Maskaleris *et al.*, 1966 (thin layer) (M8)]

Isotope dilution procedures

[Keiser *et al.*, 1963 (K5); Stanley *et al.*, 1966 (S21)]

TABLE 1 (*Continued*)

Procedures for measurement of labeled hydroxyproline
[Prockop *et al.*, 1961 (P13); Peterkofsky and Prockop, 1962 (P3); Juva and Prockop, 1966 (J11); LeRoy *et al.*, 1966 (L12)]
Enzymatic procedures
[Rosano, 1966 (R7)]
Procedures for primary amino acid removal (leaving imino acids)
[Hamilton and Ortiz, 1950 (H1); Satake *et al.*, 1960 (S1)]

[a] *p*-Dimethylaminobenzaldehyde.

collagen and the measurement of hydroxyproline on a sound basis for the first time. The previously described colorimetric procedures (L3, W1, M2) gave low values for the hydroxyproline content of gelatin, requiring large corrections. Why have over 60 modifications or separate procedures been introduced in the ensuing 25 years? The answer lies in the increasingly complex biological mixtures in which hydroxyproline is being measured.

The intermediates of the oxidative reaction used in virtually all the colorimetric procedures have not been completely characterized. Neuman and Logan did not define the products of oxidation in their procedure. Radhakrishnan and Meister obtained spectrophotometric and chromatographic evidence for the production of pyrrole-2-carboxylic acid from hydroxyproline under oxidative conditions similar to those of the Neuman and Logan procedure (R1). In accompanying enzymatic studies, pyrrole-2-carboxylic acid was derived from the unstable intermediate Δ^1-pyrroline-4-hydroxy-2-carboxylic acid. These studies led Prockop and Udenfriend to propose the reaction shown in Fig. 1 as adequate to explain the behavior of the oxidation products in their multistage procedure (P11). Bergman and Loxley, on the other hand, have presented spectral data to suggest that the oxidation products of hydroxyproline are neither pyrrole nor pyrrole-2-carboxylic acid (B9). This disagreement does not detract from the many useful methods available; it might be easily settled with the techniques of organic chemistry (e.g., the mass spectrometer). The various colorimetric procedures may produce several oxidative intermediates with virtually identical spectral characteristics on chromophore formation.

Neuman and Logan realized that the presence of significant quantities of tyrosine (and presumably other aromatic compounds as well) would

interfere with their procedure; they assumed that hydroxyproline would be purified prior to assay. Subsequent investigators have applied correction factors, changed the concentration of reagents, used extraction procedures, or introduced separation techniques to remove interfering materials that detract from the specificity of the original method. Indeed, it is specificity and not sensitivity that has prompted the many subsequent modifications, since a microadaptation of the Neuman and Logan method is perhaps the most sensitive procedure available (G9).

Peroxide was the original oxidant, but its incomplete removal quenched the chromophore reaction. Stegemann introduced the oxidant Chloramine-T, which has been widely adopted because it is simple to neutralize and does not interfere with chromophore formation (S22). Using Chlor-

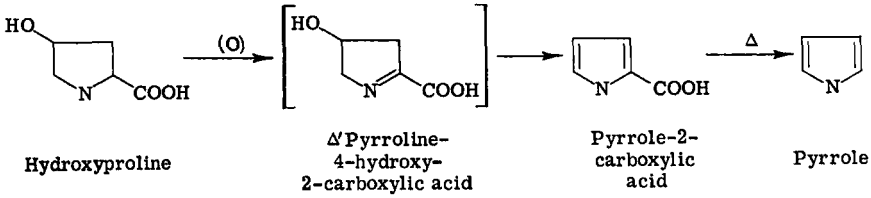

FIG. 1. Series of reactions involved in the colorimetric assay of hydroxyproline, demonstrating the oxidation and decarboxylation of hydroxyproline to pyrrol (S14).

amine-T as oxidant, Prockop and Udenfriend introduced a technique of differential extraction of pyrrole into an organic solvent and chromophore formation in that solvent system (P11). This procedure has become most widely used for urinary hydroxyproline determinations, where the ratio of hydroxyproline to total amino acids is about 1:100.

2.3. CHROMATOGRAPHIC PROCEDURES

On the basis of the original observations of Stein and Moore that amino acids can be separated by ion-exchange chromatography, many chromatographic procedures have been used prior to the colorimetric determination of hydroxyproline. In general, this type of procedure would seem necessary where the ratio of hydroxyproline to total amino acids is considerably less than 1:100. In blood plasma hydrolyzates, for example, where the ratio is 1 part of hydroxyproline to 5000 parts of amino acid, the existing colorimetric procedures were found to allow the formation of interfering chromophores, requiring a chromatographic–colorimetric procedure adapted for multiple simultaneous determinations (L9) (see Section 4.2). More recent chromatographic techniques, such as thin layer and gas–liquid systems, have not yet been used with a wide variety

of biological materials; consequently no precise recommendations can be made as to their application (see Table 1).

2.4. Isotope Dilution Procedures

This type of procedure, although laborious, circumvents many of the pitfalls of the colorimetric reaction (such as variable recovery and interference), and is helpful in authenticating a new procedure or in applying a known method to an unknown situation where complex mixtures are confronted for the first time. The author has had experience with the procedure of Keiser (K5) and found it extremely accurate and reproducible.

2.5. Radioactive Hydroxyproline Determination

Many of the chromatographic procedures are applicable to the measurement of labeled hydroxyproline; in general they do not allow for multiple simultaneous determinations. Since collagen hydroxyproline is labeled only by administering its precursor, proline, in labeled form, the difficulties in radioactive studies have arisen largely because the nonhydroxyproline metabolic products of proline or proline itself have not been rigorously separated from hydroxyproline or hydroxyproline-derived pyrrole. This is the purpose of the more recently introduced modifications for labeled hydroxyproline assay (J11, L12). It is too early to know whether one or both of these will suffice for general use.

2.6. Other Methods

A new approach to hydroxyproline assay is a recently introduced enzymatic procedure based on the ability of a multiple-enzyme system in adapted *Pseudomonas* strains of bacteria to degrade hydroxyproline (R7). Also, a standard colorimetric procedure has been adapted for use in an AutoAnalyzer apparatus (G5). Automatic amino acid analyzers can be easily programmed to isolate hydroxyproline from its more usual place in the shoulder of the aspartic acid peak (see M15).

3. Urinary Hydroxyproline

3.1. Chemical Nature

Westall first noted the presence of hydroxyproline in normal urine; it was present only in peptide form and associated with proline. He concluded that this bound hydroxyproline was a degradation product of collagen (W2), a hypothesis later confirmed by the study of enzymatic digests of collagen (G6). The types of hydroxyproline peptide present in the urine and their relation to growth were reviewed by Smiley and

Ziff; this review includes the literature on urinary hydroxyproline published through 1963 (S15). Although at least twelve different urinary hydroxyproline peptides have been isolated, 90–95% of total urinary hydroxyproline can be accounted for by the three peptides—Pro-Hypro (50–60%), Glu-Hypro-Glu (15%), and Gly-Pro-Hypro (15%) (K7, M11, M12). Pro-Hypro and Gly-Pro-Hypro have been shown to be frequent repeating sequences in the collagen molecule (G6). Also, free hydroxyproline constitutes 3% or less of total urinary hydroxyproline in the absence of dietary hydroxyproline.[3] A small proportion (5–10%) of urinary hydroxyproline is nondialyzable, suggesting larger compounds than the small peptides noted above (Z2). Krane has recently characterized a polypeptide in the urine of a patient with Paget's disease that has a molecular weight of about 8000 and contains hydroxyproline, glycine, and hydroxylysine (M24). This observation raises the interesting possibility that larger intermediates of collagen metabolism may be present in urine, and thus available for precise structural and metabolic investigations. Along these lines some urinary glycopeptides have recently been shown to contain hydroxyproline (B17, C4, C5).

3.2. Clinical Variables

The first clinical studies of urinary hydroxyproline were those of Ziff *et al.* in 1956 (Z2), using the method of Wiss (W7). In the ensuing decade, there have been approximately 150 published reports of urinary hydroxyproline determinations in a variety of clinical disorders. Considering the diversity of methodology and the numerous clinical variables, there is in general a high degree of agreement in these studies. The important variables will be considered individually.

3.2.1. *Effect of Diet*

The ingestion of collagen or gelatin, in quantities no greater than a large helping of Jello or gelatin-base ice cream, has been shown to increase urinary hydroxyproline excretion into the "abnormal" range (S14). Even without apparent gelatin feeding, the level of urinary hydroxyproline in a given subject is considerably more constant if sources of gelatin and collagen, such as meat, mayonnaise, and soft candies, are removed. Figure 2 demonstrates this effect of diet in a single normal adult. It is suggested that, when precise quantitative data on endogenous urinary hydroxyproline are desired, the diet should be care-

[3] Free hydroxyproline is measured in an aliquot of urine prior to hydrolysis; total hydroxyproline is that amount present after complete hydrolysis (usually acid). The difference between the free and total measurements is bound or peptide hydroxyproline.

fully chosen to eliminate sources of collagen and gelatin on the days of collection. The determination of free hydroxyproline in urine is a useful check on the presence of dietary hydroxyproline, since free hydroxyproline is elevated (>3%) along with peptide hydroxyproline after the ingestion of collagen products (P14).

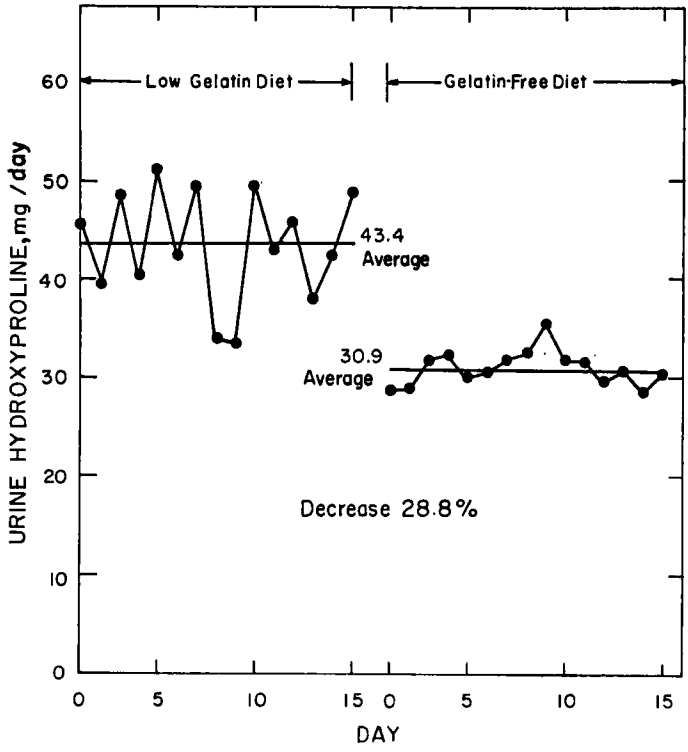

FIG. 2. Comparison of the daily urinary excretion of hydroxyproline by a normal adult during the ingestion of two different diets. Low-gelatin diet consists of a regular diet from which gelatin-rich foods, such as gelatin, Jello, ice cream, and soft candies, have been eliminated. Gelatin-free diet is a meat-free diet in which all animal protein is derived from milk, eggs, and cheese (S14).

3.2.2. *Effect of Sex*

In several published studies of urinary hydroxyproline excretion in normal adult subjects, males were noted to excrete larger quantities of total hydroxyproline (Table 2). When absolute hydroxyproline excretion was corrected for either surface area or creatinine excretion, no differences between males and females were noted. This observation suggests that

TABLE 2
URINARY HYDROXYPROLINE IN NORMAL ADULTS

	Number of subjects	Urinary hydroxyproline		References
		Average	Range	
Uncorrected (mg/24 hours)				
Adults (mixed)	524	28	9–70	[a]
Males	49	33	17–59	(A2, B6, B16, K19)
Females	37	27	11–50	(A2, B6, B16, K19)
Corrected for surface area (mg/m²/24 hours)				
Adults (mixed)	42	18	±6[b]	(G3)
Males	83	17		(A4, L1)
Females	65	16		(A4, L1)
Corrected for creatinine excretion (μg/mg of creatinine)				
Adults (mixed)	12	19	±4.6[b]	(C11)
	20		10–30	(K4)
Males	13	25	19–32	(A2)
Females	6	28	22–36	(A2)

[a] Normal adult determinations were compiled from the following references: B5, B20, C11, D9, G3, G11, H7, H9, J8, J9, J10, K14, K31, K34, L1, L7, L8, M9, M10, N1, N6, P9, R2, S12, Z2.

[b] Standard error of mean.

normal hydroxyproline levels, already shown to be quite constant in the individual subject, probably reflect total collagen mass rather than hormonal or other physiological differences between males and females.

3.2.3. *Effect of Age*

The age of the subject is a major factor in the interpretation of urinary hydroxyproline levels (S15). Measurements in subjects during active growth have shown elevations to 15-fold the normal adult level (J9). For this reason, it would seem reasonable to divide subjects under the age of 25 into several groups, each requiring its own "growth rate"-matched controls. Also, more variability is observed in the urinary hydroxyproline levels of these younger subjects than in those of adults. Consequently, attempts have been made to correct the actual hydroxyproline excretion for some parameter of size, such as body surface area. Table 3 outlines the published normal urinary hydroxyproline levels in infants, children, adolescents, and young adults. Both uncorrected and corrected data are

TABLE 3
URINARY HYDROXYPROLINE IN NORMAL CHILDREN

	Number of subjects	Urinary hydroxyproline		References
		Average	Range	
Uncorrected (mg/24 hours)				
Infants (0–1 year)	70	33	9–61	(A2, C8, J3, K16)
Children (1–10 years)	88	67	15–210	(C8, J3, J9, K16, R2)
Adolescents (11–16 years)	86	143	45–700	(K16, J3, J9, R2)
Young adults (17–24 years)	18	50	31–120	(J9, R2)
Corrected for surface area (mg/m²/24 hours)				
Infants				
(0–1 year)	31	105	40–190	(J3, K16)
(1–6 months)	43	130	90–200	(J5)
(7–11 months)	22	80	57–104	(J5)
Children				
(1–10 year)	128	64	40–120	(J3, J5, K16)
(0–10 years)	—	60 (m)[a]	±5[b]	(A4)
(0–10 years)	—	71 (f)	±9	(A4)
Adolescents				
(11–14 years)	78	72	36–110	(J3, J5, K16)
(11–20 years)	—	64 (m)	±15	(A4)
(11–20 years)	—	28 (f)	±5	(A4)
Corrected for creatinine excretion (μg/mg of creatinine)				
Children (3–13 years)	28		50–195	(K25)

[a] m for males, f for females.
[b] Standard error of mean.

included. The "averages" given have no statistical meaning, since it was at times not possible to determine the number of observations used to calculate a published mean.

The difficulty in obtaining complete 24-hour urine collections in the pediatric age group has prompted the introduction of a correction based on the concomitant determination of creatinine (W4). This type of determination, i.e., the hydroxyproline/creatinine ratio, might obviate the necessity of 24-hour urine collections in adult studies as well, and, if done on a fasting urine sample, might also eliminate the necessity of dietary restriction. Since creatinine is often determined to assess the completeness of 24-hour collections, the technical load would not be

greatly increased; on the other hand, the necessity of controlling the variables of patient cooperation in collection and diet might be eliminated. This would broaden the scope of hydroxyproline determinations considerably. The question is currently under investigation (A2).

3.2.3.1. *Infants (0–1 year)*. An increase in the excretion of free hydroxyproline in early infancy has been reported from several laboratories (C8, M20). The significance of this finding is unclear; it may represent a delayed maturation of the enzyme required for the degradation of free hydroxyproline. An inborn lack of this enzyme has been reported by Efron *et al.* (E2). The total absolute urinary hydroxyproline excretion in infancy is similar to that observed in children in general, as shown in Table 2; when corrected for body surface area, however, the excretion is the highest of any group of normal subjects.

3.2.3.2. *Children (1–10 years)*. There is large variation in normals in this age group, perhaps because of the variable growth rates in children and the early age at which females begin the prepubertal growth spurt. When the data are separately presented, normal female children have a higher urinary hydroxyproline excretion than males of comparable age (Table 2).

3.2.3.3. *Adolescents (11–16 years)*. The highest absolute hydroxyproline excretion of any group of normal subjects is seen in the rapidly growing adolescent. These levels are as high as any seen in disease processes yet studied, with the possible exception of widespread bone disease such as Paget's or severe hyperphosphatasia.

3.2.3.4. *Young Adults (17–24 years)*. There is a gradual reduction in hydroxyproline excretion in subjects between ages 17 and 24. In the individual case, however, it is not uncommon to see an occasional normal young adult, age 21–23, whose hydroxyproline excretion is 80–100 mg/day, 2–3 times the older adult average. This factor limits the value of urinary hydroxyproline in the diagnosis of disorders such as Marfan's syndrome, since the patient in whom this diagnosis is considered is often between ages 17 and 24 (see J10).

3.2.3.5. *Adults (24 years or older)*. The normal adult level of urinary hydroxyproline is fairly well defined, as shown in Table 2. In 32 studies surveyed, reporting data from 515 normal subjects, 25 studies found no normal adults excreting more than 50 mg/day; three studies, no more than 60 mg (A2, B6, L7); three studies, no more than 70 mg (J8, L8, R2); and one study, no more than 90 mg (B20). If three subjects were eliminated from this large group, all normal values would be less than 70 mg/day. In the author's experience, the upper limit of normal urinary hydroxyproline excretion, in adults of age 25 or older on diets free of meat

or gelatin, is 50 mg/day, with the large majority of normals excreting less than 40 mg/day. The possibility cannot be excluded that dietary hydroxyproline ingestion has increased the range of several studies listed.

3.3. Value in Diagnosis and Management

3.3.1. *Children*

The wide variation in normal urinary hydroxyproline levels in children has been discussed above. The most consistent of the abnormalities of urinary hydroxyproline in children, as shown in Table 4, is the decreased excretion associated with defective growth. Whether the cause of growth retardation is hormonal, genetic, or environmental (malnutrition or chronic disease and/or drug therapy), urinary hydroxyproline excretion is decreased. When the hydroxyproline excretion is corrected for surface area or creatinine excretion, this measurement seems to correlate well with the extent of growth retardation (W4). In spite of the fact that creatinine excretion is also reduced in malnutrition, Whitehead found an

TABLE 4
Urinary Hydroxyproline in Childhood Disease

Diagnosis	Number of patients	Urinary hydroxyproline (mg/24 hour)		Ref.
		Average	Range	
(1) Dwarfism, pituitary	2	25	14–48	(J3)
	5	45[a]	31–77[a]	(J5)
(2) Dwarfism, congenital	3	38	12–59	(J5)
(3) Dwarfism, other causes	8	11	5–17	
	18	56[a]	18–100[a]	(J5)
(4) Hypothyroidism	6	10	5–14	(K16)
(5) Rickets				
vitamin D-deficient (severe)	6	23	8–62	(C8)
vitamin D-resistant	2	203	199–207	(K16)
(6) Malnutrition	19	13	5–30	(P4, W4)
(7) Kwashiorkor	12	8	±1[b]	(A3)
(8) Pseudohypertrophic muscular dystrophy	9	76	27–170	(K9)
(9) Hyperthyroidism	5	144	103–188	(K16)
(10) Acute rheumatic fever	55	"High"	Up to 164	(P8)
(11) Idiopathic hyperphosphatasia	1	1283	—	(S9)
(12) Thermal burns	11	—	65–250	(K25)

[a] In mg of hydroxyproline/m²/24 hours.
[b] Standard error of mean.

excellent correlation between the "hydroxyproline index" [(hydroxyproline/creatinine) × body wt. in kg] and the degree to which malnourished children fell below standard weight–height expectations (W4; see also P4). This type of measurement would seem promising as an index of growth rate. It is currently in use to study the effect of dietary supplements on the growth of chronically undernourished subjects (W4).

Hydroxyproline excretion is not diagnostically helpful in delineating the type of growth retardation present, as can be seen from the large overlap in several types of dwarfism shown in Table 4. However, it is useful in assessing the response to therapy, obviating the wait to determine absolute growth. The response in patients with growth retardation to both human growth hormone (B15, C6, C7, D9, J3, J5, M10) and thyroid hormone (B6, C6, D3, J3, K19) has been studied by the measurement of hydroxyproline excretion. Even though it is difficult to be certain of pathological elevations in this age group, elevated excretion has been reported in hyperthyroidism, acute rheumatic fever, and a case of idiopathic hyperphosphatasia. Because of the wide variation in urinary hydroxyproline in normal children and the difficulty of establishing an abnormality in the individual case, the comparison in each study with age-matched controls would seem essential.

3.3.2. *Adults*

3.3.2.1. *Bone Disorders.* The most consistent abnormalities of urinary hydroxyproline in adult patients are shown in Table 5. Distinct elevations occur in a variety of disorders that seem to have as a common denominator an increased metabolism of bone. Since bone contains 40% of total body collagen (S16) and is generally more active in remodeling its structure than other connective tissue, such as skin and tendon, it is not surprising that bone disorders produce the highest urinary hydroxyproline levels. It seems likely that even such apparently diverse states as pregnancy, malabsorption, and paraplegia have increased bone turnover as the basis for elevated hydroxyproline excretion.

Much has been written about the value of urinary hydroxyproline elevations in separating bone formation from bone resorption. Correlations have been attempted clinically between urinary hydroxyproline, on the one hand, and serum alkaline phosphatase, serum and urinary calcium, calculations of calcium turnover such as the bone formation rate and bone resorption rate, bone histology, and bone radiology. Most authors feel that the level of urinary hydroxyproline reflects bone destruction (B6, B16, D8, D9, G1, K26, L8, S16) more than bone formation (H9, K20). This does not seem to be a very useful argument clinically, because bone formation and destruction are usually occurring simultaneously in

TABLE 5
URINARY HYDROXYPROLINE IN ADULT DISEASE

Diseases	Number of patients	Urinary hydroxyproline (mg/24 hours)		References
		Average	Range	
Bone				
Paget's disease of bone	62	202	20–998	(C11, D9, F2, G3, K31, L6, L7, M22, S13)
Metastatic bone disease (neoplastic)	238	79	10–340	(B16, G1, G11, H9, K24, K31, L6, L7, N1, P9, R8, R9)
Pulmonary hypertrophic osteoarthropathy	7	61	41–75	(H9)
Idiopathic hypertrophic osteoarthropathy	1	120	—	(M16)
Osteoporosis	65	31	8–73	(G1, K24, K31, L7, R3, R10)
Fractures	9	44	11–65	(C11, N2)
Endocrine				
Hyperthyroidism	76	109	10–440	(B6, D9, K19, K24, K31, L6, L7, R11)
Hypothyroidism	6	11	4–15	(K19, L6)
Hyperparathyroidism				
Primary	22	88	12–477	(F8, J8, K20, K31, L6, N6)
Secondary	6	90	59–119	(J8, L6)
Acromegaly	20	82	15–169	(B6, K31, L1, L6)
Hypogonadism	3	70	66–76	(B6)
Normal pregnancy	25	74	31–127	(B14, K22)
Cushing's syndrome	3	12	10–18	(K24, L1)

Connective tissue				
Marfan's syndrome	7	161	44–416	(J10, R4, S12)
Ehlers-Danlos syndrome	6	25	15–34	(M16)
Rheumatoid arthritis	73	36	8–124	(G2, L1, L7, R8, R9, S18, T3, Z2)
Scleroderma	31	40	6–84	(F1, L7, R5, S18)
Dermatomyositis	2	49	21–77	(K9)
Periarteritis nodosa	3	58	46–71	(S18)
Ankylosing spondylitis	2	23	8–35	(L7)
Skin				
Psoriasis	29	55	12–130	(B20, K34)
Stasis dermatitis	5	62	33–108	(S18)
Discoid lupus erythematosus	3	38	26–55	(S18)
Thermal burns	21	—	50–895	(E3, K21, K25)
Other				
Malabsorption states	6	139	40–242	(K38, L1)
	17	60[a]	14–124[a]	(C11)
Sarcoidosis	9	49	±16(SD)	(M9)
Acute paraplegia	3	115	105–126	(K27)
Carcinoid syndrome	4	34	16–43	(M16)

[a] In μg of hydroxyproline/mg of creatinine.

bone studied microscopically from a wide variety of bone lesions. Moreover, bone destruction usually stimulates bone formation, and vice versa; thus, the two processes are difficult to separate in the intact patient.

In spite of these inherent shortcomings, the measurement of urinary hydroxyproline offers a useful index of bone matrix metabolism to add to the spate of techniques of bone mineral measurement used to study the endocrinology and metabolism of bone (B8).

3.3.2.2. *Endocrine Disorders.* The spontaneous elevations of urinary hydroxyproline seen in several endocrine disorders prompted studies in normal subjects given exogenous thyroid and parathyroid hormones (S14). Increases in urinary hydroxyproline excretion were observed with both hormones within several hours of parenteral administration, suggesting a direct, rapid response to the hormone. On the other hand, the response to growth hormone, usually studied in subjects with retarded growth, is slower and of smaller degree, requiring days to weeks to exhibit definite elevations (J3). Attempts to change the endogenous levels of parathyroid hormone with calcium infusion can be measured indirectly by hydroxyproline excretion, and such determinations show promise of practical diagnostic value in the difficult area of hypercalcemic and hyperparathyroid states (B5).

Experimental data, using both pulse-labeled collagen hydroxyproline measurements (P15) and excretion data following parathyroid extract administration (H5), suggest that the largest source of urinary hydroxyproline normally and after parathyroid extract is mature insoluble collagen. Similar data following thyroid and growth hormone administration would be of interest, to see if the three hormones which elevate urinary hydroxyproline stimulate the same pool of collagen.

3.3.2.3. *Connective Tissue Disorders.* The first clinical study of collagen metabolism in the intact subject was the measurement of urinary hydroxyproline in patients with rheumatoid arthritis and other connective tissue disorders (Z2). These and subsequent observations in the rheumatic diseases have revealed largely normal hydroxyproline excretion, with occasional patients in each study showing elevations sufficient to raise the average slightly. Separate reports of consistent elevations in acute rheumatic fever (P8) and rheumatoid arthritis (H7) disagree with the majority of studies. Some observers feel that the patients with severe active systemic inflammatory disease will be more likely to excrete excess hydroxyproline, suggesting that urinary hydroxyproline may be slightly responsive to any generalized inflammatory process (R5, S18). If this is the case, urinary hydroxyproline is certainly an insensitive "acute-phase reactant," and it is only in the severe generalized inflammatory states that "nonspecific" elevations occur. Nonetheless, such a

mechanism might be the explanation for the occasional elevations seen in disorders of connective tissue.

3.3.2.4. *Skin Disorders.* The evidence which implicates bone as the source of urinary hydroxyproline is necessarily indirect, and evidence of the contribution that skin, tendon, or cartilage makes to urinary hydroxyproline is not available. The finding of occasional elevations in patients with disorders limited to the skin, such as psoriasis and stasis dermatitis, might suggest a minor role for skin collagen as a source of urinary hydroxyproline (S18). In general, however, the normal levels in most skin conditions would seem to discourage the use of urinary hydroxyproline determinations in the diagnosis or management of skin disorders.

3.3.2.5. *Other Disorders.* Elevated urinary hydroxyproline in malabsorption states may be secondary to the well-known development of osteomalacia in such situations (K38, L1). Sarcoidosis is known to involve bone (M9), and loss of bone is known to accompany paraplegia or enforced bed rest (K27) (or the space age counterpart, sustained "weightlessness"). Thus, all the elevations of this miscellaneous group can be explained on the basis of associated bone disorders. They were not so included in Table 5 because of insufficient data.

4. Plasma Hydroxyproline

4.1. Chemical Nature

Hydroxyproline is present in human plasma in three forms; free, peptide, and protein-bound, as shown in Table 6. It was not appreciated until recently that 80% of plasma hydroxyproline is present in protein form; this material is nondialyzable, precipitable with the usual protein precipitants, and of sufficiently large molecular weight to be excluded by Sephadex G-200 gel filtration (>200,000). This plasma hydroxyproline-containing protein (hypro-protein) has been shown to be resistant to enzymatic digestion by Pronase, a nonspecific protease that digests virtually all other plasma proteins. This is a property shared by the soluble collagen precursors, of which tropocollagen is the parent molecule; it suggests that hypro-protein may be collagen or collagen-like (L9). What relation hypro-protein has to a hydroxyproline-containing globulin, found in an elderly patient with anemia by Mandema *et al.* (M3), is not known. Frey *et al.* have recently presented indirect immunological evidence that the hypro-protein shares antigenic determinants with acid-soluble human skin collagen (F12), and that it migrates in the α_2–β-globulin region on acetolized cellulose column electrophoresis (F10).

During the initial studies of hypro-protein, the levels were compared in plasma and serum prepared simultaneously from the same normal

TABLE 6
HYDROXYPROLINE IN HUMAN PLASMA[a]

Form (no. of assays)	Hydroxyproline (μg/ml plasma)	
	Mean	Range
Free (6)	1.5	1.3–1.8
Peptide (5)	0.6	0.4–0.8
Protein-bound (73)	8.0	5.5–9.7

[a] All determinations were on fresh fasting plasma samples from normal young adult volunteers, age 21–29. Free hydroxyproline was measured by collecting the supernatants and washings after the ethanol precipitation of protein from 2 ml plasma, removing the ethanol by evaporation at 50°C, and applying the residue in water (acidified to pH 1) to a column. Elution and colorimetric measurement were as described for the hypro-protein assay. Supernatants and washings after the ethanol precipitation of protein from 2 ml of plasma were hydrolyzed overnight in alkali after the evaporation of ethanol. They were then assayed by the hypro-protein assay to obtain total nonprecipitable hydroxyproline. The value for free hydroxyproline was subtracted from the total value to obtain peptide hydroxyproline. Details of the hypro-protein assay are published elsewhere (L9).

subject. The serum levels were always 10–20% lower than those of plasma, and the losses in serum were variable; consequently the clinical studies were carried out on plasma. Oxalate or citrate is the preferred anticoagulant because of significant hypro-protein losses in the insoluble precipitate that forms on freezing and thawing heparinized plasma.

4.2. METHOD OF ASSAY

In attempts to ascertain a satisfactory method for hypro-protein assay, the existing methods being used for urine and plasma determinations were compared and widely divergent values were obtained, as shown in Table 7. By the use of an isotope dilution procedure (K5), it was found that the then existing methods (all but hypro-protein assay in Table 7) were measuring hydroxyproline as well as variable amounts of interfering material. The spectral characteristics of the interfering chromophore were found to differ from those of authentic hydroxyproline, as shown in Figs. 3 and 4. A useful guide to the presence of interference of this type is the absorbancy at the peak of the interference chromophore, 450 mμ. With this information, a chromatographic–colorimetric procedure was devised specifically for the measurement of hypro-protein (L9). Kibrick *et al.* have independently confirmed the presence and normal level of hypro-protein (K10).

Recent studies by Frey (F10) have shown that similar values for

TABLE 7
VALUES FOR PLASMA PROTEIN HYDROXYPROLINE OBTAINED BY VARIOUS PROCEDURES[a]

Procedure	No. of determinations	Hydroxyproline (μg/ml)	
		Average	Range
Neuman and Logan (N4)			
Human serum proteins[b]	18	79	53–105
Whole fowl serum (K35)*	100	115	81–132
Prockop and Udenfriend (P11)			
Whole human plasma	7	20	18–22
Whole rat plasma (B2)*	31	24	19–25
Human plasma proteins	11	16	12–18
Whole human plasma after nitrous acid treatment (H1)	7	16	15–19
Hypro-protein assay (L9)			
Human plasma proteins	21	8.1	7.0–9.7

[a] All assays were carried out in the author's laboratory except those marked with an *asterisk*, for which the values represent data taken from the reference cited.

[b] J. C. Houck, by the method of Martin and Axelrod (M5), personal communication (1963).

hypro-protein can be obtained by procedure II of Woessner (W8). No evidence is given as to the presence or absence of the interfering chromophore in this method. Lower levels for protein hydroxyproline are reported by Cebecauer and Trnavský (C1), using the procedure of Stegemann (S22). A paper chromatographic method following hydrolysis has been used by Titaev in the study of total serum hydroxyproline in acute rheumatic fever; normal levels were not given in the one of two publications of Titaev (T1, T2) available to the author.

4.3. DIAGNOSTIC SIGNIFICANCE

4.3.1. *Free Hydroxyproline*

The level of free hydroxyproline in the blood is relatively constant in fasting subjects with normal renal function, 1.3–1.8 μg/ml of plasma in the author's study (Table 6), and 0.6–3.9 μg/ml of plasma in a larger group of normal subjects in a study of Baumann *et al.* (B3). The latter study does not mention the fasting state of their subjects, which may explain the wider variation. Laitinen *et al.* found the free plasma hydroxyproline to vary only between 0.6 and 1.5 μg/ml in 95 subjects of various ages on a gelatin-free diet (L1). Kibrick *et al.* found 2.3 μg/ml of free

hydroxyproline in pooled normal human serum (K10). Using a technique of chromatographic separation, Oye found an average of 0.9 in twelve fasting normal young adults, with a range of 0.7–1.2 μg/ml (O5). With paper chromatography, Dubovsky found 1.6 μg/ml (SD = 0.6) in the deproteinized plasma of 20 normal subjects whose postprandial state was not mentioned (D7). These studies taken together suggest that normal free plasma hydroxyproline is 1–2.5 μg/ml, and that subjects with values in excess of this should be suspected of having recently ingested collagen or gelatin hydroxyproline.

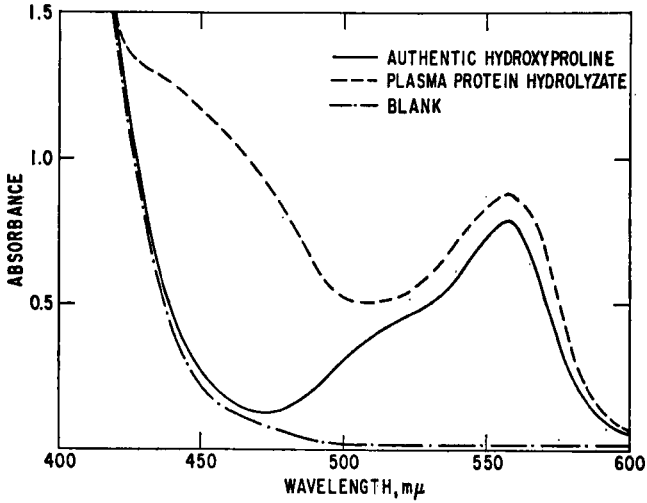

FIG. 3. Absorption spectra of chromophores from 12.5 μg of authentic hydroxyproline and protein hydrolyzate of 1 ml of plasma. Chromophores were concentrated by adding 0.6 ml of water to the final ethanol–sulfuric acid–toluene–Ehrlich's reagent mixture of the colorimetric procedure, shaking the mixture, and separating the water–ethanol–acid phase; the latter was then diluted to 3.0 ml with ethanol (L9).

Unfortunately, many of the published studies do not mention the recent dietary intake of their subjects. This is important because of the exquisite sensitivity of plasma free hydroxyproline to the ingestion of even small amounts of collagen or gelatin. This has been well studied by Prockop and Keiser (P14) and Baumann (B3). It is recommended that at least a 12-hour fast precede the blood taking if free hydroxyproline is to be measured.

Although no determinations could be found, abnormal quantities of free hydroxyproline can be suspected in early infancy on the basis of the observed elevated free hydroxyproline levels in the urine and the presumptive mechanism of decreased metabolic breakdown and overflow

excretion. This mechanism has been elegantly substantiated by Efron in a retarded child with an inborn lack of "hydroxyproline oxidase" and massive quantities of free hydroxyproline in the plasma and urine (E2). In this regard a common tubular reabsorption mechanism has been demonstrated by Schriver *et al.* for hydroxyproline, proline, and glycine, and it might be expected that an aminoaciduria with this pattern of excretion could occur (S5). Slight elevations of free hydroxyproline in plasma were observed by Dubovsky *et al.* in severe renal failure, but

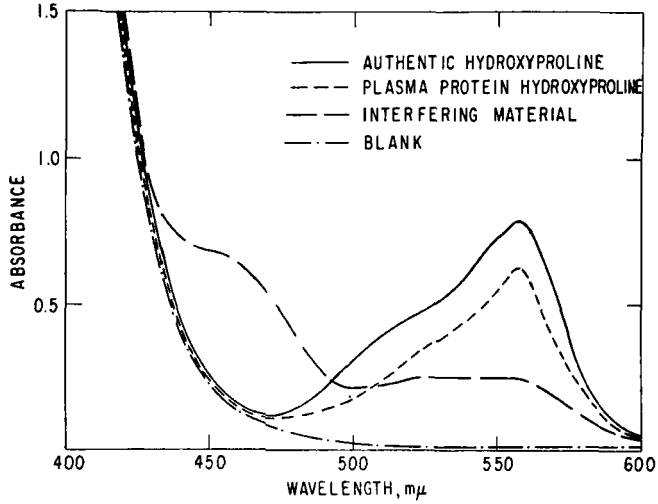

FIG. 4. Absorption spectra of chromophores from hydroxypyroline and interfering material separated chromatographically from plasma protein hydrolyzates. The chromophores obtained were concentrated (as described in the legend of Fig. 3) and their spectra determined. (For details of chromatographic separation, see L9.)

these are small compared to the enormous elevation of plasma peptide hydroxyproline reported by him; it seems possible that the slight change in free hydroxyproline might be an artifact of the evaporation procedure used (degrading small quantities of the peptides) (D7). Also, in the presence of renal failure, dietary elevations of free hydroxyproline would persist longer than in subjects with normal kidneys. Slight elevations were observed by Kivirikko *et al.* in eleven patients with hyperthyroidism (K14).

Thus, the major abnormalities of plasma free hydroxyproline appear to result from (1) the ingestion of hydroxyproline, collagen, or gelatin in any form, and (2) an inability to metabolize hydroxyproline from either prematurity or an inborn error of metabolism. Theoretically, a

defect in the renal tubular reabsorption of hydroxyproline could lead to low plasma levels, but this situation has not been observed.

Hurych and Chvapil have implicated free tissue hydroxyproline as a breakdown product of newly formed soluble collagens in labeled studies in animals. These implications have not been studied in man (H13).

4.3.2. *Peptide Hydroxyproline*

The hydroxyproline peptides, as well as peptides in general, are cleared rapidly by the kidney, and their plasma level is low when renal function is unimpaired. Their clearance rate after hydroxyproline loading has been found to equal or slightly exceed the clearance of inulin in two normal subjects, causing Benoit *et al.* to suggest that hydroxyproline peptides were secreted by the renal tubules (B7). As was found with free hydroxyproline, the peptides are also influenced by the ingestion of collagen or gelatin products. Prockop and Keiser demonstrated that hydroxyproline in peptide form was absorbed by the gastrointestinal tract and rapidly excreted, the first demonstration of intestinal absorption of a peptide or protein (P14). The possibility of using this as a model system in the study of protein transport across the intestinal wall in cases of food allergy has not been explored.

Marked elevations of plasma peptide hydroxyproline have been observed by Dubovsky *et al.* in chronic renal failure (D7). The highest levels were seen when uremia and severe bone disease occurred together. Removal of the parathyroids in four patients (three primary and one secondary hyperparathyroidism) was associated with return to normal levels when renal function was normal, and significant reduction when renal failure was present. It seems possible that plasma peptide hydroxyproline might be a rapidly changing and sensitive indicator of bone metabolism which could be studied even with renal impairment, a major obstacle to most current techniques in the diagnosis of hyperparathyroid states.

4.3.3. *Plasma Protein Hydroxyproline (Hypro-Protein)*

A broad clinical survey of the levels of plasma hypro-protein has been published (L10). The consistent elevations are shown in Table 8. No instances of a decreased level or absence were observed. Elevations of plasma hypro-protein were found in a large number of neoplastic and inflammatory conditions; in this regard plasma hypro-protein levels were comparable to the erythrocyte sedimentation rate and other "acute-phase reactants." These observations led to the study of induced elevations of hypro-protein in normal subjects by the production of fever and inflammation with the administration of etiocholanolone, a pyrogenic

TABLE 8
ELEVATIONS OF PLASMA HYPRO-PROTEIN[a]

Disease	Number of patients	Protein-bound hydroxyproline ug/ml	
		Mean	Range
Hodgkin's disease, afebrile	6	18	13–22
Sjögren's syndrome	3	17	11–21
Familial Mediterranean fever	5	16	14–18
Idiopathic recurrent fever	4	15	8–23
Osteochondrosarcoma	4	15	12–18
Acute febrile states[b]	3	15	13–16
Advanced renal disease	5	14	11–16
Carcinoid syndrome with heart lesions	3	14	13–14
Hyperthyroidism	3	14	12–15
Breast cancer with metastases	2	14	13–14
Paget's disease	2	13	12–13
Rheumatoid arthritis	8	11	8–16
Ehlers-Danlos syndrome	4	11	9–16
Acromegaly	4	11	8–16
Normal adult	54	8	5–10

[a] Procedure of LeRoy *et al.* (L9).

[b] Thrombophlebitis with pulmonary embolus, systemic viral infection, infectious mononucleosis.

steroid. Conversely, the production of fever alone with endotoxin did not lead to elevated hypro-protein levels (L13). It was also demonstrated that the elevations seen in Hodgkin's disease, the highest hypro-protein levels of the entire study, were well correlated with the activity of the disease, and a response to therapy was associated with a return to normal levels; subsequent relapse was uniformly associated with elevated hypro-protein levels (L11). Although it may prove to be useful in the management of patients with Hodgkin's disease or similar disorders, there seems to be nothing specific about elevated hypro-protein levels in patients with lymphomas.

5. Tissue Hydroxyproline

5.1. INTRODUCTION

The experience with hydroxyproline measurement in tissue hydrolyzates differs distinctly from that with urine and plasma hydroxyproline determinations. In tissues, methods have not been as carefully evaluated; results have been much more variable both within and between the

laboratory animals and commercially important species studied; and the effects of age, diets, drugs, and hormones have been in such disagreement between studies that generalizations would be of no value.

5.2. Method of Assay

The most critical feature of tissue hydroxyproline determination is the specificity of the method used. For tissues very high in collagen content and very low in plasma or globular protein content (i.e., skin, tendon, bone, lens) there is a minimal formation of interfering chromogen (see Figs. 3 and 4, and Section 4.2), and analyses by simple colorimetric procedures are satisfactory. On the other hand, in the analysis of viscera and in the presence of plasma proteins there is significant formation of interfering chromophore, and a simple procedure is no longer specific for hydroxyproline. The author's studies on plasma and the studies of Hutterer and Singer (H14) on rat liver suggest that the simplest method to check the specificity of a procedure is to monitor the spectral characteristics of the chromophore produced either by a recorded spectrum (400–600 mμ) or by reading at OD_{450} for interference and OD_{560} for authentic hydroxyproline (see Fig. 4). If the absorbancy at 450 mμ is significant, a purification or separation step prior to colorimetric assay is imperative. Such a modification was found to reduce variability and improve recoveries in unpublished studies measuring the hydroxyproline of canine arteries in the author's laboratory. It would be desirable for future studies employing tissue hydroxyproline measurements to include spectral or other evidence of the absence of interfering chromophore formation. This is even more critical in the area of the radioactive hydroxyproline determinations of tissues, to ensure that the radioactivity measured was a part of hydroxyproline (J11, L12).

5.3. Studies in Experimental Animals

The list of tissue hydroxyproline measurements in experimental animals is long; in general three types of investigation have been carried out:

(1) Hydroxyproline has been used to assess the collagen content (and hence the commercial desirability) of various types of meat and hides as well as the effect of age, breeding, diet, anatomical location, and the processing or tanning methods used. Several of the methods in Table 1 are for this purpose (B1, B18, D1, G4, L19, W6, Z1); these studies would seem to be of value for the meat and leather industries.

(2) Extensive studies have been carried out in most of the small laboratory animals available, measuring hydroxyproline in the various organs as an index of total collagen content. Among the experimental

situations studied were the effects of age, sex, diet, most of the polypeptide and steroid hormones, drugs, and the experimental induction of disease. These measurements are often coupled with an assay of a constituent of mucopolysaccharides in order to study overall connective tissue changes. Few such studies have been carried out in man; they will be considered below. It is uncertain whether the data in experimental animals are of value in predicting tissue responses in man; the laboratory animal data will not be considered in detail here (B19, C2, C3, F11, H10, J4, K11, K13, K17, K35–K37, M6, M13, N5, N8, S17, S19, W5).

(3) The most instructive types of tissue hydroxyproline measurement have been those aimed at an assessment of the metabolism or the turnover of collagen in various areas. These have been of two types. (a) Tissues have been extracted in solutions of increasing solvating ability, and hydroxyproline, in both free and bound forms, has been measured in both the extracting solutions and the insoluble residue. The most "soluble" (extractable is preferred because these components are probably not in solution as they exist *in vivo*) forms of hydroxyproline, and hence collagen, have been shown to be the most recently synthesized and the least cross-linked subunits of the collagen fiber (J4, M6). Such studies have been critical to the understanding of collagen chemistry that exists today (see H3, H4). Studies utilizing the extraction principle have recently been carried out, using human skin biopsies from a variety of clinical disorders (*vide infra*). (b) Tissue hydroxyproline has been labeled by the administration of isotopically labeled proline, shown by the studies of Stetten to be the precursor of collagen hydroxyproline (S24), and the specific activity of tissue hydroxyproline has been used to compare the metabolism of collagen in various organs and sites directly (H5, H13, K15, K18, L18). Although the methodology is much more tedious, this type of study holds increasing promise in the study of the dynamic aspects of collagen and connective tissue. There have been virtually no detailed studies with labeled proline in man.

5.4. Studies in Man

Disorders of the skin and of the blood vessels have been studied by the measurement of hydroxyproline in these tissues (B11, C9, K2, S11, S25, V1); skin samples have been used as an index of the general state of maturation of collagen in various disorders (H6). Hydroxyproline measurements in cardiac muscle have been used to quantify the degree of fibrosis from various cardiac diseases (C9, M19). Measurements have been made in amniotic fluid (K12), in the uterus at various stages (H2, W9), and in the whole body of infants (P5).

5.4.1. *Skin*

A decrease in hydroxyproline per unit area has been found in senile degeneration of the skin (S11). These authors are critical of earlier data showing an increase in skin collagen with age, because of the earlier use of dry skin weight or hexosamine/collagen ratios. It is difficult to decide which denominator is more accurate biologically at this point (C10). Verzar, using a denaturation technique, has shown that young scar tissue in skin of older persons behaves as young collagen for the first few years following its formation (V1). Fleischmajer has found the hydroxyproline content of the skin to be normal in eight patients with scleroderma, leading him to propose a defect in the ground substance (F9).

Harris and Sjoerdsma have made a detailed analysis of skin collagen obtained from small punch biopsies of patients with a variety of skin and systemic disorders (H6); this type of study allows the detailed as well as the sequential study of human skin collagen in disease. These investigators measured water content, total collagen, "soluble" or acid-extractable collagen, and the ratio of α and β subunits present in the extractions. Their data are shown in condensed form in Table 9. The skin of patients with keloids, homocystinuria, and thyrotoxicosis showed increased levels of "soluble" collagen; the skin of patients with scleroderma showed a decreased "soluble" collagen fraction. This study demonstrates the feasibility of a direct approach to the study of skin collagen metabolism in man.

5.4.2. *Uterus*

The 6–8-fold increase in total collagen in the uterus with pregnancy, as well as the rapid loss of this relatively inert substance postpartum, has made uterine tissue a favorite for the study of rapid connective tissue turnover and the mechanisms involved, particularly the mechanism of rapid catabolism of such large amounts of insoluble collagen (H2, W9). The availability of human uterine tissue has enhanced the interest in these studies; however, they are not yet strictly pertinent to the understanding of disease and will not be further considered here (see W9).

5.4.3. *Heart and Blood Vessels*

Cardiac muscle hydroxyproline, measured by a simple colorimetric procedure with inadequate specificity in this situation, increased slightly beyond age 35–40, while a similar rise was seen in the hydroxyproline of aortic tissue (C9). Other studies using identical methods showed no change with age in aortic and pulmonary artery hydroxyproline (B11, K2). The influence of both age and cardiac hypertrophy on cardiac

TABLE 9
SKIN COLLAGEN PROFILE IN MAN[a]

Condition (no. of subjects)	Water content (%)	Total collagen (μg of hypro/mg dry wt.)	"Soluble" collagen[b] (%)	Collagen subunits (α:β ratio)[c]
Control subjects (34)	60–69	95–129	1.4–3.5	0.8–1.2
Skin diseases				
Keloid (6)	74–80	104–124	4.6–12.1	1.3–1.9
Mycosis fungoides (4)	63–75	36–78	2.3–9.8	1.0–1.7
Scar tissue (5)	64–78	78–115	2.0–8.5	1.0–2.0
Heritable diseases of connective tissue				
Marfan's syndrome (3)	61–66	102–128	2.3–2.8	0.9–1.1
Osteogenesis imperfecta (2)	57–68	99–124	1.3–2.8	1.0
Ehlers-Danlos syndrome (3)	60–65	93–121	2.0–6.1	1.0–1.2
Homocystinuria (4)	62–68	101–127	1.7–7.5	1.1–1.6
Endocrine disease				
Growth hormone deficiency (3)	60–67	97–129	0.6–2.1	1.0–1.2
Thyrotoxicosis (1)	62	101	6.9	1.1
Connective tissue diseases				
Rheumatoid arthritis (6)	62–65	97–118	1.7–3.6	1.1–1.2
Scleroderma (8)	58–67	72–115	0.7–1.2	1.0–1.7

[a] Data of Harris and Sjoerdsma (H6).
[b] Acetic acid extract of minced skin, % of total hydroxyproline.
[c] Determined by disc gel electrophoresis.

hydroxyproline has also been studied; when hydroxyproline was related to total heart muscle protein (nitrogen), no changes with age or hypertrophy were noted (M19). The collagen content of the internal saphenous vein was found to be about 50% (S25); thus, the veins represent a high-collagen tissue in which a simple colorimetric assay would probably be satisfactory without prior hydroxyproline separation.

5.4.4. *Other Tissues*

The concentration of free and total hydroxyproline in human amniotic fluid was observed to decrease in pregnancy between the fourth month and term (K12). The amino acid composition of collagen from human bone, tendon, dura mater, and postmenopausal uterus was found to be similar (H2). In whole body measurements in malnourished infants, there was no change in collagen protein despite severe total protein depletion (P5). A distinctively high content of hydroxylysine has been noted in human renal glomeruli (L17). Also, the glomerular basement membrane, including initially all the hydroxyproline and hydroxylysine, is solubilized by collagenase, suggesting that an unusual collagen-like material may be an integral part of the renal glomerulus. To what degree alterations of this material can affect renal function is not yet known.

It is clear from these studies that measurements of tissue hydroxyproline in man have not progressed to the stage of hydroxyproline measurements in urine or plasma. With the availability of more specific techniques, it seems likely that the study of tissue hydroxyproline will play an increasing role in our understanding of disorders in which fibrosis and altered connective tissue are prominent.

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REFERENCES

A1. Alexeenko, L. P., and Orekhovich, V. N., A method for proline and hydroxyproline determination by means of ninhydrin reaction in acid medium after starch chromatography. *Dokl. Akad. Nauk SSSR* **133,** 690–693 (1960).

A2. Allison, D. J., Walker, A., and Smith, Q. T., Urinary hydroxyproline: creatinine ratio of normal humans at various ages. *Clin. Chim. Acta* **14,** 729–734 (1966).

A3. Anasuya, A., and Narasinga, B. S., Urinary excretion of hydroxyproline in kwashiorkor. *Indian J. Med. Res.* **54,** 849–857 (1966).

A4. Anderson, J., Bannister, D. W., and Tomlinson, R. W. S., Total urinary hydroxyproline excretion in normal human subjects. *Clin. Sci.* **29,** 583–587 (1965).

B1. Baker, L. C., Lampitt, L. H., and Brown, K. P., Connective tissue of meat. II. Determination of hydroxyproline. *J. Sci. Food Agr.* **4,** 165–172 (1953).

B2. Bates, W. K., McGowen, J., and Talmage, R. V., Influence of the parathyroids on plasma hydroxyproline levels. *Endocrinology* **71,** 189–195 (1962).

B3. Baumann, H., Fritze, E., and Kallweit, C., Der Hydroxyprolingehalt des Blutserums bei Gesunden und bei Silikosekranken. *Klin. Wochschr.* **42,** 786–788 (1964).

B4. Bekhor, I. J., and Bavetta, L. A., Studies on determination of hydroxyproline. *Anal. Chem.* **33,** 1807 (1961).

B5. Bell, N. H., Colwell, J. A., Saval, F., del Greco, F., and Casey, D. E., Effects of calcium infusion on urinary hydroxyproline and phosphorus: Evaluation as a diagnostic test. *J. Clin. Endocrinol. Metab.* **26,** 677–682 (1966).

B6. Benoit, F. L., Theil, G. B., and Watten, R. H., Hydroxyproline excretion in endocrine disease. *Metab. Clin. Exptl.* **12,** 1072–1082 (1963).

B7. Benoit, F. L., Theil, G. B., and Watten, R. H., Evidence for renal tubular secretion of hydroxyproline peptides. *Clin. Res.* **12,** 266 (1964).

B8 Bentzel, C. J., Carbone, P. P., and Rosenberg, L., The effect of prednisone on. calcium metabolism and Ca^{47} kinetics in patients with multiple myeloma and hypercalcemia. *J. Clin. Invest.* **43,** 2132–2145 (1964).

B9. Bergman, M., and Loxley, R., Two improved and simplified methods for the spectrophotometric determination of hydroxyproline. *Anal. Chem.* **35,** 1961–1965 (1963).

B10. Bergmann, M., Complex salts of amino acids and peptides. II. Determination of *l*-proline with the aid of rhodanilic acid. The structure of gelatin. *J. Biol. Chem.* **110,** 471–479 (1935).

B11. Bertelsen, S., Hexosamine, hydroxyproline and calcium levels in the tunica media of the human aorta and pulmonary artery. *Acta Pharmacol. Toxicol.* **18,** 359–369 (1961).

B12. Blau, K., and Darbre, A., Gas chromatography of volatile amino acid derivatives. II. Leucine, cysteine, proline, hydroxyproline, methionine, phenylalanine, aspartic acid and glutamic acid. *J. Chromatog.* **17,** 445–449 (1965).

B13. Blomfield, J., and Farrar, J. F., Factors affecting the determination of hydroxyproline. *Anal. Chem.* **36,** 950–952 (1964).

B14. Bocci, F., and Forconi, S., Idrossiprolinuria in gravidanza. *Atti Accad. Fisiocrit. Siena, Sez. Med. Fis.* [13] **12,** 1319–1325 (1963).

B15. Bonadonna, G., Sonenberg, M., and Merlino, M. J., Total urinary hydroxyproline excretion in diabetics before and after hypophysectomy and after growth hormone in adult hypopituitarism. *Metab., Clin. Exptl.* **14,** 832–835 (1965).

B16. Bonadonna, G., Merlino, M. J., Myers, W. P. L., and Sonenberg, M., Urinary hydroxyproline and calcium metabolism in patients with cancer. *New Engl. J. Med.* **275,** 298–305 (1966).

B17. Bourrillon, R., and Vernay, J. L., Deux glycopeptides a hydroxyproline dans l'urine humaine normale. *Biochim. Biophys. Acta* **117,** 319–330 (1966).

B18. Bowes, J. H., Hydroxyproline as a measure of hide substance. *J. Soc. Leather Trades Chemists* **43,** 203–211 (1959).

B19. Bright, N. H., Effect of diphenylhydantoin on proline and hydroxyproline excretion in the rat. *Proc. Soc. Exptl. Biol. Med.* **120,** 463–465 (1965).

B20. Brunish, R., and Sørensen, B., Urinary excretion of acid mucopolysaccharides and hydroxyproline in psoriasis. *Dermatologica* **130,** 165–172 (1965).
C1. Cebecauer, L., and Trnavský, K., The study of collagen metabolism in the human body. *Vnitrni Lekar.* **12,** 339–344 (1966).
C2. Cheek, D. B., Powell, G. K., and Scott, R. E., Growth of muscle mass and skeletal collagen in the rat. I. Normal growth. *Bull. Johns Hopkins Hosp.* **116,** 378–387 (1965).
C3. Cheek, D. B., Powell, G. K., and Scott, R. E., Growth of muscle mass and skeletal collagen in the rat. II. The effect of ablation of the pituitary, thyroid or testes. *Bull. Johns Hopkins Hosp.* **116,** 387–395 (1965).
C4. Cherian, M. G., and Radhakrishnan, A. N., A new glycopeptide containing hydroxyproline and sarcosine in human urine. *Biochim. Biophys. Acta* **101,** 241–244 (1965).
C5. Cherian, M. G., and Radhakrishnan, A. N., Isolation and characterization of a new glycopeptide containing hydroxyproline from human urine. *Indian J. Biochem.* **3,** 101–105 (1966).
C6. Chiumello, G., and del Guercio, M. J., Modificazioni della idrossiprolinuria nel trattamento dell'ipotiroidismo e del nanismo ipopituitarico. *Minerva Pediat.* **17,** 1069–1073 (1965).
C7. Chiumello, G., Vaccari, A., and Sereni, F., Bone growth and metabolic studies of premature infants treated with human growth hormone. *Pediatrics* **36,** 836–842 (1965).
C8. Christiaens, L., Biserte, G., Fontaine, G., and Farriaux, J.-P., Etude de la proline et de l'hydroxyproline urinaire en pediatrie. *Ann. Pediat., Semaine Hop.* **41,** 789–797 (1965).
C9. Clausen, B., Influence of age on connective tissue. Hexosamine and hydroxyproline in human aorta, myocardium, and skin. *Lab. Invest.* **11,** 229–234 (1962).
C10. Clausen, B., Influence of age on connective tissue. Uronic acid and uronic acid-hydroxyproline ratio in human aorta, myocardium, and skin. *Lab. Invest.* **11,** 1340–1345 (1962).
C11. Crabbe, P., and Isselbacher, K. J., Urinary hydroxyproline excretion in malabsorption states. *Gastroenterology* **48,** 307–311 (1965).
C12. Cucco, A., Determination of hydroxyproline in collagen and leather. *Cuoio, Pelli, Mat. Concianti* **38,** 214 (1962); *J. Soc. Leather Trades Chemists* **47,** 238–239 (1963) (abstr.).
D1. Dahl, O., and Persson, K-A., Hydroxyproline, methodological studies of analysis. *Acta Chem. Scand.* **17,** 2499–2503 (1963).
D2. Dakin, H. D., Amino-acids of gelatin. *J. Biol. Chem.* **44,** 499–529 (1920).
D3. Danowski, T. S., Rodnan, G. P., Sarver, M. E., and Moses, C., Hydrocortisone and/or desiccated thyroid in physiologic dosage. XII. Effects of thyroid hormone excesses on urinary solutes and steroids. *Metab., Clin. Exptl.* **13,** 729–738 (1964).
D4. Darbre, A., and Blau, K., Trifluoroacetylated amino acid esters: The stability of the derivatives of cysteine, hydroxyproline, serine, threonine and tyrosine. *Biochim. Biophys. Acta* **100,** 298–300 (1965).
D5. Deasy, C., Occurrence of pyrrole-2-carboxylic acid in hide hydrolysates and its bearing on the determination of hydroxyproline. *J. Am. Leather Chemists Assoc.* **56,** 357–361 (1961).
D6. Deyl, Z., and Rosmus, I., Chromatography of collagen breakdown products. *Biokhimiya* **29,** 545–547 (1964).
D7. Dubovsky, J., Pacovsky, V., and Dubovska, E., Plasma hydroxyproline and

proline in primary hyperparathyroidism and in chronic uremia. *Clin. Chim. Acta* **12,** 230–232 (1965).

D8. Dull, T. A., Causing, L., and Henneman, P. H., Urinary total hydroxyproline as an index of connective tissue turnover in bone. *J. Clin. Invest.* **41,** 1355 (1962).

D9. Dull, T. A., and Henneman, P. H., Urinary hydroxyproline as an index of collagen turnover in bone. *New Engl. J. Med.* **268,** 132–134 (1963).

D10. Dunn, M. S., and Murphy, E. A., Chromatographic purity of amino acids. *Anal. Chem.* **33,** 997–998 (1961).

E1. Editorial, Iminoacidopathies. *New Engl. J. Med.* **272,** 1349–1350 (1965).

E2. Efron, M. L., Bixby, E. M., and Pryles, C. V., Hydroxyprolinemia. II. Rare metabolic disease due to deficiency of the enzyme "hydroxyproline oxidase." *New Engl. J. Med.* **272,** 1299–1309 (1965).

E3. Estes, F. L., and Blocker, T. G., Amino acid excretion after thermal burn. *Texas Rept. Biol. Med.* **24,** 54–66 (1966).

F1. Faglia, G., and Norbiato, G., Metabolismo del collagene ed idrossiprolinuria mella sclerodermia. *Atti Acad. Med. Lombarda* **20,** 278–280 (1965).

F2. Faglia, G., and Norbiato, G., Significato della valutazione dell'idrossiprolina urinaria in alcune osteopatie. *Atti Acad. Med. Lombarda* **20,** 286–289 (1966).

F3. Fels, I. G., Determination of hydroxyproline in liver. *Clin. Chem.* **4,** 62–65 (1958).

F4. Filliat, M., Brunet, M., Cotte, J., Romagny, G., Rebouillat, J., Site, J., and Gilly, R., Hydroxyproline urinaire chez l'enfant. Valeurs normales et pathologiques. Etude preliminaire portant sur 200 dosages. *Pediatrie* **20,** 925–931 (1965).

F5. Filliat, M., Cotte, J., and Brunet, M., Méthode de dosage de l'hydroxyproline urinaire. *Pathol. Biol., Semaine Hop.* [N.S.] **14,** 762–767 (1966).

F6. Firschein, H. E., and Shill, J. P., The determination of total hydroxyproline in urine and bone extracts. *Anal. Biochem.* **14,** 296–304 (1966).

F7. Fischer, E., Ueber eine neue Aminosäure aus Leim. *Ber. Deut. Chem. Ges.* **35,** 2660 (1902).

F8. Flanagan, B., and Nichols, G., Jr., Metabolic studies of human bone *in vitro*. II. Changes in hyperparathyroidism. *J. Clin. Invest.* **44,** 1795–1804 (1965).

F9. Fleischmajer, R., The collagen in scleroderma. *Arch. Dermatol.* **89,** 437–441 (1964).

F10. Frey, J., Etude d'une méthode d'exploration et du taux normal de l'hydroxyproline du sérum. *Biochim. Biophys. Acta* **111,** 440–446 (1965).

F11. Frey, J., Henry, J.-C., Gras, J., and Roussat, D., Etude comparée de l'hydroxyprolinémie, de la cholesterolemie et des groups basiques des protéines sériques chez le lapin soumis a un régime hypercholésterolé. *Compt. Rend.* **260,** 6720–6722 (1965).

F12. Frey, J., Hoa, N., Gras, J., Henry, J.-C., and Gudefin, Y., Immunoelectrophoresis reveals collagen solubility in human serum. *Science* **150,** 751–752 (1965).

F13. Frey, J., The value of the determination of serum hydroxyproline and urinary excretion hydroxyproline in the study of disturbances of collagen metabolism. *J. Med. Lyon* **45,** 1547–1556 (1964).

G1. Gennari, C., Rubegni, M., Forconi, S., and Romano, S., Coorelazione tra idrossiprolinuria, fosfatasemia alcalina e dinamica del ^{45}Ca. *Boll. Soc. Ital. Biol. Sper.* **41,** 508–512 (1965).

G2. Georgescu, C., Contributii la studiul tulburarilor metabolismului aminoacidic in poliartrita rheumatoida. *Med. Interna* **17,** 1491–1498 (1965).

G3. Goidanich, I. F., Lenzi, L., and Silva, E., Urinary hydroxyproline excretion in normal subjects and in patients affected with primary diseases of bone. *Clin. Chim. Acta* **11,** 35–38 (1965).

G4. Goll, D. E., Bray, R. W., and Hoekstra, W. G., Age-associated changes in muscle composition. The isolation and properties of a collagenous residue from bovine muscle. *J. Food Sci.* **28,** 503–509 (1963).
G5. Grant, R. A., Estimation of hydroxyproline by the AutoAnalyzer. *J. Clin. Pathol.* **17,** 685–686 (1964).
G6. Grassmann, W., Hannig, K., and Nordwig, A., Über die apolaren Bereiche des Kollagenmoleküls. *Z. Physiol. Chem.* **333,** 154–158 (1963).
G7. Grau, R., Fleischmann, O., and Schweiger, A., Determination of connective tissue (content of meat) by means of the hydroxyproline method and determination of thickening substances. *Fleischwirtschaft* **15,** 295–297 (1963); *Chem. Abstr.* **59,** 12089 (1963).
G8. Gray, D. O., Blake, J., Brown, D. H., and Fowden, L., Chromatographic separation of amino acid isomers. *J. Chromatog.* **13,** 276–277 (1964).
G9. Grunbaum, B. W., and Glick, D., Studies in histochemistry. XLV. Determination of hydroxyproline in microgram amounts of tissue. *Arch. Biochem. Biophys.* **65,** 260–267 (1956).
G10. Gruson, M., Ryckewaert, A., Hioco, D., Lanham, C., and De Seze, S., Hydroxyproline excretion and bone metastases. *Rev. Rhumat.* **33,** 23–29 (1966).
G11. Gruson, M., Ryckewaert, A., Hioco, D., Lanham, C., and De Seze, S., L'hydroxyprolinurie au cours des cancers secondaires des os. *Pathol. Biol. Semaine Hop.* [N.S.] **14,** 210–215 (1966).
G12. Guest, G. H., and McFarlane, W. D., A study of the colorimetric determination of pyrrole with isatin and the application of the method to biological materials. *Can. J. Res.* **B17,** 133–138 (1939).
G13. Guest, G. H., A note on the colorimetric determination of proline. *Can. J. Res.* **B17,** 143–144 (1939) (abstr.).
H1. Hamilton, P. B., and Ortiz, P. J., Proline and hydroxyproline: Determination of the sum of their α-nitrogen. *J. Biol. Chem.* **187,** 733–742 (1950).
H2. Harding, J. J., The amino acid composition of human collagens from adult dura mater and post-menopausal uterus. *Biochem. J.* **86,** 574–576 (1963).
H3. Harding, J. J., The unusual links and cross-links of collagen. *Advan. Protein Chem.* **20,** 109–190 (1965).
H4. Harrington, W. F., and von Hippel, P. H., The structure of collagen and gelatin. *Advan. Protein Chem.* **16,** 1–138 (1961).
H5. Harris, E. D., Jr., and Sjoerdsma, A., Effect of parathyroid extract on collagen metabolism. *J. Clin. Endocrinol. Metab.* **26,** 358–359 (1966).
H6. Harris, E. D., Jr., and Sjoerdsma, A., Collagen profile in various clinical conditions. *Lancet* **II,** 707–711 (1966).
H7. Hartmann, F., Die Hydroxyprolin aus scheidung in Urin von Normalpersonen und Patientin mit rheumatischen Erkrankungen. *Klin. Wochschr.* **44,** 1053–1058 (1966).
H8. Hosley, H. F., and Taft, E., Hydroxyproline excretion in cancer. *Proc. Am. Assoc. Cancer Res.* **6,** 30 (1965) (abstr.).
H9. Hosley, H. F., Taft, E. G., Olson, K. B., Gates, S., and Beebe, R. T., Hydroxyproline excretion in malignant neoplastic disease. *Arch. Internal Med.* **118,** 565–571 (1966).
H10. Houck, J. C., and Patel, Y. M., Proposed mode of action of corticosteroids on the connective tissue. *Nature* **206,** 158–160 (1965).
H11. Hruza, Z., and Hlavackova, V., Age changes in hydroxyproline excretion following stress. *Gerontologia* **11,** 76–81 (1965).

H12. Hurych, J., and Chvapil, M., Present day problems of collagen research. V. Methods of hydroxyproline determination (in Czech). *Kozarstvi* **12,** 317–323 (1962).

H13. Hurych, J., and Chvapil, M., The role of free hydroxyproline in the biosynthesis of collagen. *Biochim. Biophys. Acta* **107,** 91–96 (1965).

H14. Hutterer, F., and Singer, E. J., A modified method for hydroxyproline determination. *Anal. Chem.* **32,** 556–558 (1960).

I1. Iachan, A., Roitman, R., and Perrone, J. C., The determination of proline and hydroxyproline. *Anais Acad. Brasil. Cienc.* **31,** 53–55 (1959).

J1. Jackson, S. H., The urinary excretion of "free" and "peptide" amino acids by a patient with thermal burns. *Clin. Chim. Acta* **12,** 389–393 (1965).

J2. Jagenberg, O. R., The urinary excretion of free amino acids and other amino compounds by the human. *Scand. J. Clin. Lab. Invest.* **11,** Suppl. 43, 1–183, (1959).

J3. Jasin, H. E., Fink, C. W., Wise, W., and Ziff, M., Relationship between urinary hydroxyproline and growth. *J. Clin. Invest.* **41,** 1928–1935 (1962).

J4. Jasin, H. E., and Ziff, M., Relationship between soluble collagen and urinary hydroxyproline in the lathyritic rat. *Proc. Soc. Exptl. Biol. Med.* **110,** 837–841 (1962).

J5. Job, J.-C., Sizonenko, P., Pakey, J.-P., Pakey, P., Bourdon, R., and Rossier, A., L'hydroxyproline urinaire chez l'enfant normal et dans les retards de croissance. *Arch. Franc. Pediat.* **23,** 679–691 (1966).

J6. Johnston, C. C., Jr., Hydroxyprolinuria and the osseous effects of parathyroid hormone. *J. Lab. Clin. Med.* **64,** 873 (1964) (abstr.).

J7. Johnston, C. C., Jr., and Deiss, W. P., Jr., Parathyroid hormone and urinary hydroxyproline. *Metab., Clin. Exptl.* **14,** 523–529 (1965).

J8. Johnston, N. G., Lee, C. A., and Lloyd, H. M., Urinary hydroxyproline and the bone in primary and secondary hyperparathyroidism. *Metab., Clin. Exptl.* **15,** 1084–1091 (1966).

J9. Jones, C. R., Bergman, M. W., Kittner, P. J., and Pigman, W. W., Urinary hydroxyproline excretion in normal children and adolescents. *Proc. Soc. Exptl. Biol. Med.* **115,** 85–87 (1964).

J10. Jones, C. R., Bergman, M. W., Kittner, P. J., and Pigman, W. W., Urinary hydroxyproline excretion in Marfan's syndrome as compared with age matched controls. *Proc. Soc. Exptl. Biol. Med.* **116,** 931–933 (1964).

J11. Juva, K., and Prockop, D. J., Modified procedure for the assay of H^3- or C^{14}-labeled hydroxyproline. *Anal. Biochem.* **15,** 77–83 (1966).

K1. Kamynina, R. F., and Poverennyi, A. M., Urinary hydroxyproline and acid polysaccharide in rheumatic children. *Vopr. Revmatizma* **1,** 17–20 (1966).

K2. Kanabrocki, E. L., Fels, I. G., and Kaplan, E., Calcium, cholesterol, and collagen levels in human aortas. *J. Gerontol.* **15,** 383–387 (1960).

K3. Katz, F. H., and Kappas, A., The effect of natural estrogens on hydroxyproline excretion. *J. Clin. Invest.* **44,** 1063 (1965).

K4. Katz, F. H., and Kappas, A., The effect of natural estrogens on hydroxyproline excretion in man. *Collagen Currents* **6,** 88–89 (1965).

K5. Keiser, H., LeRoy, E. C., Udenfriend, S., and Sjoerdsma, A., Collagen-like protein in human plasma. *Science* **142,** 1678–1679 (1963).

K6. Keiser, H. R., Gill, J. G., Jr., Sjoerdsma, A., and Bartter, F. C., Relation between urinary hydroxyproline and parathyroid function. *J. Clin. Invest.* **43,** 1073–1082 (1964).

K7. Kibrick, A. C., Hashiro, C. Q., and Safier, L. B., Hydroxyproline peptides of urine in arthritic patients and controls on a collagen-free diet. *Proc. Soc. Exptl. Biol. Med.* **109,** 473–478 (1962).

K8. Kibrick, A. C., Hashiro, C. Q., Schutz, R. S., Walters, M., and Milhorat, A. T., Prolylhydroxyproline in urine: Its determination and observations in muscular dystrophy. *Clin. Chim. Acta* **10,** 344–351 (1964).

K9. Kibrick, A. C., Hashiro, C., Walters, M., and Milhorat, A. T., Hydroxyproline excretion in urine of patients with muscular dystrophy and other muscle diseases. *Proc. Soc. Exptl. Biol. Med.* **115,** 662–665 (1964).

K10. Kibrick, A. C., Kitagawa, G., Maskaleris, M. L., Gaines, R., Jr., and Milhorat, A. T., Hydroxyproline in human blood: Forms in which it is present. *Proc. Soc. Exptl. Biol. Med.* **119,** 622–625 (1965).

K11. Kivirikko, K. I., and Liesmaa, M., Effect of cortisone and age on the hydroxyproline and proline concentrations of blood and urine in the rat. *Acta Endocrinol.* **27,** 441–445 (1958).

K12. Kivirikko, K. I., Koivusalo, M., and Koivusalo, P., Free and bound hydroxyproline in the human amniotic fluid in early and full-term pregnancy. *Ann. Chir. Gynaecol. Fenniae* **52,** 356–360 (1963).

K13. Kivirikko, K. I., Koivusalo, M., Laitinen, O., and Liesmaa, M., Effect of thyroxine on the hydroxyproline in rat urine and skin. *Acta Physiol. Scand.* **57,** 462–467 (1963).

K14. Kivirikko, K. I., Koivusalo, M., Laitinen, O., and Lamberg, B.-A., Hydroxyproline in the serum and urine of patients with hyperthyroidism. *J. Clin. Endocrinol. Metab.* **24,** 222–223 (1964).

K15. Kivirikko, K. I., and Laitinen, O., Urinary ^{14}C-hydroxyproline in thyroxine-treated rats after the administration of ^{14}C-proline. *Acta Chem. Scand.* **19,** 1781–1782 (1965).

K16. Kivirikko, K. I., and Laitinen, O., Clinical significance of urinary hydroxyproline determinations in children. *Ann. Paediat. Fenniae* **11,** 148–153 (1965).

K17. Kivirikko, K. I., and Laitinen, O., Effect of cortisone on the hydroxyproline in the serum and urine of young rats. *Acta Physiol. Scand.* **64,** 356–360 (1965).

K18. Kivirikko, K. I., Laitinen, O., Aer, J., and Halme, J., Studies with ^{14}C-proline on the action of cortisone on the metabolism of collagen in the rat. *Biochem. Pharmacol.* **14,** 1445–1451 (1965).

K19. Kivirikko, K. I., Laitinen, O., and Lamberg, B.-A., Value of urine and serum hydroxyproline in the diagnosis of thyroid disease. *J. Clin. Endocrinol. Metab.* **25,** 1347–1352 (1965).

K20. Klein, L., Albertsen, K., and Curtiss, P. H., Jr., Urinary hydroxyproline in hyperparathyroidism: A study of three cases with and without bone lesions. *Metab., Clin. Exptl.* **11,** 1023–1027 (1962).

K21. Klein, L., Curtiss, P. H., Jr., and Davis, J. H., Collagen breakdown in thermal burns. *Orthopedic Surg., Surg. Forum* **13,** 349–361 (1962).

K22. Klein, L., Urinary hydroxyproline during late pregnancy and postpartum involution. *Metab., Clin. Exptl.* **13,** 386–390 (1964).

K23. Klein, L., Urinary hydroxyproline and calcium infusion studies in hyperparathyroidism. *Clin. Res.* **12,** 353 (1964).

K24. Klein, L., Curtiss, P. H., Jr., and Campbell-Jacobs, R., Urinary hydroxyproline, calcium, and serum alkaline phosphatase levels following fracture in man. *J. Bone Joint Surg.* **46A,** 919 (1964) (abstr.).

K25. Klein, L., and Davis, J. H., Correlation of urinary hydroxyproline to severity of burn. *Surg. Forum, Proc.* **15,** 465–467 (1964).

K26. Klein, L., Lafferty, F. W., Pearson, O. H., and Curtiss, P. H., Jr., Correlation of urinary hydroxyproline, serum alkaline phosphatase and skeletal calcium turnover. *Metab., Clin. Exptl.* **13,** 272–284 (1964).

K27. Klein, L., and van den Noort, S., Urinary hydroxyproline and serum alkaline phosphatase in acute paraplegia. *Clin. Res.* **13,** 327 (1965).

K28. Klein, L., and Weiss, P. H., Induced connective tissue metabolism in vivo: Reutilization of pre-existing collagen. *Proc. Natl. Acad. Sci. U.S.* **56,** 277–284 (1966).

K29. Knorring, J., von, and Wegelius, O., Kardiosklerosens invirkan pa myokardiets halt av hydroxiprolin och hexosamin. *Finska Lakaresallskap. Handl.* **108,** 150–155 (1964).

K30. Ko, C.-T., An improved method for the photometric determination of hydroxyproline. *Sheng Wu Hua Hsueh Yu Sheng Wu Wu Li Hsueh Pao* **4,** 476–480 (1964).

K31. Kocher, P. P., Vuille, R., Rovarino, D., and Courvoisier, B., Etude de l'hydroxyprolinurie dans les ostéopathies. *Helv. Med. Acta* **32,** 480–486 (1965).

K32. Koevoet, A. L., The determination of hydroxyproline in urine. *Clin. Chim. Acta* **12,** 232–234 (1965).

K33. Kohler, H. F., and Pechet, M. M., The inhibition of bone resorption by thyrocalcitonin. *J. Clin. Invest.* **45,** 1033 (1966).

K34. Korting, G. W., and Holzmann, H., Zur Hydroxyprolin-Ausscheidung im Harn von Hautkranken. *Klin. Wochschr.* **43,** 361–364 (1965).

K35. Kowalewski, K., Serum hexosamine and hydroxyproline in pullets fed atherogenic diet. *Arch. Intern. Physiol. Biochim.* **70,** 631–635 (1962).

K36. Kowalewski, K., Effect of thiouracil and/or thyroxine on hexosamine and hydroxyproline in blood and tissues of rats. *Arch. Intern. Physiol. Biochim.* **72,** 632–638 (1964).

K37. Kowalewski, K., Effect of growth hormone and of an anabolic steroid on excretion of hydroxyproline in the urine of rats. *Acta Endocrinol.* **50,** 321–328 (1965).

K38. Kowlessar, O. D., Haeffner, L. J., and Bronstein, H. D., Evidence for aminoaciduria and peptiduria in adult celiac disease. *J. Clin. Invest.* **43,** 1274 (1964).

L1. Laitinen, O., Nikkila, E. A., and Kivirikko, K. I., Hydroxyproline in the serum and urine. Normal values and clinical significance. *Acta Med. Scand.* **179,** 275–284 (1966).

L2. Lampitt, L. H., Baker, L. C., and Brown, K. P., Connective tissue of meat. I. Separation and determination. *J. Sci. Food Agr.* **3,** 367–378 (1952).

L3. Lang, K., Eine Mikromethode zur Bestimmung des Prolins und des Oxyprolins. *Z. Physiol. Chem.* **219,** 148–154 (1933).

L4. Leach, A. A., Notes on a modification of the Neuman and Logan method for the determination of the hydroxyproline. *Biochem. J.* **74,** 70–71 (1960).

L5. Leach, A. A., The determination of gelatin in blood and urine. *Anal. Biochem.* **2,** 529–531 (1961).

L6. Lee, C. A., and Lloyd, H. M., Urinary hydroxyproline in diseases involving bone and calcium metabolism. *Med. J. Australia* **1,** 992–995 (1964).

L7. Le Jeune, E., Ruitton, P., Noel, G., Volle, L., Bouvier, M., Bertrand, J. N., and Graber-Duvernay, B., Hydroxyproline excretion in rheumatic diseases. *Rev. Rhumat.* **33,** 30–38 (1966).

L8. Lenzi, F., Ravenni, G., Rubegni, M., and Del Giovane, L., The significance of urinary hydroxyproline in osteoporosis. *Panminerva Med. (English Ed.)* **5,** 155–158, (1963).

L9. Le Roy, E. C., Kaplan, A., Udenfriend, S., and Sjoerdsma, A., A hydroxyproline-containing, collagen-like protein in plasma and a procedure for its assay. *J. Biol. Chem.* **239,** 3350–3356 (1964).

L10. LeRoy, E. C., and Sjoerdsma, A., Clinical significance of a hydroxyproline-containing protein in human plasma. *J. Clin. Invest.* **44,** 914–919 (1965).
L11. LeRoy, E. C., Carbone, P. P., and Sjoerdsma, A., Elevated plasma levels of a hydroxyproline-containing protein in Hodgkin's disease and their relation to disease activity. *J. Lab. Clin. Med.* **67,** 891–897 (1966).
L12. LeRoy, E. C., Harris, E. D., and Sjoerdsma, A., A modified procedure for radioactive hydroxyproline assay in urine and tissues after labeled proline administration. *Anal. Biochem.* **17,** 377–382 (1966).
L13. LeRoy, E. C., and Wolff, S. M., Variations in plasma levels of a hydroxyproline-containing protein with fever and inflammation in man. *Arthritis Rheumat.* **9,** 281–287 (1966).
L14. Leuchs, H., Synthese von Oxy-pyrrolidin-carbon-säuren (Oxy-prolinen). *Ber. Deut. Chem. Ges.* **38,** 1937 (1905).
L15. Leuchs, H., and Brewster, J. F., Die Synthese des natürlichen, aus Eiweissstoffen gewonnenen Oxy-prolins. (Über Pyrrolidin-Abkömmlinge IV.) *Ber. Deut. Chem. Ges.* **46,** 986 (1913).
L16. Leuchs, H., and Bormann, K., Darstellung der drei mit dem natürlichen Oxyprolin stereoisomeren Formen. (Über Pyrrolidin-Abkömmlinge, V.) *Ber. Deut. Chem. Ges.* **52,** 2086 (1919).
L17. Lidsky, M. D., and Sharp, J. T., A distinctive hydroxylysine: hydroxyproline ratio in glomeruli. *Arthritis Rheumat.* **8,** 453 (1965).
L18. Lindstedt, S., and Prockop, D. J., Isotopic studies on urinary hydroxyproline as evidence for rapidly catabolized forms of collagen in the young rat. *J. Biol. Chem.* **236,** 1399–1403 (1961).
L19. Lollar, R. M., Hydroxyproline analysis as a tool for tannery chemists. *J. Am. Leather Chemists' Assoc.* **53,** 2–23 (1958).
M1. McDonald, J. E., Mamola, N., and Henneman, P. H., Urinary hydroxyproline as an index of bone resorption. *Clin. Res.* **13,** 329 (1965) (abstr.).
M2. McFarlane, W. D., and Guest, G. H., A new colorimetric method for the determination of hydroxyproline and its application to gelatin hydrolyzates. *Can. J. Res.* **B17,** 139–142 (1939).
M3. Mandema, E., van der Schaaf, P. C., and Huisman, T. H. J., Investigation on the amino acid composition of a macroglobulin and a cryoglobulin. *J. Lab. Clin. Med.* **45,** 261–269 (1955).
M4. Marcucci, F., and Mussini, E., Separation of proline and hydroxyproline derivatives by thin-layer chromatography. *J. Chromatog.* **18,** 431–432 (1965).
M5. Martin, C. J., and Axelrod, A. E., A modified method for determination of hydroxyproline. *Proc. Soc. Exptl. Biol. Med.* **83,** 461–462 (1953).
M6. Martin, G. R., Mergenhagen, S. E., and Prockop, D. J., Influence of scurvy and lathyrism (odoratism) on hydroxyproline excretion. *Nature* **191,** 1008–1009 (1961).
M7. Martin, T. J., Robinson, C. J., and MacIntyre, I., The mode of action of thyrocalcitonin. *Lancet* **I,** 900–902 (1966).
M8. Maskaleris, M. L., Sevendal, E. S., and Kibrick, A. C., Carbobenzoxy derivatives of amino acids and peptides: Instant thin-layer chromatography as hydrobromides. *J. Chromatog.* **23,** 403–409 (1966).
M9. Massaro, D., Handler, A. E., Katz, S., and Young, R. C., Jr., Excretion of hydroxyproline in patients with sarcoidosis. *Am. Rev. Respirat. Diseases* **93,** 929–933 (1966).
M10. Matiasevic, D., and Gershberg, H., Studies on hydroxyproline excretion and corticosteroid-induced dwarfism: Treatment with human growth hormone. *Metab., Clin. Exptl.* **15,** 720–729 (1966).

M11. Mechanic, G., Skupp, S. J., Safier, L. B., and Kibrick, A. C., Isolation of two peptides containing hydroxyproline from urine of a patient with rheumatoid arthritis. *Arch. Biochem. Biophys.* **86,** 71–76 (1960).

M12. Meilman, E., Urivetzky, M. M., and Rapoport, C. M., Urinary hydroxyproline peptides. *J. Clin. Invest.* **42,** 40–50 (1963).

M13. Mikkonen, L., Lampiaho, K., and Kulonen, E., Effect of thyroid hormones, somatotrophin, insulin and corticosteroids on synthesis of collagen in granulation tissue both in vivo and in vitro. *Acta Endocrinol.* **51,** 23–31 (1966).

M14. Milazzo, F., La determinazione della idrossiprolina urinaria in alcune affezioni ossee primitive a livello del massiccio facciale. *Arch. Ital. Otol., Rinol. Laringolo.* **77,** 45–53 (1966).

M15. Miller, E. J., and Piez, K. A., An accelerated single-column procedure for the automatic analysis of amino acids in collagen and elastin hydrolyzates. *Anal. Biochem.* **16,** 320–326 (1966).

M16. Mitoma, C., Smith, T. E., Davidson, J. D., Udenfriend, S., DaCosta, F. M., and Sjoerdsma, A., Improvements in methods for measuring hydroxyproline. *J. Lab. Clin. Med.* **53,** 970–976 (1959).

M17. Miyada, D. S., and Tappel, A. L., Colorimetric determination of hydroxyproline. *Anal. Chem.* **28,** 909–910 (1956).

M18. Moehler, K., and Antonacopoulus, N., Chemically determining the connective tissue in meat and meat preparations (in German) *Z. Lebensm.-Untersuch. -Forsch.* **106,** 425–440 (1957); *Chem. Abstr.* **52,** 6660 (1958).

M19. Montfort, I., and Perez-Tamayo, R., The muscle-collagen ratio in normal and hypertrophic human hearts. *Lab. Invest.* **11,** 463–470 (1962).

M20. Morrow, G., III, Kivirikko, K. I., and Prockop, D. J., Hydroxyprolinemia and increased excretion of free hydroxyproline in early infancy. *J. Clin. Endocrinol. Metab.* **26,** 1012–1014 (1966).

M21. Moskowitz, R. W., Klein, L., and Katz, D., Urinary hydroxyproline levels in an aged population. A study of non-osteoporotic and osteoporotic patients. *Arthritis Rheumat.* **8,** 61–68 (1965).

M22. Mühlbach, von R., Beziehungen zwischen der Harnausscheidung von Hydroxyprolin und der alkalischen Serumphosphatase beim M. Paget als Ausdruck von Veränderungen im Kollagenstoffwechsel. *Beitr. Orthopaed.* **13,** 189–196 (1966).

M23. Mühlbach, von R., Hirthe, D., and Lindenhayn, K., The elimination of bound hydroxyproline as an expression of alterations of the organic matrix of cartilage and bone. *Z. Arzneimittel Fortbild.* **60,** 597–602 (1966).

M24. Munoz, A. J., and Krane, S. M., Characterization of urinary polypeptides containing hydroxyproline from patients with Paget's disease of bone. *Arthritis Rheumat.* **9,** 868 (1966) (abstr.).

M25. Mussini, E., and Marcucci, F., Separation of prolines and hydroxyprolines by gas chromatography. *J. Chromatog.* **20,** 266–269 (1965).

M26. Myhill, D., and Jackson, D. S., Separation of proline and hydroxyproline using thin-layer chromatography. *Anal. Biochem.* **6,** 193–198 (1963).

N1. Nakagawa, M., and Tamaki, T., Urinary hydroxyproline excretion in orthopedic disease. *Nagoya J. Med. Sci.* **27,** 228–235 (1965).

N2. Nakagawa, T., Hydroxyproline in urine in orthopedic diseases (in Japanese). *J. Japan. Orthoped. Assoc.* **38,** 79–81 (1964).

N3. Nellas, C. L., Crawford, N., and Scherbel, A. L., Pancreatic collagenase therapy for severe, progressive systemic sclerosis. Effect on skin and on hydroxyproline content in urine. *Clin. Pharmacol. Therap.* **6,** 367–371 (1965).

N4. Neuman, R. E., and Logan, M. A., The determination of hydroxyproline. *J. Biol. Chem.* **184,** 299–306 (1950).
N5. Ney, R. L., Au, W. Y. W., Kelly, G., Radde, I., and Bartter, F. C., Actions of parathyroid hormone in the vitamin D-deficient dog. *J. Clin. Invest.* **44,** 2003–2009 (1965).
N6. Ney, R. L., Gill, J. R., Jr., Keiser, H. R., and Bartter, F. C., Regulation of hydroxyproline excretion in hyperparathyroidism. *J. Clin. Endocrinol. Metab.* **26,** 815–820 (1966).
N7. Nicholls, C. H., Makisumi, S., and Saroff, H. A., Gas chromatography of the methyl esters of the amino acids as the free base and by dissociation of their acid salts. *J. Chromatog.* **11,** 327–330 (1963).
N8. Nimni, M. E., De Guia, E., and Bavetta, L. A., Collagen, hexosamine and tensile strength of rabbit skin during aging. *J. Invest. Dermatol.* **47,** 156–158 (1966).
N9. Norbiato, G., Faglia, G., and Silvestrini, F., Induced modifications, by the human somatotropic hormone, on urinary elimination of hydroxyproline in subjects affected by dwarfism of pituitary and thyroid origin. *Metabolismo* **1,** 51–61 (1966).
O1. Oeriu, S., and Tanase, I., Séparation et détermination de la proline, de l' hydroxyproline et de la 3-hydroxyproline des produits biologiques par chromatographie sur papier. *Bull. Soc. Chim. Biol.* **47,** 979–985 (1965).
O2. Ogle, J. D., Arlinghaus, R. B., and Logan, M. A., 3-Hydroxyproline, a new amino acid of collagen. *J. Biol. Chem.* **237,** 3667 (1962).
O3. Okuda, H., Noguchi, S., Fujii, S., and Yamamura, Y., Quantitative determination of hydroxyamino acids and peptides containing hydroxyamino acids at the N-terminal. *Tokushima J. Exptl. Med.* **9,** 137–143 (1962).
O4. Onitsuka, T., A method for the determination of hydroxyproline in tissues. *Seikagaku* **32,** 857–863 (1960).
O5. Oye, I., The amount of free hydroxyproline in human blood serum. *Scand. J. Clin. Lab. Invest.* **14,** 259–261 (1962).
P1. Partridge, S. M., and Elsden, D. F., Rapid methods for the determination of glucosamine, galactosamine and hydroxyproline. *Biochem. J.* **80,** 34P (1961).
P2. Pashley, D. H., Claycomb, C. K., and Summers, G. W., Liberation of hydroxyproline from gelatin by acid hydrolysis. *Anal. Biochem.* **15,** 154–156 (1966).
P3. Peterkofsky, B., and Prockop, D. J., A method for the simultaneous measurement of the radioactivity of proline-C^{14} and hydroxyproline-C^{14} in biological materials. *Anal. Biochem.* **4,** 400–406 (1962).
P4. Picou, D., Alleyne, G. A. O., and Seakins, A., Hydroxyproline and creatinine excretion in infantile protein malnutrition. *Clin. Sci.* **29,** 517–523 (1965).
P5. Picou, D., Halliday, D., and Garrow, J. S., Total body protein, collagen and non-collagen protein in infantile protein malnutrition. *Clin. Sci.* **30,** 345–351 (1966).
P6. Piez, K. A., Irreverre, F., and Wolff, H. L., The separation and determination of cyclic imino acids. *J. Biol. Chem.* **223,** 687–697 (1956).
P7. Pil'v, I. I., and Titaev, A. A., Procedure for the determination of hydroxyproline and proline in biological liquids. I. Determination of hydroxyproline. *Lab. Delo* **9,** 22–25 (1963).
P8. Pil'v, I. I., Collagen metabolism during the active phase of rheumatic fever in children. *Pediatriya* **43,** 47–49 (1964).
P9. Platt, W. D., Doolittle, L. H., and Hartshorn, J. W. S., Urinary hydroxyproline excretion in metastatic cancer of bone. *New Engl. J. Med.* **271,** 287–290 (1964).
P10. Porretta, A., and Giannone, L., The determination of hydroxyproline in meat. *Biol. Abstr.* **43,** 9221 (1963).

P11. Prockop, D. J., and Udenfriend, S., A specific method for the analysis of hydroxyproline in tissues and urine. *Anal. Biochem.* **1,** 228–239 (1960).

P12. Prockop, D. J., and Sjoerdsma, A., Significance of urinary hydroxyproline in man. *J. Clin. Invest.* **40,** 843–849 (1961).

P13. Prockop, D. J., Udenfriend, S., and Lindstedt, S., A simple technique for measuring the specific activity of labeled hydroxyproline in biological materials. *J. Biol. Chem.* **236,** 1395–1398 (1961).

P14. Prockop, D. J., Keiser, H. R., and Sjoerdsma, A., Gastrointestinal absorption and renal excretion of hydroxyproline peptides. *Lancet* **II,** 527–528 (1962).

P15. Prockop, D. J., Isotopic studies on collagen degradation and the urine excretion of hydroxyproline. *J. Clin. Invest.* **43,** 453–460 (1964).

R1. Radhakrishnan, A. N., and Meister, A., Conversion of hydroxyproline to pyrrole-2-carboxylic acid. *J. Biol. Chem.* **226,** 559–571 (1957).

R2. Ravenni, G., Rubegni, M., and Del Giovane, L., L'idrossiprolinuria nel suggetto normale. *Boll. Soc. Ital. Biol. Sper.* **38,** 263–266 (1962).

R3. Ravenni, G., Rubegni, M., and Del Giovane, L., L'idrossiprolinuria nell' osteoporosi. *Boll. Soc. Ital. Biol. Sper.* **38,** 266–267 (1962).

R4. Ravenni, G., Rubegni, M., Gennari, C., and Forconi, S., L'idrossiprolinuria nella sindrome di Marfan. *Boll. Soc. Ital. Biol. Sper.* **41,** 352–354 (1965).

R5. Rodnan, G. P., and Cammarata, R. J., Urinary excretion of hydroxyproline in progressive systemic sclerosis (diffuse scleroderma). *Arthritis Rheumat.* **6,** 294 (1963) (abstr.).

R6. Rogers, C. J., Kimmel, J. R., Hutchin, M. E., and Harper, H. A., A hydroxyproline method of analysis for a modified gelatin in plasma and urine. *J. Biol. Chem.* **206,** 553–559 (1954).

R7. Rosano, C. L., Enzymic method for determination of hydroxyproline. *Anal. Biochem.* **15,** 341–345 (1966).

R8. Rubegni, M., Ravenni, G., and Del Giovane, L., Aumento dell'eliminazione urinaria dell'idrossiprolina nei processi osteolitici. *Boll. Soc. Ital. Biol. Sper.* **38,** 877–878 (1962).

R9. Rubegni, M., Ravenni, G., and Del Giovane, L., L'eliminazione urinaria dell'-idrossiprolina nell'artrite reumatoide. *Boll. Soc. Ital. Biol. Sper.* **38,** 879–880 (1962).

R10. Rubegni, M., Ravenni, G., and Puccetti, F., Alterazione dell'eliminazione urinaria degli aminoacidi nell'osteoporosi. *Giorn. Gerontol.* **10,** 427–430 (1962).

R11. Rubegni, M., Gennari, C., Ravenni, G., Forconi, S., and Bencini, M., L'idrossiprolinuria nelle matlattie della tiroide. *Boll. Soc. Ital. Biol. Sper.* **41,** 355–357 (1965).

S1. Satake, K., Okuyama, T., Ohashi, M., and Shinoda, T., The spectrophotometric determination of amine, amino acid, and peptide with 2,4,6-trinitrobenzene 1-sulfonic acid. *J. Biochem. (Tokyo)* **47,** 654–660 (1960).

S2. Satwekar, K., and Radhakkrishnan, A. N., Free and bound amino acids in human urine from normal subjects and patients with tropical sprue. *Clin. Chim. Acta* **12,** 394–402 (1965).

S3. Schultz-Haudt, S. D., Aarli, J., Nilsen, A. L., and Unhjem, O., Hydroxyproline-containing glycopeptides of some human and animal tissues. *Biochim. Biophys. Acta* **101,** 292–299 (1965).

S4. Scriver, C. R., Glycyl-proline in urine of humans with bone disease. *Can. J. Phys. Pharmacol.* **42,** 357–364 (1964).

S5. Scriver, C. R., Efron, M. L., and Schafer, I. A., Renal tubular transport of proline,

hydroxyproline, and glycine in health and in familial hyperprolinemia. *J. Clin. Invest.* **43,** 374–385 (1964).

S6. Scriver, C. R., Goldbloom, R. B., and Roy, C. C., Hypophosphatemic rickets with renal hyperglycinuria, renal glucosuria, and glycyl-prolinuria. A syndrome with evidence for renal tubular secretion of phosphorus. *Pediatrics* **34,** 357–371 (1964).

S7. Scriver, C. R., and Wilson, O. H., Possible locations for a common gene product in membrane transport of imino-acids and glycine. *Nature* **202,** 92–93 (1964).

S8. Scriver, C. R., and Goldman, H., Renal tubular transport of proline, hydroxyproline, and glycine. II. Hydroxy-L-proline as substrate and as inhibitor *in vivo*. *J. Clin. Invest.* **45,** 1357–1363 (1966).

S9. Seakins, J. W. T., Peptiduria in an unusual bone disorder. *Arch. Disease Childhood* **38,** 215–219 (1963).

S10. Serafini-Cessi, F., and Cessi, C., An improved method for determination of hydroxyproline in protein hydrolyzates. *Anal. Biochem.* **8,** 527–528 (1964).

S11. Shuster, S., and Bottoms, E., Senile degeneration of skin collagen. *Clin. Sci.* **25,** 487–491 (1963).

S12. Sjoerdsma, A., Davidson, J. D., Udenfriend, S., and Mitoma, C., Increased excretion of hydroxyproline in Marfan's syndrome. *Lancet* **II,** 994–995 (1958).

S13. Sjoerdsma, A., and LeRoy, E. C., Collagen metabolites in human blood and urine. *Excerpta Med. Intern. Congr. Ser.* **82,** 78–84 (1964).

S14. Sjoerdsma, A., Udenfriend, S., Keiser, H., and LeRoy, E. C., Hydroxyproline and collagen metabolism. Clinical implications. *Ann. Internal. Med.* [N.S.] **63,** 672–694 (1965).

S15. Smiley, J. D., and Ziff, M., Urinary hydroxyproline excretion and growth. *Physiol. Rev.* **44,** 30–44 (1964).

S16. Smith, D. A., and Nordin, B. E. C., The relation between calcium balance and hydroxyproline excretion in osteoporosis. *Proc. Roy. Soc. Med.* **57,** 868–870 (1964).

S17. Smith, Q. T., and Allison, D. J., Skin and femur collagens and urinary hydroxyproline of cortisone-treated rats. *Endocrinology* **77,** 785–791 (1965).

S18. Smith, Q. T., Rukavina, J. G., and Haaland, E. M., Urinary hydroxyproline in various diseases. *Acta Dermat.-Venereol.* **45,** 44–49 (1965).

S19. Smith, Q. T., and Allison, D. J., Changes of collagen content in skin, femur and uterus of 17 β-estradiol benzoate-treated rats. *Endocrinology* **79,** 486–492 (1966).

S20. Stalder, K., Bestimmung von Hydroxyprolin im Harn. *Z. Anal. Chem.* **212,** 196–199 (1965).

S21. Stanley, E. M., Dawson, K. B., Field, E. O., and Glicksman, A. S., An isotope dilution method for determining the absolute amounts and specific activities of proline and hydroxyproline in tissues. *Intern. J. Appl. Radiation Isotopes* **17,** 467–477 (1966).

S22. Stegemann, H., Mikrobestimmung von Hydroxyprolin mit Chloramin-T und p-dimethylaminobenzaldehyd. *Z. Physiol. Chem.* **311,** 41–45 (1958).

S23. Stěpán, J., Marsikova, L., and Prokopova, D., On the problem of the interference of chloroquine with the regulatory mechanisms in man. *Med. Pharm. Exptl.* **12,** 381–387 (1965).

S24. Stetten, M. R., and Schoenheimer, G., The metabolism of *l*(—)-Proline studied with the aid of deuterium and isotopic nitrogen. *J. Biol. Chem.* **153,** 113–132 (1944).

S25. Svejcar, J., Prerovsky, I., Linhart, J., and Kruml, J., Content of collagen, elastin, and water in walls of the internal saphenous vein in man. *Circulation Res.* **11,** 296–300 (1962).

T1. Titaev, A. A., Disturbed connective tissue metabolism in rheumatic children. *Vopr. Revmatizma* **1,** 13–16 (1962).
T2. Titaev, A. A., Determination of collagen-like proteins in blood serum. *Lab. Delo* **9,** 5–7 (1963).
T3. Trnavský, K., Cebecauer, L., and Vrsanska, A., Serum hydroxyproline and hydroxyproline excretion in juvenile rheumatoid arthritis. *Z. Rheumaforsch.* **25,** 264–268 (1966).
T4. Troll, W., and Cannan, R. K., A modified photometric ninhydrin method for the analysis of amino and imino acids. *J. Biol. Chem.* **200,** 803–811 (1953).
U1. Udenfriend, S., Formation of hydroxyproline in collagen. *Science* **152,** 1335–1340 (1966).
V1. Verzar, F., Ageing of newly formed collagen. *Experientia* **18,** 473–474 (1962).
W1. Waldschmidt-Leitz, E., and Akabori, S., Bestimmung von oxyprolin. Zur method nach K. Lang. *Z. Physiol. Chem.* **224,** 187–192 (1934).
W2. Westall, R. G., The amino acids and other ampholytes of urine. 3. Unidentified substances excreted in normal human urine. *Biochem. J.* **60,** 247–255, (1955).
W3. Westfall, B. B., Peppers, E. V., Sanford, K. K., and Earle, W. R., The amino acid content of the ultrafiltrate from horse serum. *J. Natl. Cancer Inst.* **15,** 27–35 (1954).
W4. Whitehead, R. G., Hydroxyproline creatinine ratio as an index of nutritional status and rate of growth. *Lancet* **II,** 567–570 (1965).
W5. Widdowson, E. M., and Whitehead, R. G., Plasma amino-acid ratios and urinary hydroxyproline excretion in rats deficient in protein and calories. *Nature* **212,** 683–686 (1966).
W6. Wierbicki, E., and Deatherage, F. E., Hydroxyproline as an index of connective tissue in muscle. *Agr. Food Chem.* **2,** 878–882 (1954).
W7. Wiss, von O., Über eine Mikrobestimmung des Oxyprolins in Blut und Harn; Beeinflussung des Oxyprolin-gehaltes durch Verabreichung von Prolin. *Helv. Chim. Acta* **32,** 149–153 (1949).
W8. Woessner, J. F., Jr., The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. *Arch. Biochem. Biophys.* **93,** 440–447 (1961).
W9. Woessner, J. F., and Brewer, T. H., Formation and breakdown of collagen and elastin in the human uterus during pregnancy and post-partum involution. *Biochem. J.* **89,** 75–82 (1963).
Z1. Zaides, A. L., Mikhailov, A. N., and Pushenko, O. I., A modified method for hydroxyproline assay. *Biokhimiya* **29,** 5–7 (1964).
Z2. Ziff, M., Kibrick, A., Dresner, E., and Gribetz, H. J., Excretion of hydroxyproline in patients with rheumatic and non-rheumatic diseases. *J. Clin. Invest.* **35,** 579–587 (1956).
Z3. Ziff, M., The significance of urinary hydroxyproline peptide excretion. *Proc. Roy. Soc. Med.* **59,** 9–10 (1966).

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ISOENZYMES OF HUMAN ALKALINE PHOSPHATASE

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I. Introduction

By isoenzyme we refer to a biochemically distinct class of catalytically active proteins with the same specificity of bond cleavage or alteration which may occupy several zones following electrophoresis. If one cannot distinguish by biochemical means one enzyme zone from another, both catalyzing the same reaction, the two proteins are variants of the same isoenzyme.

The subject of isoenzymes[1] of human alkaline phosphatase has been a perplexing one (Fig. 1), for the reason that the enzyme bands separated by electrophoresis on starch gel have had no clear complete interpretation with regard to organ source, biochemical individuality, or genetic constitution of the individual. Particularly disturbing is the fact that a number of tissues can each yield up to three and four bands that generally merge with one another in mixtures and become unrecognizable with respect to tissue origin. In the case of alkaline phosphatase we are obligated to rely on a combination of organ source, biochemical properties, and electrophoretic distinctions as guidelines for working in this field. Thus, placental alkaline phosphatase is an isoenzyme of alkaline phosphatase because it can be distinguished from other alkaline phosphatases by biochemical means. The different molecular forms of this isoenzyme which can be produced and separated by physical means are referred to simply as variants of placental alkaline phosphatase. Moreover, the limitations of interpretation of isozyme bands in general are now being recognized even with lactic dehydrogenase, which appeared to be the least complicated enzyme species (R23, V10, V11) and has been the most intensively studied.

[1] Second Conference on "Multiple Molecular Forms of Enzymes," N. Y. Academy of Sciences, December 1–3, 1966. Interpretation of the biological significance of isozyme bands is complicated by the fact that nongenetic variants in unpredictable numbers can arise. These include isozymes produced as artifacts in the preparation of the specimen and even on the starch gel, aggregates of monomeric forms, and conformers (Kaplan) that are essentially configurational isomeric proteins. Accordingly, the need is recognized for biochemical criteria with which to identify isozymes of the same basic molecular species.

Apart from isoenzymes, hyperphosphatasemia in diseases of liver and bone has made the serum alkaline phosphatase determination the most frequently requisitioned enzyme assay for over 30 years. Obviously, it has also fixed for the great majority (C17) the expectation that liver and bone normally constitute the major sources of serum alkaline phosphatase. Uneasiness rarely is expressed in the literature regarding the patients with bone and liver disease whose enzyme values are *not* elevated, or in the cases of hyperphosphatasemia where neither liver nor bone disorders exist.

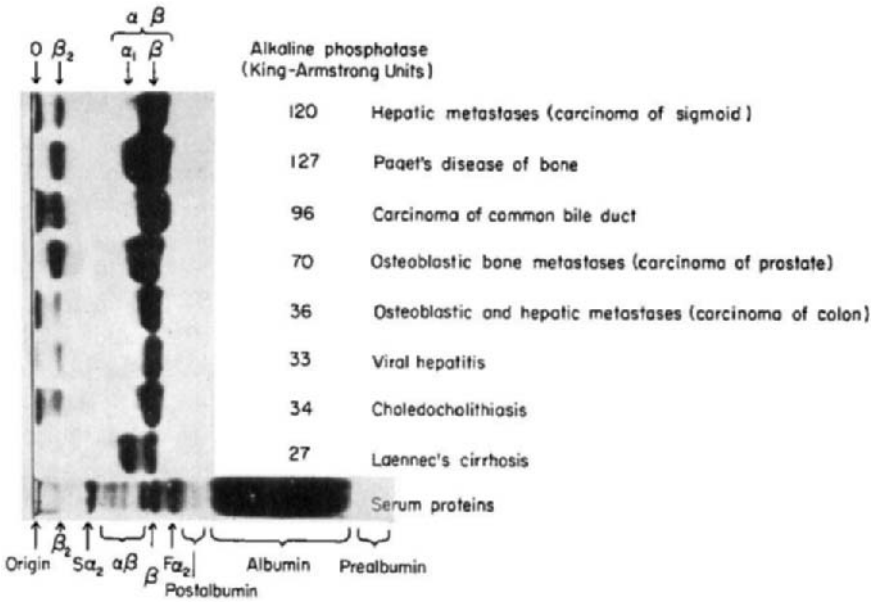


FIG. 1. Starch-gel zymogram of serum alkaline phosphatase in hepatobiliary and osteoblastic bone diseases [according to Taswell and Jeffers (T2)].

In view of this uneasiness and on the strength of experimental observations, to be described, the authors have found it necessary in this chapter to consider the question whether or not serum alkaline phosphatase in normal healthy nonpregnant individuals may be essentially a mixture of unmodified and metabolically modified intestinal alkaline phosphatases, whose relative proportions are predetermined by genetic and physiological factors. The basis of this statement and its implications constitute a major point of this chapter.

Clearly, in this volume concerned with advances in clinical chemistry, methodology of the alkaline phosphatase isoenzyme assay merits atten-

tion, and especially the automated procedures for L-phenylalanine-sensitive alkaline phosphatase (LPSAP) that are rapidly displacing manual techniques. Such isoenzyme methodology is based on conditions arrived at by biochemical studies, and therefore this review encompasses the extensive enzyme kinetic studies in the literature and in this laboratory, with particular attention to the mechanism of catalysis and uncompetitive inhibition.

Also included are analyses of studies reporting distinctive immunochemical properties as well as physical properties of enzymes prepared from several organs. Separate sections are concerned with electrophoretic data and heat sensitivity.

An understanding of the biological significance of alkaline phosphatase now requires a knowledge of ultrastructure and genetics. Tremendous advances have been made in our information of cell ultrastructure and of enzyme components of cell membranes. This knowledge is basic to a grasp of the physiological role which alkaline phosphatases may play in absorption (of fat, amino acids, and sugars), osteogenesis, and pregnancy, apparently unrelated processes which may have the same significance for the enzyme at the level of cell ultrastructure and function.

No less striking contributions have come from the field of molecular genetics, which has produced the DNA, RNA, mRNA, and tRNA sequence for explaining the genetic control of protein synthesis. In relation to human serum alkaline phosphatase, the literature now contains many references to its association with ABO blood groups. In addition, the inheritance of alkaline phosphatase in *Escherichia coli* and *Aspergillus niger,* etc., has provided phenomena more readily amenable to genetic studies.

A biochemical evalution of human alkaline phosphatase is postponed until the above considerations have been presented. In our view, the most reasonable analytical approach is based on the measurement of L-phenylalanine-sensitive and -insensitive moieties along with their respective heat stabilities. To this may be added information gathered from starch-gel electrophoresis with native and heated serum and from the presence of L-phenylalanine-sensitive bands on the gels following electrophoresis. Experiments of a different type can be included, in which the serum is incubated with neuraminidase and susceptibility of the glycoprotein is established following electrophoresis. Finally, the data on L-phenylalanine inhibition of heat-sensitive and -insensitive moieties appear to make sense, if the population of normal subjects is divided into one with the slow-moving intestinal band and one without it. It is from this consideration and other indirect and direct inferences that the intestine is

given serious consideration as the major source of human serum alkaline phosphatase in healthy individuals.

2. Methodology

Historically (D11, K1), β-glycerophosphate was the first substrate for alkaline phosphatase (B24); the liberated inorganic phosphate was measured by the classical Fiske-Subbarow procedure (F18). The introduction next of the phenyl phosphate substrate by King and Armstrong (K11) carried with it the advantages of a more rapid hydrolysis, and the opportunity to measure phenol liberation by the Folin-Ciocalteu method. More convenient colorimetric reagents for phenol followed later, which do not require deproteinization, such as Powell and Smith's amino-antipyrine–alkaline ferricyanide reagents (P22) and diazo reagent (S48). Also, other substrates have made their appearance, e.g., *p*-nitrophenyl phosphate (B16), phenolphthalein diphosphate (H22), indoxyl phosphate (T7), 4-methylumbelliferyl phosphate (F1), phenolphthalein monophosphate (B2), naphthyl phosphate (M32), and phosphoenol pyruvate (S34). Superimposed on these experiences with alkaline phosphatase assay has been automatic instrumentation, which is now commanding much attention. Consequently, the treatment of this section of the chapter will be concerned more with the evaluation of fundamentals of manual and of automated procedures than of details in technique.

Adherence to sound principles of the assay of serum enzymes in general (F5–F7) is required for both manual and automatic techniques. Thus, one should insure that the release of product that is being measured is a linear function of the amount of alkaline phosphatase. The reaction must be carried out at the optimal pH characteristic of the enzyme with that particular buffer and substrate. The period of hydrolysis and substrate concentration should be adjusted in such a way that an initial zero-order rate of hydrolysis is obtained. The standard curve should obey Lambert-Beer's law. Arbitrary and variable dilutions of high-titer sera are not recommended.

In relation to the variable of dilution, Nath and Ghosh (N7, N8) demonstrated (Table 1) that, contrary to serum acid phosphatase, alkaline phosphatase activity in native human serum is increased up to 80% when diluted with either water or buffer.

The above observations call for the adoption of a serum alkaline phosphatase assay method in which either an undiluted human serum is used or a constant dilution of sera is maintained. This is necessary to obtain comparable and reproducible results with different serum specimens. Fishman (F5, F6) avoided this difficulty by using a solution of

TABLE 1

EFFECT OF DILUTION ON ALKALINE PHOSPHATASE ACTIVITY OF HUMAN SERUM[a]

Specimen no.		1	2	3	4	5	6
Undiluted serum	$A \times 10^7$	20	23	370	122.7	37.3	34.1
Water-diluted serum[b]	$A \times 10^7$	25.2	29.7	393.6	131.4	51	53.7

[a] The numbers are derived from the first-order velocity constants of the hydrolysis of phenyl phosphate. Taken from Nath and Ghosh (N7).

[b] Serum was diluted three-fold. Here, the values are obtained by multiplying the measured activities by three.

another protein (bovine serum albumin) or heat-inactivated human sera for dilution of native high-titer sera. This is an important modification because in many diseases, like rickets, infectious hepatitis, and other hyperphosphatasemic conditions, the alkaline phosphatase level in serum or plasma is significantly enhanced to a point that exceeds the capacity of the photometer. Obviously it then becomes necessary for the accurate measurement of the total activity to dilute the native serum with a suitable diluent.

Also, before one can choose the substrate concentration for a particular method, the Michaelis constant must be determined. Usually, if the enzyme does not exhibit inhibition at high substrate concentration, it may be reasonable to use a working molar concentration of the substrate equal to 3 or 4 times the K_m value so that the enzyme can manifest its highest activity. As in the case of β-glucuronidase (F17), the conditions developed for a particular isoenzyme (of alkaline phosphatase) in one tissue may not be applicable for another isoenzyme in another tissue or for those present in serum. Hence reevaluation of every reaction condition (e.g., substrate concentration, optimum pH) is necessary for each isoenzyme. *Vide infra* 2.1.1.

2.1. MANUAL METHODS

The different methods in use may be broadly divided into two categories: one based on the measurement of released phosphate, and the other on the determination of the liberated organic moiety.

When liberated phosphate is allowed to react with ammonium molybdate at a specified concentration and pH, ammonium phosphomolybdate is formed which can be reduced by a large number of reductants to give blue-colored solutions. The nature of the reducing agents used to convert

ammonium phosphomolybdate to molybdenum blue varies from one method to another. For instance, the reductants employed in the Fiske-Subbarow technique by Bodansky (B23), Shinowara *et al.* (S30), Dryer *et al.* (D23), and Lowry-Lopez (L19) are 1,2,4-aminonaphtholsulfonic acid, stannous chloride, *N*-phenyl-*p*-phenylenediamine, and ascorbic acid, respectively. However, some of the methods mentioned use such a high concentration of acid in the ammonium molybdate reagent that acid-labile phosphates cannot be used as substrates. To circumvent this difficulty, Lowry and Lopez (L19) employed very low concentrations of acids in the ammonium molybdate and ascorbic acid mixture, an extremely sensitive color reagent. However, this method and the others did not overcome the problems of instability of the color produced and the necessity of carrying out the color reaction at a definite fixed pH. Nath and Ghosh (N7), by taking a number of colorimetric readings at 5-minute intervals and finding out the zero-time reading from the linear rate of increment of the intensity, successfully performed the Lowry-Lopez method under conditions of variable pH and despite the time-dependent intensity of the color.

Moreover, the advantage of a method involving the assay of inorganic phosphate is that it can use any organic phosphate as a substrate for alkaline phosphatase. Thus Fishman *et al.* (F11), in their search for organ-specific substrates for alkaline phosphatase, used this method, employing a wide variety of phosphates of phenol, salicylic acid, glucose, ethanolamine, serine, threonine, choline, phenolphthalein, propanediol, α- and β-naphthols, menthol, borneol, methanol, and octanol, each of them being studied with alkaline phosphatase preparations in blood, bone, intestine, lung, liver, and kidney. This method was employed to assay serum alkaline phosphatase activity with *p*-methylphenyl phosphate (N4, N13), *m*-methoxyphenyl phosphate, *m*-methylphenyl phosphate (N9, N10), 16β-methyl-9α-fluoroprednisolone phosphate (N12, N13) and alkyl phosphates like *n*-amyl and isoamyl phosphate (N3) in Nath's fundamental and clinical studies on the substrate specificity of this enzyme.

The second category of methods concerns the estimation of the liberated organic phenolic group, based either on its inherent chromogenicity, e.g., *p*-nitrophenyl phosphate (L19), phenolphthalein diphosphate (H22), and phenolphthalein monophosphate (B2), or on its conversion to a pigment product by coupling with a diazotized amine (G13, S48) or by allowing it to react with some specific reagent (K10, P22). The chromogenic substrates in the former class have the advantage that they need no other special reagent for developing the color. A disadvantage to *p*-nitrophenyl phosphate is its limited stability and the fact that

high concentrations of bilirubin and hemoglobin interfere with the accuracy of the method, as the absorption maxima of these substances are close to that of *p*-nitrophenolate. Methods for measuring alkaline phosphatase continue to appear (F26, J5, K27).

The most sensitive and reproducible manual procedure in the latter group, in which the diazo coupling reaction is utilized, is that of Fishman *et al.* (F14, S48). In this method, the liberated phenol from phenyl phosphate is coupled with 5-nitroanisole-2-diazoniumnaphthalene-1,5-disulfonate (Fast Red B salt, Hilton-Davis Chemical Co., Cincinnati, Ohio) (Fig. 2).

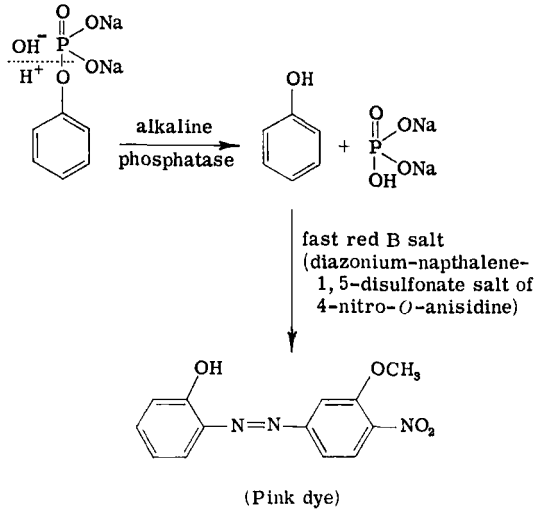


FIG. 2. Diazotization color reaction for determining phenol [according to Stolbach *et al.* (S48)].

The pink azo dye formed is completely soluble in alcoholic borate solution; the color was remarkably stable even up to 24 hours at room temperature. The method is so sensitive that a fraction of a microgram of phenol can be estimated with ease. It is very economical of enzyme, requiring only 0.1 ml of properly diluted serum or tissue preparations, and the tedious deproteinization step becomes unnecessary.

2.1.1. *Procedure for Serum, Intestinal, and Placental Alkaline Phosphatases—Substrate Phenyl Phosphate*

Buffered (50 m*M* carbonate–bicarbonate) phenyl phosphate (2.0 ml) of suitable concentration and pH (i.e., 18 m*M* at pH 9.8 with intestinal and serum alkaline phosphatase, and 72 m*M* at pH 10.7 with placental

alkaline phosphatase) was allowed to react at 37°C with 0.1 ml of the previously diluted enzyme specimen. After a definite interval of time (15 minutes) the enzyme reaction was terminated by adding 2.0 ml of 1.5 *M* formaldehyde, which eliminates all further enzyme activity. The mixture was then treated with 4 ml of saturated ethanolic borate solution and 0.5 ml of a 0.5% solution of Fast Red B salt. The orange-pink colored solution was read in a photometer at 490 mμ against a similarly prepared blank without the enzyme. From a previously established phenol calibration curve the readings were converted to μmoles of phenol, and the unit is defined as 1 μmole of phenol/ml/min.

α-Naphthyl phosphate can also be used as a substrate for the enzyme, the assay being done by coupling the released α-naphthol with the same diazo reagent used for phenol. However, because this color reaction is less sensitive for α-naphthol than for phenol, the measurement of inorganic phosphate is required as described below.

2.1.2. *Procedure for Estimation of Alkaline Phosphatase Activity with α-Naphthyl Phosphate as Substrate*

The buffered (50 m*M*, carbonate–bicarbonate) α-naphthyl hydrogen phosphate solution (3 ml), the pH (9.8 in the case of intestinal enzyme) of which was adjusted to the desired value, was treated with properly diluted enzyme solution (1 ml). The digest, which contained 5 m*M* of substrate, was incubated at 37°C for a suitable interval of time, depending on the concentration of the enzyme under investigation. The enzymatic hydrolysis was arrested by adding 1 ml of 30% trichloroacetic acid, and the mixture with the protein precipitate was then centrifuged for 15 minutes. The clear protein-free supernatant (2 ml) was treated with 0.4 ml of 0.008 *M* ammonium molybdate solution and 4 ml of *p*-semidine reagent (D23, G5). The resultant blue color of the solution was stable up to 1 hour, and its optical density was read in a Klett-Summerson photometer with a No. 69 filter against a similarly prepared blank, in which trichloroacetic acid was added prior to addition of the enzyme in the digest. The results of alkaline phosphatase activity, expressed as μmoles of phosphorus/ml/min, were obtained from a standard phosphate calibration curve prepared under the described conditions.

Since a number of methods are in widespread use, it is worthwhile to review comparative studies of alkaline phosphatase activity measured by these different methods. Schwartz *et al.* (S24) established that a linear relation exists between the results of the Bodansky and King-Armstrong methods. Laurent and Norberg (L5) also demonstrated a linear relation between values on serum performed with a modified Bessey-Lowry compared to a modified King-Armstrong method. However, Gelb *et al.*

(G4) warned of inconstancy in the ratio of Bodansky values to those of the King-Armstrong method as measured in the Technicon AutoAnalyzer (*vide infra*). Deren *et al.* (D13) made a comparative study of four different methods of determining alkaline phosphatase in sera from a variety of pathological and normal cases. They reported that the Bodansky (B23), King-Armstrong (K11), Bessey-Lowry (B16), and Klein (K16) assay methods for serum alkaline phosphatase are equally sensitive in the diagnosis of various diseases. In their opinion, however, the results of the first three methods are proportional to each other, whereas there is no reasonable conversion factor to express the relation between Klein phenolphthalein diphosphate and the other three methods.

The organic nonphosphate moiety may be fluorogenic, which would tend to increase the sensitivity of measurement (G17, M32).

2.2. Automation

It is widely agreed that automated techniques (B35, C16, K3, K15, M9, S46, T4) will be almost universally employed for serum alkaline phosphatase in the near future, especially in the routine laboratory. It should again be emphasized that, as with the manual methods, attention must be paid to the state of the serum, and to the employment of conditions which ensure a linear rate of hydrolysis and of color reactions that are both stable and sensitive. Moreover, the widely used AutoAnalyzer superimposes a continuous flow hydrodynamics that permits the enzyme reaction to result from a steady rate of provision of the substrate, buffer, and enzyme and an equally steady rate of color pigment production. The readings obtained from the continuous recording colorimeter are uninterpretable unless correlated with tracings obtained in a similar manner for both standard phenol and standard enzyme solutions and for serum in the absence of substrate.

The question of absolute chemical standards versus standardized enzyme preparations has been debated in a recent symposium.[2] The phenol curves of the AutoAnalyzer provide an absolute means of expressing the optical density of the enzyme specimen as phenol. However, it is assumed implicitly that the enzyme reaction in the AutoAnalyzer is exactly the same as in the manual method, and that the determination of phenol, in itself, is the only concern of the analyst. We contend that this assumption is not justified a priori for the reasons that (1) enzyme inactivation may occur in the time interval the specimen is located on the turntable before it enters the flow line; this would be true in the

[2] Technicon Symposium; Automation in Analytical Chemistry, New York, October 16–18, 1966.

case of a very heat-sensitive isoenzyme; (2) the mechanical forces of pumping enzyme solutions through long tubes with narrow orifices may be deleterious to fragile enzymes or isoenzymes; and (3) AutoAnalyzer incubation times are much shorter than manual methods require. Disparate findings between AutoAnalyzer and manual data on the same specimen may be explained by the presence of heat-sensitive enzymes.

Enzyme standards, whose units are established carefully by a manual method using the incubation time of the AutoAnalyzer procedure, provide a direct means of translating AutoAnalyzer optical density values into enzyme units. This latter practice avoids the introduction of unique AutoAnalyzer units with different levels of normal and standard deviation, an event to be avoided if possible.

The reader should not assume that all desirable methods for alkaline phosphatase can be easily adapted to the AutoAnalyzer. The diazo method for phenol, which possesses the greatest sensitivity, yields a pigment that attaches itself to the walls of the plastic tubing, an event that greatly disturbs the continuous flow dynamics and, in consequence, the height and smoothness of the peaks seen on colorimeter recordings. The measurement of inorganic phosphate requires protein precipitation, a process that is inefficient with the AutoAnalyzer. Also, the employment of a dialyzing cell to remove protein from the flow is only as successful as the adequacy of the dialyzing membranes and the efficiency of flow dialysis. Alkaline phosphatase is not being generally determined automatically at this time, by the measurement of inorganic phosphate.

Comment now is to be centered on the AutoAnalyzer total alkaline phosphatase method, using phenyl phosphate as substrate and the Powell and Smith aminoantipyrine–ferricyanide reagents, formulated in Fig. 3.

The liberated phenol condenses with 4-aminoantipyrine and, in the presence of oxidizing conditions (alkaline ferricyanide), gives rise to an organic product in which the phenol residue contains the chromophoric quinonoid group.

This color reaction has now provided measurements of total alkaline phosphatase and the L-phenylalanine-sensitive isoenzyme.

2.3. Differential Isoenzyme Analysis

From the biochemical point of view, there is merit in measuring the moiety of the serum alkaline phosphatase activity that is inhibited by L-phenylalanine. This moiety is henceforth referred to as LPSAP, L-phenylalanine-sensitive alkaline phosphatase. The conditions to be chosen should provide the maximum expression of LPSAP and the extent of inhibition of intestine and placenta should be as great as possible at a concentration of L-phenylalanine that does not at the same time inhibit

alkaline phosphatase of liver and bone. Data that bear on these points are now presented.

In these experiments, Inglis and Fishman found that recovery of intestinal alkaline phosphatase added to individual sera and tissue mixtures is frequently more complete when D minus L values are employed in contrast to R-total minus L values as defined in footnote [b] in Table 2.

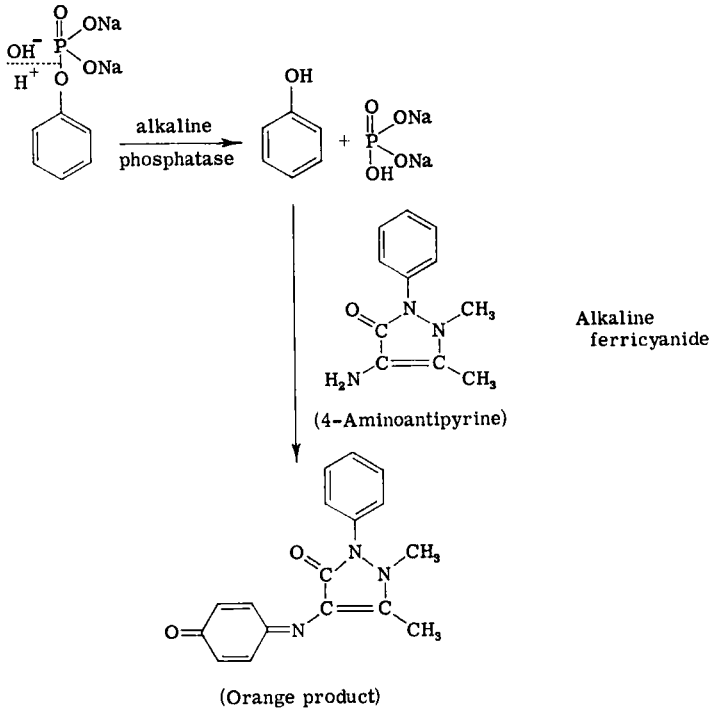


FIG. 3. Alkaline ferricyanide 4-aminoantipyrine phenol color reaction [according to Powell and Smith (P22)].

Moreover, R — L values often give negative results with sera in contrast to D — L values (Table 3), which are never negative. For these reasons, D-phenylalanine is included in the control digest whenever LPSAP is measured.

As reported earlier (F16, G8), placenta and intestine are two sources of alkaline phosphatase that are equally sensitive to L-phenylalanine. As can be expected, gastric and duodenal contents contain LPSAP as well. Preparations made so far from the following tissues contain much smaller amounts of LPSAP: liver, bone, kidney, lung, and spleen, usually of the order of 0–15% (Table 4).

Under standard conditions, using 0.018 *M* phenylphosphate and 0.005 *M*

TABLE 2
RECOVERY OF INTESTINAL ALKALINE PHOSPHATASE ADDED TO SERUM AND TO TISSUE PREPARATIONS[a]

Material	R[b]	L[b]	D[b]	R − L		D − L	
				Found	Expected	Found	Expected
Serum[c]	3.35	3.05	3.50	0.3	—	0.45	—
Mixture 1	4.65	2.65	5.50	2.00	2.45	2.85	2.62
Mixture 2	3.60	2.10	3.80	1.50	1.47	1.70	1.60
Mixture 3	2.15	1.55	2.35	0.60	0.75	0.80	0.93
Mixture 4	3.25	2.10	3.70	1.15	1.30	1.60	1.55
Tissue[d]	21.34	17.48	18.7	3.86	—	1.22	—
Mixture 5	18.80	10.40	18.98	8.40	9.23	8.58	8.12
Mixture 6	13.30	8.69	13.4	4.61	5.92	4.71	4.50
Mixture 7	15.32	11.15	14.9	4.17	5.23	3.75	3.43

[a] These values are derived from phenol liberated from phenylphosphate under conditions previously described (F14). Values are in Bodansky-Shinowara units.

[b] R (regular) is the enzyme activity in the absence of phenylalanine, L that in the presence of 0.005 *M* L-phenylalanine, and D that in the presence of 0.005 *M* D-phenylalanine.

[c] The serum was mixed with various known amounts of intestinal alkaline phosphatase in heat-inactivated serum to provide mixtures 1 to 4.

[d] Combination of liver and bone alkaline phosphatases to which various amounts of intestinal alkaline phosphatase were added to provide mixtures 5, 6, and 7.

L-phenylalanine, the percent inhibitions of human intestinal liver and bone alkaline phosphatases are 77, 8, and 10 (Table 4), respectively.

An attempt was made to find conditions in which human intestinal alkaline phosphatase would exhibit L-phenylalanine inhibition higher than 77%, by changing the substrate and inhibitor concentrations. The results in Table 5 illustrate that the employment of higher inhib-

TABLE 3
COMPARISON OF R − L AND D − L SERUM ALKALINE PHOSPHATASE VALUES

Serum specimen	Alkaline phosphatase (Bodansky-Shinowara[a] units/100 ml)				
	R	L	D	R − L	D − L
1	3.85	4.10	4.45	−0.25	0.35
2	3.60	3.60	4.25	0	0.65
3	1.80	1.80	1.90	0	0.10
4	13.60	14.10	15.60	−0.5	1.50
5	1.50	1.60	1.80	−0.1	0.20
6	2.60	2.75	3.00	−0.15	0.25
7	5.90	5.65	6.80	0.25	1.15

[a] See footnote [a] to Table 2.

TABLE 4

SENSITIVITY OF ALKALINE PHOSPHATASE OF TISSUE AND BODY FLUIDS TO L-PHENYLALANINE[a]

	Source[b]									
	Duodenal juice	Bowel juice	Placenta	Intestine	Lung	Kidney	Bone	Bile	Spleen	Liver
No. of observations	5	5	10	20	4	3	6	5	8	7
Mean L-phenylalanine inhibition (%)[c]	81	79	79	77	14.9	14.3	10.3	9.0	8.2	7.7
Standard deviation	±1.0	±1.2	±2.0	±2.8	±1.7	±4.0	±6.3	±2.4	±3.6	±1.9

[a] 5 m*M* at pH 9.3, 50 m*M* carbonate–bicarbonate buffer, with 18 m*M* phenylphosphate as substrate. Control digest contains 5 m*M* D-phenylalanine.

[b] Individual values for two specimens of stomach were 14 and 13%, and of pancreas were 9 and 12%.

[c] $\frac{D - L}{D} \times 100$.

TABLE 5
L-PHENYLALANINE INHIBITION OF ALKALINE PHOSPHATASE PREPARATIONS OF HUMAN INTESTINE, BONE, AND LIVER[a]

	Tissues					
	Intestine		Bone		Liver	
Number	% Inhibition	Mean with SD	% Inhibition	Mean with SD	% Inhibition	Mean with SD
(1)	91	91 ± 1.7	13	18 ± 5.6	17	16 ± 3.1
(2)	91		16		18	
(3)	89		21		15	
(4)	89		12		12	
(5)	91		24		13	
(6)	91		25		14	
(7)	—		10		19	
(8)	—		21		12	
(9)	—		22		12	

[a] Using 2.5 m*M* phenyl phosphate and 7 m*M* inhibitor at pH 9.3 (50 m*M* carbonate-bicarbonate buffer).

itor concentration (0.007 *M*) and lower phenyl phosphate concentration (0.0025 *M*) resulted in 91% inhibition of the intestine, but under the new conditions the bone and liver enzymes underwent inhibition to a greater extent (18 and 16%, respectively) than in Table 4. L-Phenylalanine concentrations greater than 0.005 *M* had no additional advantage in studies on differential organ inhibition.

Contrast between the high LPSAP of placenta and intestine versus the slight LPSAP of liver and bone is marked in analytical studies with the AutoAnalyzer (Fig. 4).

The standard enzyme preparation for measurement of LPSAP is a highly-purified specimen of human placental alkaline phosphatase made according to Ghosh *et al.* (G8). It is introduced as a standard in every tenth sample cup, after having been diluted in pooled heat-inactivated serum. The extent of inhibition is 77 ± 2.0% with the 40 samples/hour rate, and this value agrees with the manual results. Moreover, the type of inhibition for both placental and intestinal sources is uncompetitive (*vide infra*). The results of the AutoAnalyzer are expressed in KA (King-Armstrong) units, and of the manual method in Bodansky-Shinowara units as defined previously (F14). The two are related in linear fashion in Fig. 5, which yields the conversion factor of KA AutoAnalyzer units to Bodansky-Shinowara units of 3.0.

The phenomenon of L-phenylalanine inhibition will be considered at

length in a later section. At this juncture, it is sufficient to state that the alkaline phosphatase activity in the presence of the noninhibitor D-phenylalanine is accepted as representing total activity. The activity measured in the presence of the inhibitor L-phenylalanine yields the uninhibited moiety, the difference between the results with D- and L-phenylalanines representing L-phenylalanine-sensitive alkaline phosphatase (LPSAP). Occasional sera may show a slight activation by D-phenylalanine. According to our experience (F7), this is attributable to

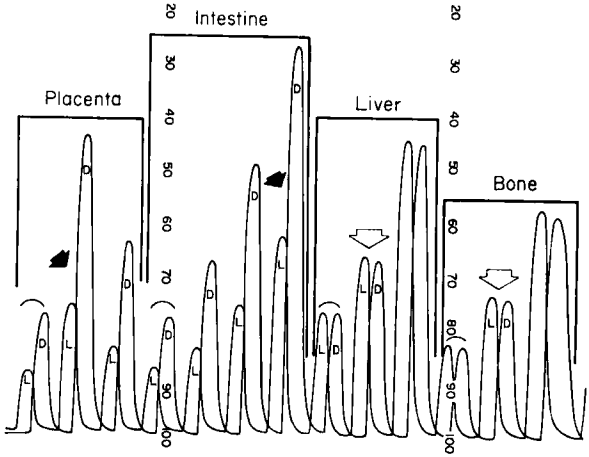


FIG. 4. Differential AutoAnalyzer alkaline phosphatase tracings. In each pair of peaks the tracings of the line containing L-phenylalanine precedes the one containing D-phenylalanine in the buffered substrate [according to Green *et al.* (G16)].

a competitive activation of alkaline phosphatase that has undergone a conformational alteration in structure (F7). The D-total value, therefore, is considered the maximal activity of the serum alkaline phosphatase.

The detailed procedure including reagents is now described (F8).

Automated Procedure for LPSAP. The manifold illustrated in Fig. 6 is constructed where possible with glass tubing. Incubation time begins with the entry of serum into the buffer substrate stream identified as D or L, through the H-3 cactus, and ends with mixture with the stream of the 4-aminoantipyrine (usually 10 minutes with 40-foot coils immersed in the incubator). Following the entry of ferricyanide solution 30 seconds later, 3-mm internal diameter mixers produce a good rate of color development. The sample rate is 20/hour, and a 1:2 wash ratio is employed to accomplish full baseline return of the recording pen. Serum blanks are run in the same way, but substrate is omitted from the D and L streams. Optical density is read at 505 mμ.

Reagents and Standards. Phenol standards are prepared by diluting the stock phenol standard with clear pooled heat-inactivated sera to yield concentrations of 5–200 μg of phenol/ml. Tracings exhibited by standards are converted from peak percent light transmittance to optical density; this value is corrected by its individual serum blank and plotted to yield a linear curve relating optical density to micrograms of standard phenol. Baselines are set at 98 and 98.5% transmittance. The peaks of the tracings for serum blanks are also converted to optical density and read as micrograms of phenol, which are substituted into the formula

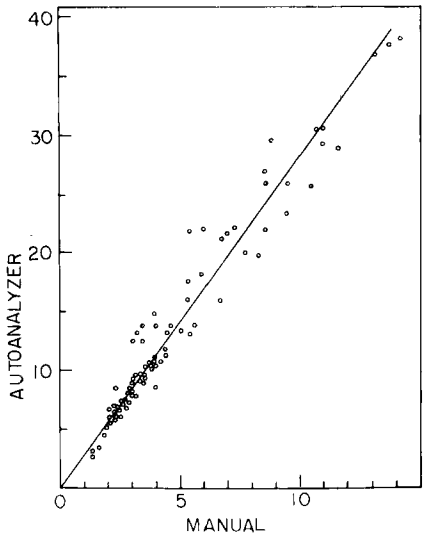


FIG. 5. Relation between serum alkaline phosphatase determined in the Auto-Analyzer and by the manual method. AutoAnalyzer units are King-Armstrong, and manual units are Bodansky-Shinowara (F14).

provided in the N-29 Technicon methodology. This formula yields the King-Armstrong unit for alkaline phosphatase based on a 15-minute incubation period. The difference in units between the D-total alkaline phosphatase activity and the activity measured in the presence of L-phenylalanine yields the L-phenylalanine-sensitive alkaline phosphatase (LPSAP).

Pregnancy sera are excluded from sera pooled for heat inactivation hours at 55°C).

Carbonate–bicarbonate buffer, pH 9.8: sodium carbonate (2.65 g) and sodium bicarbonate (2.11 g) are dissolved and adjusted to pH 9.8 with *N* sodium hydroxide or hydrochloric acid. The mixture is diluted to 1 liter with distilled water.

L-*Phenylalanine-buffered substrate:* L-phenylalanine (0.826 g, Calbiochem) and 4.0 g of disodium phenyl phosphate are dissolved in 1 liter of carbonate–bicarbonate buffer and adjusted to pH 9.8.

D-*Phenylalanine-buffered substrate:* D-phenylalanine (0.826 g, Calbiochem) is substituted for L-phenylalanine, 4.0 g of disodium phenylphosphate are added, and solution is completed in 1.0 liter of carbonate–bicarbonate buffer and adjusted to pH 9.8.

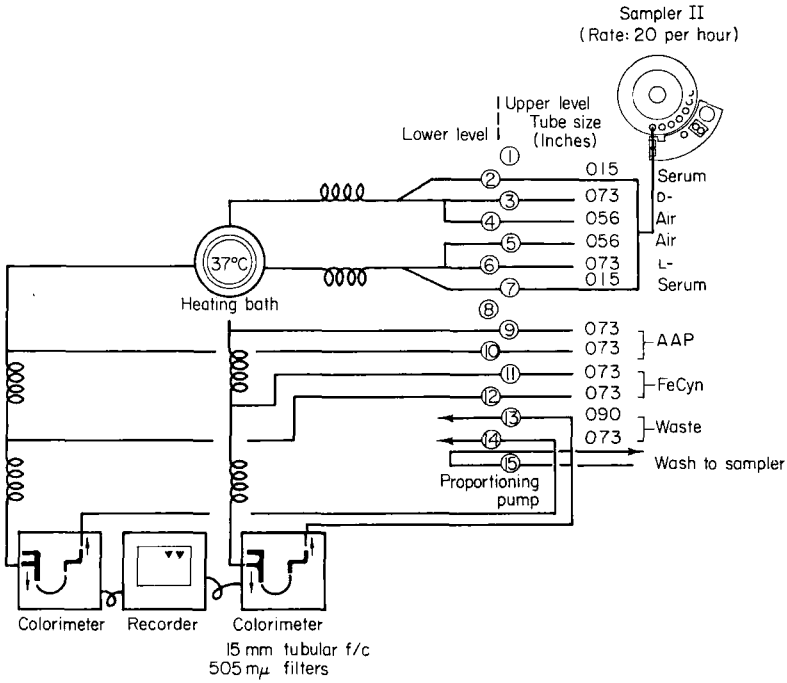


FIG. 6. AutoAnalyzer Manifold for L-phenylalanine-sensitive alkaline phosphatase (F8). Waste lines 13 and 14 are both 0.90 in. and line 15 is 0.073 in.

4-Aminoantipyrine: 4-aminoantipyrine (1 g) is dissolved in 1 liter of distilled water.

Potassium ferricyanide: Potassium ferricyanide (5.0 g) is dissolved in 1 liter of distilled water.

Precautions. All reagents are filtered after preparation, stored in the refrigerator, and warmed to room temperature before use. The substrate solutions should be made up only in a quantity sufficient for a daily run. At the end of the day, the AutoAnalyzer is "scrubbed" with a low suds-forming detergent (Heikol) followed by copious rinsing with distilled water. This maneuver eliminates "noise" from the tracings.

The LPSAP often represents a single protein component (the slow-moving band) in individuals with blood types B and O, as demonstrated by ourselves (F9, K25, S49) and others (A17, B10, R7). It may also be associated with enzyme bands at other locations in gel zymograms (K25).

3. Biochemical Studies on Alkaline Phosphatases of Tissues

This section of the chapter is divided into six parts dealing in turn with kinetics, isoenzyme isolation, immunochemical and electrophoretic properties, heat sensitivity, and neuraminidase action. The purpose is to bring to the reader our present state of knowledge of those basic biochemical and biophysical properties that relate specifically to isoenzymes and their recognition.

3.1. Kinetic Studies and Mechanism of Enzyme Action

Although a considerable literature records numerous studies in this field (A19, B17, C12, J3, L9, P16, S4, S5), many have been performed on preparations of tissues from other than human sources. In conformity with the subject of this chapter and to avoid species differences, most attention will be directed to human tissue alkaline phosphatases and in particular their variants. The stereospecific L-phenylalanine inhibition has provided the impetus to study its molecular mechanism, which necessarily requires an understanding of the mechanism of catalysis. It is expected that discovery of other stereospecific inhibitors will follow and that they may have even greater utility than L-phenylalanine. However, since it is the first such unique inhibitor, this section of the chapter will receive extensive treatment after a consideration of some basic kinetic information.

3.1.1. *Substrate Specificity and K_m*

The alkaline phosphatase activity under consideration is that of a monophosphohydrolase (International Enzyme Classification 3.1.3.1).

A degree of organ specificity for substrate is to be expected. Thus, a substrate preference known is the ability of intestinal alkaline phosphatase to hydrolyze *o*-carboxyphenylphosphate more rapidly than other substrates (F11, F13). Further, placental alkaline phosphatase is stated to hydrolyze *p*-nitrophenyl phosphate less rapidly than β-glycerophosphate (S1). Although ethanolamine phosphate is hydrolyzed preferentially by rat liver alkaline phosphatase, such a preference was missing from human liver preparations (F11).

The use of two substrates plus a specific inhibitor can be of great value in identifying organ-specific isoenzymes, as shown by Green *et al.* (G16)

in the interpretation of tartrate-sensitive moieties of the serum acid phosphatase, employing phenyl phosphate and α-naphthyl phosphate.

Michaelis Constants. Roche (R19) and Sarles (S6) suggested that the measurement of the affinity constant of the substrate for alkaline phosphatase might be a valid means of characterizing the organ sources in hyperphosphatasemia. Later, Moss and King (M36) were able to characterize isoenzymes in different tissues by the determination of Michaelis constants with β-naphthyl phosphate as substrate. The tissue difference in K_m values, although small, was found to be reproducible.

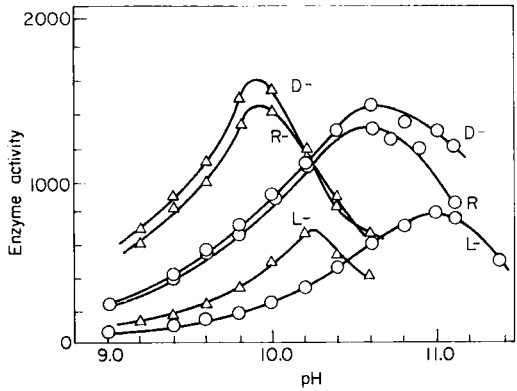


FIG. 7. pH optima for intestinal and placental alkaline phosphatases: (R) values in the absence of phenylalanine; (D) in the presence of 0.005 *M* D-phenylalanine, and (L) in the presence of 0.005 *M* L-phenylalanine. The substrate concentration was 0.018 *M* phenyl phosphate. The triangles represent the intestine, and the circles represent human placenta.

The respective values for K_m (μM) are: liver, 67; intestine, 90; bone and kidney, 103 (± 4, SD). Thus, although liver, bone, and intestine are different from each other, there is complete overlapping in the values of the bone with kidney isoenzymes.

The K_m values of different human tissues with various substrates are given in Table 6.

3.1.2. *pH Optima*

These are listed in Table 7. Just as in the case of substrate, organ-specific pH optima have been observed. Thus, for example, purified human placental alkaline phosphatase exhibits an optimum pH of 10.6 in contrast to 9.8 for the human intestinal preparation (Fig. 7).

The influence of substrate and its concentration is of obvious importance. β-Glycerophosphate is hydrolyzed at a slower rate than phenyl

phosphate and the optimum pH of its hydrolysis is lower—pH 8.8 vs. pH 9.8. Substrate concentration and pH are conveniently related in the Dixon plot of pK_m vs. pH, as in Fig. 8. For intestine, the point of inflection at pH 8.6 is attributed to a dissociable group in the free enzyme and at 9.6 to another dissociable group in the enzyme–phenyl phosphate

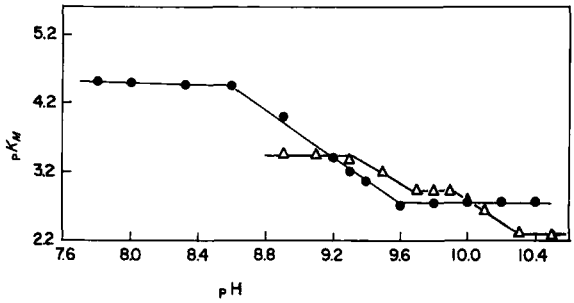


FIG. 8. Relationship between pK_m versus pH for intestine (rat) and placental (human) alkaline phosphatases. *Filled circles* represent intestine and *open triangles* human placenta. The plot for intestinal enzyme is made according to Ghosh and Fishman (G5). Ammonium sulfate fractionation was omitted during the purification of the placental enzyme.

complex. Also the maximal velocity and the Michaelis constant were pH-dependent (G5). For placenta, the pK values were 9.3 and 9.9 in the free enzyme, and changed to 9.7 and 10.3, respectively, in the ES complex. The pK data are compatible with cysteinyl-SH and lysinyl ϵ-NH_2 (C14) groups.

The nature of the buffer is a variable (B41, G5) that requires evaluation and a judicious choice. Veronal buffer, recommended by King's group (K11, K12), undergoes decomposition at 37°C so that the pH of the digest, initially 9.8, is found to be 9.3 at the end of 1-hour incubation. The popular use of 2-amino-2-methyl-1,3-propanediol and tris(hydroxymethyl)aminomethane buffers neglects the consideration that these substances serve as phosphoryl acceptors (D8) and directly enhance the rate of decomposition of the substrate. If the concentration of such a buffer is different in various laboratories, this alone could produce divergent data. In the study of amino acid inhibition, the buffer substances in the digests should not possess NH_2 groups such as in glycine and ethanolamine, otherwise the interpretation of the results cannot be attributed exclusively to the added amino acid. In particular, ammonium buffer is to

TABLE 6
THE MICHAELIS CONSTANTS OF HUMAN ALKALINE PHOSPHATASES

No.	Substrate	pH	Enzyme source	Buffer	Temp. (°C)	K_m (μM)	Reference
(1)	Phenyl phosphate	9.3	Intestine	Carbonate–bicarbonate (0.05 M)	37	160	Fishman and Ghosh[a]
(2)						140 (D)[b]	
(3)						95 (L)[c]	
(4)	Phenyl phosphate	10.0	Intestine	—	37	2100	Ahmed and King (A5)
(5)	β-Glycerophosphate	10.0	Intestine	—	37	1200	
(6)	α-Naphthyl phosphate	9.8	Intestine	Carbonate–bicarbonate (0.05 M)	37	630	Fishman and Ghosh[a]
(7)						590 (D)[b]	
(8)						240 (L)[c]	
(9)	β-Naphthyl phosphate	10.1	Intestine	Carbonate–bicarbonate (0.1 M)	37	90	Moss and King (M36)
(10)			Liver	Carbonate–bicarbonate (0.1 M)	37	67	
(11)			Bone	Carbonate–bicarbonate (0.1 M)	37	103	
(12)		10.2	Kidney	Carbonate–bicarbonate (0.1 M)	37	104	
(13)	Phenyl phosphate	10.0	Kidney	—	37	5700	Ahmed and King (A5)
(14)	β-Glycerophosphate	10.0	Kidney	—	37	25000	
(15)	Pyridoxal phosphate	9.0	Brain	Tris-acetate (0.1 M)	37	430	Saraswathi and Bacchawat (S4)
(16)	p-Nitrophenyl phosphate	9.0	Brain	Tris-acetate (0.1 M)	37	1250	
(17)	5′-AMP	9.0	Brain	Tris-acetate (0.1 M)	37	575	
(18)	Phenyl phosphate	8.9	Placenta	Carbonate–bicarbonate (0.05 M)	37	316	Fishman and Ghosh[a]
(19)		9.1	Placenta	Carbonate–bicarbonate (0.05 M)	37	313	
(20)		9.3	Placenta	Carbonate–bicarbonate (0.05 M)	37	370	
(21)		9.5	Placenta	Carbonate–bicarbonate (0.05 M)	37	590	
(22)		9.7	Placenta	Carbonate–bicarbonate (0.05 M)	37	1100	
(23)		9.8	Placenta	Carbonate–bicarbonate (0.05 M)	37	1145	
(24)		9.9	Placenta	Carbonate–bicarbonate (0.05 M)	37	1100	
(25)		10.0	Placenta	Carbonate–bicarbonate (0.05 M)	37	1890	
(26)		10.1	Placenta	Carbonate–bicarbonate (0.05 M)	37	2000	
(27)		10.3	Placenta	Carbonate–bicarbonate (0.05 M)	37	5000	
(28)		10.5	Placenta	Carbonate–bicarbonate (0.05 M)	37	5000	

(29)	β-Glycerophosphate	10.2	Placenta	Ethanolamine	38	1000	Anagnostopoulos and Matsudaira (A16)
(30)	Phenyl phosphate	10.0	Placenta	—	37	1200	Ahmed and King (A5)
(31)	β-Glycerophosphate	10.0	Placenta	—	37	1800	
(32)	*p*-Methylphenyl phosphate	10.0	Serum	Carbonate–bicarbonate (0.05 *M*)	35	955	Nath and Ghosh (N9)
(33)	*m*-Methylphenyl phosphate	10.0	Serum	Carbonate–bicarbonate (0.05 *M*)	35	765	
(34)	Phenyl phosphate	9.8	Serum	Carbonate–bicarbonate (0.05 *M*)	35	645	
(35)	*m*-Methoxyphenyl phosphate	9.8	Serum	Carbonate–bicarbonate (0.05 *M*)	35	370	
(36)	Phenyl phosphate	9.5	Serum	Barbitone (0.04 *M*)	25	350	
(37)					30	220	
(38)					35	180	
(39)					40	150	
(40)			Dilute serum (1:5)	Barbitone (0.04 *M*)	25	260	Nath and Ghosh (N8)
(41)					30	180	
(42)					32.5	100	
(43)					35	70	
(44)					40	50	

[a] This paper.
[b] In the presence of 5 m*M* D-phenylalanine.
[c] In the presence of 5 m*M* L-phenylalanine.

TABLE 7. OPTIMUM pH OF HUMAN ALKALINE PHOSPHATASES

No.	Enzyme source	Substrate	Substrate concn. (m*M*)	Buffer	Optimum pH	Reference
(1)	Intestine	Phenyl phosphate	18.0	Carbonate–bicarbonate	9.9	Fishman and Ghosh[a]
(2)		α-Naphthyl phosphate	5.0	Carbonate–bicarbonate	9.8	
(3)	Intestine	β-Naphthyl phosphate	5.0	Carbonate–bicarbonate	10.1	Moss and King (M36)
(4)	Placenta	Phenyl phosphate	18.0	Carbonate–bicarbonate	10.6	Ghosh *et al.* (G8)
(5)	Placenta	Phenyl phosphate	72.0	Carbonate–bicarbonate	10.7	Fishman and Ghosh[a]
(6)	Placenta	β-Glycerophosphate	1.0	Ethanolamine	10.2	Anagnostopoulos and Matsudaira (A16)
(7)		Phenyl phosphate	1.0	Ethanolamine	10.2	
(8)	Placenta	Phenyl phosphate	2.5	Carbonate–bicarbonate	10.3	
(9)			5.0	Ethanolamine	10.2	
(10)				Veronal	9.9	
(11)				Glycine	9.6	Ahmed and King (A5)
(12)		β-Glycerophosphate	5.0	Ethanolamine	9.7	
(13)				Veronal	9.9	
(14)				Glycine	9.6	
(15)	Serum	Phenyl phosphate	4.0	Veronal	9.5	King and Armstrong (K11)
(15a)	Serum	Phenyl phosphate	4.0	Carbonate–bicarbonate	9.8	Nath and Ghosh (N9)
(16)	Serum	β-Glycerophosphate	20.0	Barbital	9.3	Bodansky (B23, B24), Shinowara *et al.* (S30), Keay and Trew (K3)
(17)	Serum	*p*-Methylphenyl phosphate	4.0	Carbonate–bicarbonate	10.0	
(18)		*m*-Methoxyphenyl phosphate	4.0	Carbonate–bicarbonate	9.8	Nath and Ghosh (N9)
(19)		*m*-Methylphenyl phosphate	4.0	Carbonate–bicarbonate	10.0	
(20)	Liver	β-Naphthyl phosphate	5.0	Carbonate–bicarbonate	10.1	
(21)	Bone	β-Naphthyl phosphate	5.0	Carbonate–bicarbonate	10.1	Moss and King (M36)
(22)	Kidney	β-Naphthyl phosphate	5.0	Carbonate–bicarbonate	10.2	
(23)	Brain	Pyridoxal phosphate	0.33	Tris-acetate	9.0	Saraswathi and Bachhawat (S4)
(24)	Urine	Phenyl phosphate	5.0	Carbonate–bicarbonate	10.1	Delory and King (B47)

[a] This paper.

be avoided, since it is inhibitory, especially to placental alkaline phosphatase.

Our buffer preference in kinetic studies has been either 50 m*M* carbonate–bicarbonate buffer or 12.5 m*M* boric acid–borate buffer. The kinetic data have been identical with the use of both buffers. In addition, the pH of the complete digest is the same at the end of the incubation period as when the mixture was put in the incubator, and there is no inhibition detectable at the recommended buffer concentrations.

Bodansky (B32a) has dealt in detail with considerations of pH in the Shinowara (S30) and Bodansky (B23) methods.

3.1.3. *Inhibitors and Activators*

The reader is referred to standard treatises and the literature for complete listings of inhibitors of alkaline phosphatase (B14, B32, E3, E13, F4, H8, H12, M8, M29, R18, R21, S19a, S43, W5). In this section we will concern ourselves only with those inhibitors that bear on human alkaline phosphatase isoenzymes.

Studies on the cyanide inhibition of the enzyme by Cloetens (C12) and Drill and Riggs (D22) point to the presence of two alkaline phosphatases in animal tissues. We have found cyanide inhibition of intestinal alkaline phosphatase (S19) to be noncompetitive.

Historically, bile salts were first (B29) found to inhibit bone but not intestinal alkaline phosphatase, a fact that suggested organ differences in this enzyme. Somewhat later, the serum alkaline phosphatase of pregnancy was found to be similarly insensitive to bile salts.

Amino acids have been studied in relation to alkaline phosphatase, and organ differences have been observed by Bodansky (B30) and Fishman (F13). Inhibition is competitive in nature. The most recent study on the influence of L-histidine demonstrates alteration in the pH optimum (B33) of hydrolysis. Moreover, a survey of amino acid inhibitors (F11) has produced (F13) the unique stereospecific uncompetitive inhibitor, L-phenylalanine, which is discussed in detail in section 3.1.6.

Metal ions (C8) such as Co^{2+} have been studied in conjunction with amino acids and synergistic phenomena have been described (B31). Mg^{2+} ions are known to activate many preparations of alkaline phosphatase but this effect differs with the enzyme source.

In the case of amino acids and metal ions, there are many instances of the reversal of activation by higher concentrations of these substances in the digest (A7, B31).

Many phosphoryl acceptors are activators by virtue of directing the reaction toward products by the removal of inorganic phosphate in the form of the phosphate-acceptor compound.

3.1.4. *Essential Groups*

The following is a list of groups that in the past have been considered necessary for alkaline phosphatase activity in general: tyrosinyl—OH (S36, S37), —NH_2 (A15, G15, L10), —SH (A3, L10), and metal site (A3, M13, P7, V5).

In a detailed study by Fishman and Ghosh (F7), evidence has accumulated which suggests that the —NH_2 groups, including the ϵ-NH_2 of lysine, participate in substrate binding only, whereas the SH and metal site are involved more directly in the catalytic event. In the case of ϵ-NH_2 groups, *o*-methylisourea was the specific reagent used and, for α-NH_2 groups, the conditions of formaldehyde–acetamide inhibition of Fraenkel-Conrat (F22–F25) were employed. Inactivation of SH groups (B7) by *p*-hydroxymercuribenzoate, iodoacetamide, and iodine is considered not only to alter the active site but also to disturb the three-dimensional configuration of the protein. Here, the presence of substrate can protect the enzyme from inactivation by iodoacetamide and iodine, while cysteine can reverse inhibition by these substances and by *p*-hydroxymercuribenzoate. Moreover, chelating agents such as EDTA, 8-hydroxyquinoline, *o*-phenanthroline, α,α'-dipyridyl, etc., were strong inhibitors. Lastly, the effect of EDTA could be reversed by Zn^{2+} ions, an event that has been commonly observed in enzymes possessing the metal site (P7). Rat intestine was the enzyme source in this study.

Accordingly, for rat intestinal alkaline phosphatase, consideration is limited to the ϵ-amino group of lysine, the —SH of cysteine, and a metal as sites involved in the catalytic event.

Dabich and Neuhas (D1) recently suggested the existence of two groups exhibiting pK values of 8.6 and 9.6 in the active site of purified bovine alkaline phosphatase. The pK values obtained from pK_m–pH and log V_{max}/K_m curves by these workers are identical with those obtained with the intestinal enzyme (G5). They believed that at least three groups are required at the active site; they did not identify them, but suggested the use of group-specific reagents for their identification.

In earlier studies on essential groups, Sizer (S36) suggested, on the basis of iodine inactivation, that the phenolic hydroxyl group of a tyrosine residue might be essential for alkaline phosphatase activity. Later Morton (M30), in his interpretation of the Dixon (pK_m–pH) plot obtained with calf-intestinal alkaline phosphatase, pointed out that the phenolic hydroxyl group of tyrosine might be in the active site. However, he gave equal weight (M30) to the alternative possibility that the essential group could well be the ϵ-amino group of lysine, since the pK values for the tyrosine hydroxyl and the ϵ-amino group were very close to each other.

In our view, iodine inactivation in the case of rat intestinal alkaline phosphatase causes blocking of the —SH group, since other evidence for the presence of this group is strong. The ϵ-amino group of lysine is considered to be essential not merely because tyrosine can be reasonably excluded, but because rat intestinal alkaline phosphatase is inhibited by the lysine-specific *o*-methylisourea reagent and because the pK_m–pH plot shows a discontinuity at pH 9.6 (G5), the pK of the ϵ-amino group of lysine (C14).

The primary alcohol group of serine has long been implicated in the active site of alkaline phosphatase (B42, M20, Z3). Thus, Engstrom (E3–E6) and Schwartz *et al.* (S22, S23) isolated radioactive *O*-phosphorylserine in the hydrolyzate of *E. coli* alkaline phosphatase which had been incubated with labeled phosphate containing ^{32}P. Many have considered this to be evidence for a phosphoryl enzyme in the same way that aryl enzymes are viewed as catalytic intermediates. However, it is now known (P4, W9, W10) that the phosphoryl enzyme is thermodynamically stable, and that the serine side chain acts only as the phosphoryl acceptor and not as an energy-rich intermediate. Also, the pK of the serine hydroxyl (pK = 13.5) is far removed from the pK values of the dissociable groups that are present in the free enzyme and the enzyme–substrate complex. Nevertheless, there is reason to include serine in the region of the active site, but its role may not be a limiting one.

Finally, the imidazole group has not been found to be essential for alkaline phosphatase (P9).

3.1.5. *Mechanism of Catalysis*

The problem of elucidating the mechanism of catalysis by alkaline phosphatase has been attacked by a number of workers from different directions; from kinetic studies and modification of key linkages in the substrates and enzyme to the visualization of mechanism through models. Studies of related phosphohydrolases are relevant (A19, K26, L20, P11, P12).

In studies using phenyl derivatives with various substituents in the benzene ring of substrates for hydrolases such as plant acid phosphatase (N14), β-D-glucosidase (N15), α-D-glucosidase (H2), chymotrypsin (B15, C1), benzoyl cholinesterase (O1), and aryl sulfatase (D18), and for oxidizing enzyme systems such as amino acid oxidase (N19) and aldehyde dehydrogenase (D10), the rate of enzyme-catalyzed hydrolysis was greatly dependent on the electron densities in that part of the substrate molecule to which the enzyme could conceivably attach. The same conclusions arrived at by employing the σ–ρ relationship of Hammett (H4)

have been found to apply to similar findings with human serum alkaline phosphatase (N9). The results on the correlation of thermodynamic parameters (N10) and Michaelis constants (N9) with σ values of the substituents in the substrate are compatible with Koshland's induced-fit theory of enzymes (K22) and suggest a multipoint attachment between the enzyme and the substrate during complex formation, mediated through electrophilic and nucleophilic reactions between groups of opposite charge. Intramolecular electrostatic interaction between the groups (L9, L11) and configurational change during dilution (N8) are suggested.

That the presence of a bridge oxygen linking R, the organic moiety, with PO_3^{2-} is essential to make the molecule a substrate for alkaline phosphatase is apparent from the studies on its competitive inhibition by corresponding phosphonic acids in which the bridge oxygen is lacking (K19).

The justification for postulating a metal, probably zinc (B21, V5), with coordination number 4 is based on the results obtained elsewhere (M13, P6) and in this laboratory on the inhibition of the enzyme by various metal chelators. During the formation of the ES complex, this positively charged metal site may participate in forming coordinate bonds with the bridge oxygen and the electron-rich ionic oxygen of the substrate (Fig. 9). This is in harmony with Najjar's (N2) formulation of the phosphoglucomutase-G-1-P–transition complex, where a metal site is regarded as an anchoring point for the phosphoryl group of the substrate. Thus, the metal may be considered fixed in the enzyme surface by coordination with certain electron-rich atoms of the enzyme, one of which may be the sulfur of the sulfhydryl group. The other electronegative atom could be the oxygen atom of the serine hydroxyl, which is postulated to remain in the amino acid's oxazoline ring (P15, R27) in esterases.

On the basis of the foregoing considerations, one can propose a mechanism of catalysis (Fig. 9) as follows. During enzyme–substrate complex formation, one electron-rich ionic oxygen of the phosphate ester is attracted toward the positively charged —NH_3^+ group of lysine. Owing to its greater electronegativity compared to phosphorus, the double bonded oxygen acquires a partial negative charge and hence this oxygen of the substrate conceivably becomes hydrogen bonded with the hydrogen-donating site of the —SH group. Linkages to the metal site then occur as shown, and the hydrolytic cleavage is initiated by a nucleophilic attack by the hydroxyl ion of the medium on the phosphorus atom (D17) of the substrate. The phosphate is subsequently released from the phosphorylated enzyme intermediate.

It is clear that no inferences have been made regarding configuration of the protein or regarding the presence of each group on one, two, or

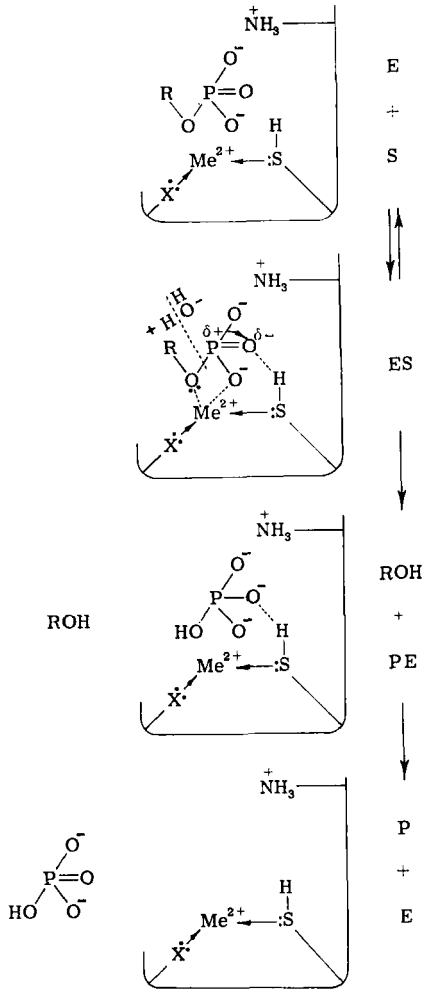


FIG. 9. A proposed mechanism for the catalysis of the hydrolysis of monophosphate esters by alkaline phosphatase.

three different polypeptide chains. Also, the role of carbohydrate units is omitted since there is no evidence that they participate in catalysis (G7).

The mechanism, insofar as ES formation is pictured, is consistent with the previous studies on alkaline phosphatase by Schwartz (S22), Portmann *et al.* (P17), Plocke *et al.* (P7), Mathies (M13), Malamy and Horecker (M4), Nath and Ghosh (N9, N10), Stein and Koshland (S44), Cohn (C15), Engstrom (E2, E7), Schlesinger and Barrett (S17), and Dabich and Neuhas (D1), and conforms to the views of Klotz (K18),

Boyer and Harrison (B37), Cunningham (C21), Vallee (V5), and Hsu *et al.* (H20). It must be recognized, however, that the present knowledge does not exclude the possibility of other modes of enzyme–substrate attachment, but this (Fig. 9) appears to be the likely one.

3.1.6. *Uncompetitive Inhibition by* L-*Phenylalanine*

The discovery of the stereospecific inhibitor, L-phenylalanine, arose from a systematic study of rat tissue alkaline phosphatases (F11) and from investigation of human intestinal and placental enzyme preparations (F13, F16).

In normal healthy subjects, only placenta and intestine, including their secretions, exhibit substantial stereospecific inhibition by L-phenylalanine (Table 4). Moreover, the extent of this inhibition is the same for both placenta and intestine, approximately 80% (G16). Other tissues exhibit much lower inhibition when it does appear. Confirmatory studies have appeared (L2, R7, M35, N20, W6).

The question of the organ specificity of the stereospecific L-phenylalanine inhibition should be examined again. The original finding in tissues and sera of nonpregnant individuals demonstrated that intestine and intestinal washings were the only sources inhibited to an extent of 77% by 0.005 *M* L-phenylalanine, the other tissues registering 0–17% inhibition. Since then, placental alkaline phosphatase has evidenced the same behavior as intestine, and there are indications of appreciable phenylalanine inhibition of other tissue fractions. It is conceivable that alkaline phosphatase exists in a certain physical (folded?) state in the membranes of the striated border, and this native molecule is inhibited most completely by L-phenylalanine. In homogenates this enzyme would appear in the microsomal fraction. Perhaps those tissues endowed with microvilli, active in absorption phenomena, that are rapidly producing alkaline phosphatase, as would be the case for intestinal mucosa and the placenta, have a disproportionately higher concentration of the L-phenylalanine-sensitive moieties. In other words, the L-phenylalanine sensitivity of alkaline phosphatase in fresh tissues may be organelle- or site-specific rather than organ-specific, and may thus be related to the turnover rate of the enzyme or even of cells. Enzyme preparations from such tissues would have disproportionally higher LPSAP. Consequently, the serum LPSAP measurement *in itself* cannot be indicative of the organ source unless additional medical and laboratory data are available. These include blood type, secretor status, pregnancy, cirrhosis, steroid therapy, starch-gel data, etc.

Certain kinetic features of L-phenylalanine inhibition will now be described for the purified human intestinal and placental preparations of alkaline phosphatase. In experiments on the effect of pH, Ghosh and Fishman (G5) observed that the degree of stereospecific L-phenylalanine inhibition of alkaline phosphatase from rat or human intestine and from human placenta is highly pH-dependent. Rat or human intestinal alkaline phosphatase exhibited maximum inhibition at pH 9.2 with phenyl phosphate as substrate, whereas the human placental alkaline phosphatase had a peak at pH 9.6 (Fig. 10).

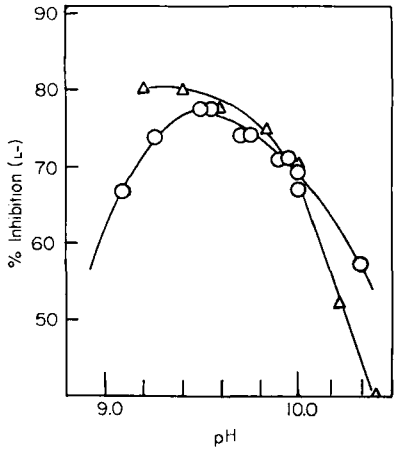


FIG. 10. Inhibition by L-phenylalanine as a function of pH. Triangles represent human intestinal and circles represent human placental alkaline phosphatase preparations.

The inhibition is also dependent on substrate concentration (Fig. 11). The L-phenylalanine inhibition of rat (G5) or of (Fig. 12) human intestinal alkaline phosphatase and of human placental (G6) enzyme is of the "uncompetitive" type, because the double reciprocal plots of velocity and substrate concentration were all straight lines parallel to those obtained without the inhibitor. Consequently, the extent of the inhibition was greatly dependent on substrate (Fig. 11) and inhibitor concentrations (Fig. 10). Detailed studies have appeared elsewhere (G5).

In order to study how L-phenylalanine affects the rate of decomposition of ES to products, k_3, the first-order velocity constant for the decomposition, was determined with and without the inhibitor, using 0.05 M carbonate–bicarbonate buffer at pH 9.2. K_m, necessary for the calculation of k_3, was obtained according to the method of Veibel and Lillelund as

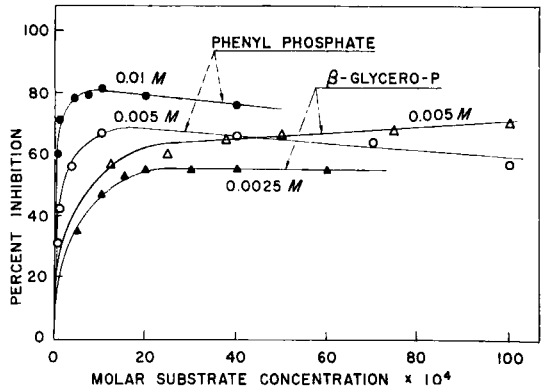


FIG. 11. Inhibition by L-phenylalanine as a function of substrate concentration. The concentrations in the figure refer to L-phenylalanine; the enzyme source was rat intestine.

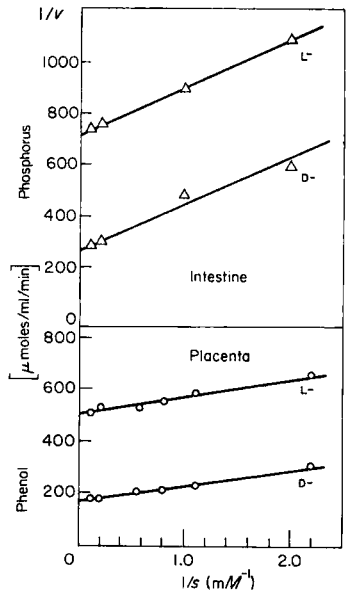


FIG. 12. Lineweaver-Burk plot relating the reciprocals of velocity and substrate concentration. For human intestine the substrate was α-naphthyl phosphate, and the L-phenylalanine concentration was 0.005 *M*. For human placenta the substrate was phenyl phosphate, and the L-phenylalanine concentration was 0.0025 *M*.

TABLE 8

DETERMINATION OF k_3[a] OF INTESTINAL ALKALINE PHOSPHATASE IN THE PRESENCE OF D- AND L-PHENYLALANINE

$e_0 \times 10^3$ (mg protein)[b]	$s_0 \times 10^4$ (M)[c]	$1/k_0$ (min^{-1})[d]		$K_m(M)$		$k_3 \times 10^3$ (min^{-1})	
		D	L	D	L	D	L
0.14	3	998.2	2584			3.4	1.1
0.14	9	2895.0	6115			2.7	1.1
0.14	18	4669.0	13960	0.00019	0.00008	3.0	1.0
0.28	3	506.4	1312			3.4	1.1
0.28	9	1339.0	3459			2.9	1.0
0.28	18	2688.0	6579	0.00017	0.00010	2.7	1.0

Mean k_3 with 0.005 M D-phenylalanine = 3.0×10^{-3}; standard error = $\pm 0.05 \times 10^{-3}$
Mean k_3 with 0.005 M L-phenylalanine = 1.05×10^{-3}; standard error = $\pm 0.01 \times 10^{-3}$

[a] k_3 = first-order rate constant for the decomposition of the enzyme–substrate complex into products based on the relation $k_3 = \dfrac{k_0 (K_m + S_0)}{e_0}$.

[b] e_0 = concentration of the enzyme protein.

[c] s_0 = initial molar concentration of the substrate.

[d] k_0 = first-order rate constant for the overall reaction.

applied to human serum alkaline phosphatase (N9) and other enzymes (N14, N15) [from the negative value of the intercept on the x axis of the straight line drawn by plotting $1/k_0$ values against the initial molar substrate concentration (s_0)]; k_0 was determined from the slope of the linear plot of the logarithm of the molar concentration of the unhydrolyzed substrate versus time by the method of least squares. The mean of the two K_m values obtained with two different enzyme concentrations was taken to calculate k_3; the necessary data are shown in Table 8. Although the absolute value of k_3 cannot be obtained without the molar concentrations of the enzyme, e_0 in milligrams of protein was used for obtaining results from comparative purposes (Table 8). The results in Table 8 indicate that the k_3 value is much less ($p = 0.001$) in the presence of L-phenylalanine than that in its absence.

The value (18,000 cal/mole) for the energy of activation for the hydrolysis of phenyl phosphate obtained from the slope of log V_{max} versus $1/T$ in the presence of L-phenylalanine is 3 times higher than that (6000 cal/mole) in its absence. Hence, so far as the energy requirement is concerned (A8, F30), L-phenylalanine appears to enhance the activation energy (G9) necessary for the reaction and thus impedes the cleavage of the substrate. The fact that the percentage inhibition of intestinal phosphatase by L-phenylalanine depends to a great extent on both substrate and inhibitor concentrations supports the postulation that EIS is formed

during the course of inhibition. The data for k_3 values and activation energies do not conflict with our hypothesis of uncompetitive inhibition and formation of a stable enzyme–inhibitor–substrate complex. They also suggest that in the Michaelis-Menten scheme (M18) the inhibitor (I) combines with ES to produce EIS,

$$\begin{array}{ccccc} & & \overset{k_1}{\rightleftarrows} & & \overset{k_3}{\rightarrow} \\ E + S & & ES & & E + \text{Products} \\ & & \underset{k_2}{\downarrow} I & & \\ & & EIS & & \end{array}$$

which either does not break down to the products at all or does so at a slower rate in comparison with that of ES. The presence of the inhibitor reduces the Michaelis constant, lowers the rate of the decomposition (k_3) of ES to products, and increases the energy of activation; all these findings are compatible with the view that the enzyme–substrate complex encountered in this work combines with the inhibitor, forming an enzyme–inhibitor–substrate complex.

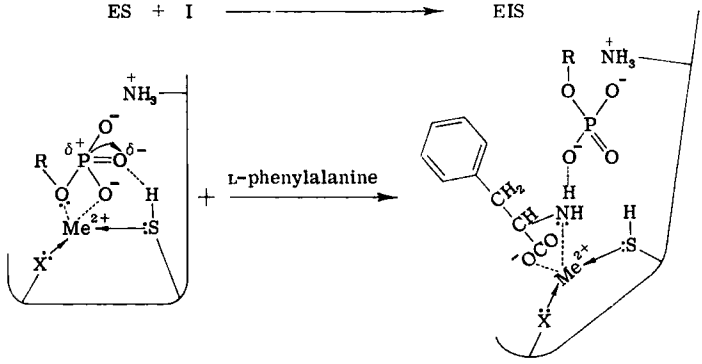


FIG. 13. Representation of the formation of an EIS complex during alkaline phosphatase inhibition by L-phenylalanine. Compare with Fig. 9.

Alternative interpretations may be considered. Thus, Frieden's (F29) Mechanism I lists four steps, each with corresponding dissociation constant, K, and decompositions of ES and EMS to products with their rate constants k_5 and k_6,

$$E + S \overset{K_1}{\rightleftarrows} ES \qquad (ES \overset{k_5}{\rightarrow} E + P) \tag{1}$$

$$E + M \overset{K_2}{\rightleftarrows} EM \tag{2}$$

$$ES + M \overset{K_3}{\rightleftarrows} EMS \qquad (EMS \overset{k_6}{\rightarrow} EM + P) \tag{3}$$

$$EM + S \overset{K_4}{\rightleftarrows} EMS \tag{4}$$

in which S is the substate, E the enzyme, and M the modifier, which is defined as binding to a site other than the active site of the enzyme.

Parallel curves of $1/v$ against $1/S$ (L16) are also characteristic of Frieden's "limiting Case 3," in which $k_6 = 0$ and $K_2 = \infty$; and of his "nonlimiting Case 1," which can occur in any set of circumstances in which $K_2/K_3 = k_5/k_6$. In the latter situation when $k_6 < k_5$, the enzyme binds the modifier more firmly in the presence of substrate than in its absence (inhibition). Additional experimentation is necessary to decide between these various alternatives.

When L-phenylalanine is present in a proper concentration, the decomposition of the normal enzyme–substrate complex (ES) is impaired because of the intervention of the inhibitor. The carboxylate group of L-phenylalanine, negatively charged ionic species of which are prevalent at the alkaline pH of the digest, may be attached to a positive center of the enzyme molecule, presumably the metal site (Me), which can also coordinate simultaneously with the amino nitrogen and accept its lone pair of electrons. As a consequence, the ionic oxygen of the phosphate becomes distant from the sulfhydryl site and the bridge oxygen becomes separated from the metal site, which is now occupied by the carboxylate group of the inhibitor. The catalytic event is thus essentially prevented.

Hypothetical Molecular Mechanism for ES and EIS Formation. The scheme in Figs. 9 and 13, although representing the general mechanism, fails to explains why L- but not D-phenylalanine is an uncompetitive inhibitor. What is required is a spatial model (L15) which could permit visualization of the positions of these two enantiomorphs of phenylalanine in the reaction mechanism. Accordingly, molecular models were built up, assuming that the NH_3^+ group is attached to lysine, that the SH belongs to cysteine, and that X is the oxygen in the oxazoline ring derived from the serine hydroxyl group (Figs. 14, 15, and 16). In constructing the models, the amino acids have been assumed to be vicinal and on the same polypeptide chain. However, the only requirement is that the essential groups occupy the desired positions in space whether or not they are present on the same or different polypeptide chains.

The model (Fig. 14) illustrates a surprisingly good fit of the substrate with the enzyme in forming the ES complex as outlined schematically in Fig. 9. Moreover, the L-phenylalanine is accommodated rather precisely in the enzyme–inhibitor–substrate complex and the interacting groups, particularly the metal site, are brought into closer apposition (Fig. 15). On the other hand, D-phenylalanine, having its phenyl group in a plane perpendicular to that of L-phenylalanine, encounters steric hindrance by the α-helix of the enzyme molecule and a poor fit results (Fig. 16).

Similar arguments as to the steric position of the benzyl group as postulated above were put forward by Awad *et al.* (A20).

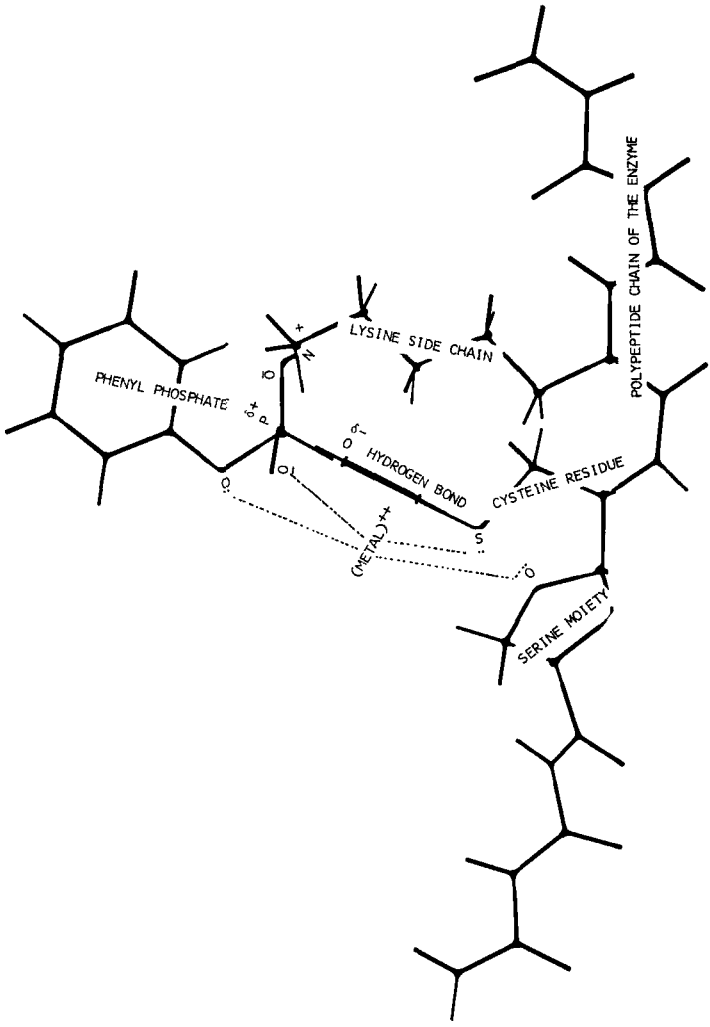


FIG. 14. Dreiding model of the enzyme substrate (ES) complex as hypothesized from Fig. 9.

The results of previous observations (F11) are compatible with the mechanism of EIS (Figs. 13 and 15). Thus the inability of β-phenyl-β-alanine and formylphenylalanine to inhibit rat intestinal alkaline phosphatase is understandable since the former compound does not have amino and carboxyl groups in the α-position, a prerequisite for the attachment to enzyme and substrate, and in the latter reagent the free amino group is blocked by the formyl radical. Also, glycylphenylalanine

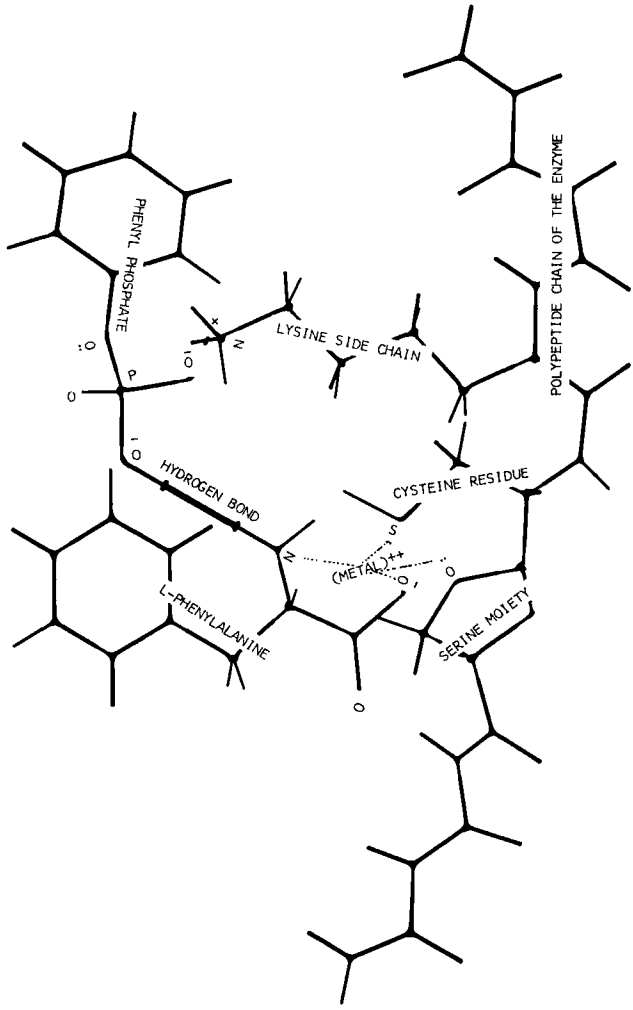


FIG. 15. Dreiding model of the enzyme–inhibitor–substrate (EIS) complex as hypothesized from Fig. 13.

and glycyltyrosine lack such α-amino groups and cannot be expected to be inhibitors. The position of the benzyl group in phenylalanine also becomes a determining factor. Thus it was observed (F11) that α-phenyl-β-alanine is not an inhibitor of rat intestinal alkaline phosphatase.

The true picture of the mechanism will be forthcoming only when the amino acid sequence of the active center of human alkaline phosphatase can be elucidated. It should be added that extensive unpublished studies

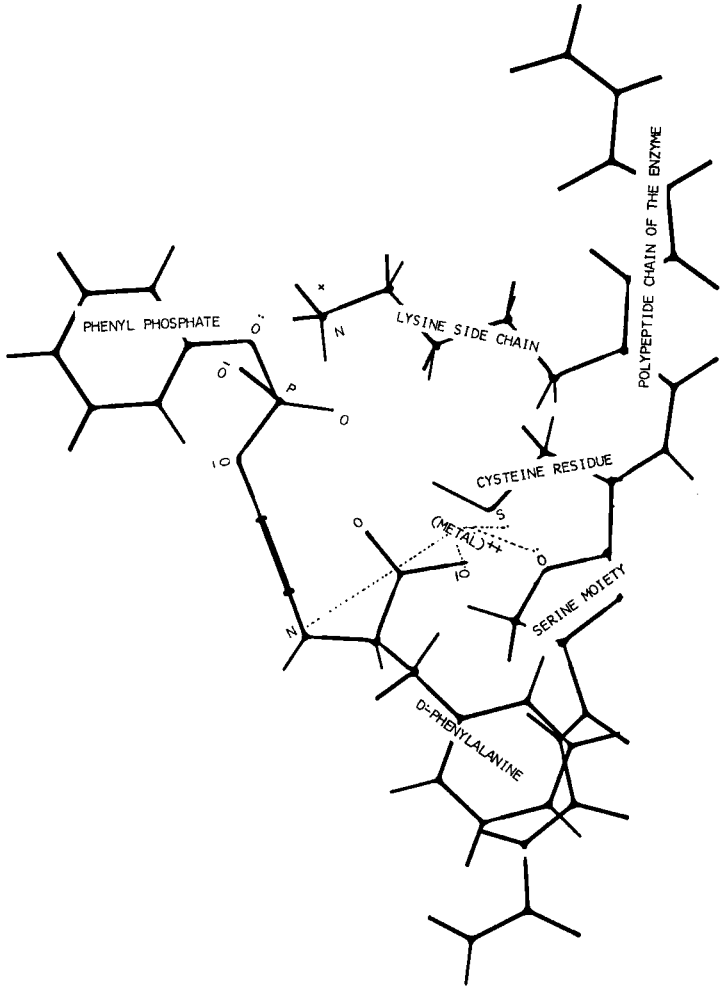


FIG. 16. Dreiding model of a hypothetical enzyme–substrate–D-phenylalanine complex.

have failed to demonstrate an allosteric phenomenon but are consistent with a homosteric mechanism of inhibition.

3.2. ISOLATION OF ISOENZYMES

This section deals particularly with the isolation of alkaline phosphatase of human serum and tissues. Studies on nonhuman sources have appeared (B17, B18, M3, M4, M28, P16, S16, S18, S19).

At the present time, an isoenzyme of alkaline phosphatase carries an

organ identity and has biochemically distinct properties. If the isoenzyme yields more than one band on electrophoresis, these enzyme bands are termed electrophoretic variants. Chromatographic variants would be obtained by chromatography.

Previous work on human alkaline phosphatases has utilized chromatography (E10) and starch-gel electrophoresis. Thus in 1956 Boman and Westlund (B34) reported the purification and separation of serum phosphatases by Dowex-2 column chromatography. Moss (M34) used gel filtration on Sephadex G-200 and DEAE-cellulose chromatography for separating 5′-nucleotidase and nonspecific alkaline phosphatase activities in human sera. In most of the studies of alkaline phosphatases in human tissues of liver (M33), intestine (M34, M35), bone (M36), kidney (B46), and urine (B44, B46, B47), crude extracts of these tissues were used and

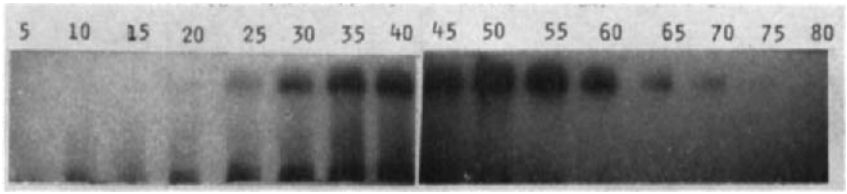


FIG. 17. Starch-gel patterns of Sephadex G-200 gel filtrates of human placental alkaline phosphatase showing the fractionation of variants A and B. The fraction numbers are indicated. The direction of migration was from bottom to top (anode).

only slight purification of the enzyme was achieved. Moss *et al.* (M36, M38) also purified alkaline phosphatases from liver, kidney, intestine, and bone by starch-gel electrophoresis. Reis (R3), Seelich and Ehrlich-Gomulka (S27), and Anagnostopoulos and Matsudaira (A16) were among the first to recognize human placenta as a rich source of alkaline phosphatase and to use it in purification. Further advances in purity were made by Ahmed and King (A4), who employed steps requiring butanol extraction, ammonium sulfate fractionation, and electrophoresis. The relevant properties of the enzyme were also investigated (A5, A6).

In this laboratory, attempts (G6, G8) have been made to purify and crystallize human placental alkaline phosphatase enzyme by a number of procedures involving homogenization with 0.05 *M* Tris buffer (pH 8.6), extraction with butanol, ammonium sulfate precipitation, exposure to heat, ammonium sulfate fractionation, dialysis, repeated ethanol fractionation, gel filtration with Sephadex G-200 (Fig. 18), continuous curtain electrophoresis on paper (Beckman Model CP), multiple TEAE-cellulose anion exchange chromatography, and equilibrium dialysis. Variant A (electrophoretically fast-moving) of human placental alkaline

phosphatase has been purified 4000-fold, and the final enzyme (variant A) was completely free (Fig. 18) from variant B (electrophoretically slow-moving) and was found to be homogeneous, judged by a number of criteria, such as sucrose density gradient centrifugation, ultracentrifugation, Sephadex-gel filtration, starch-gel electrophoresis, and Sephadex-gel electrophoresis. The final enzyme preparation showed an activity of 1130 international units per milligram of protein, which appeared to be higher than that reported by Anagnostopoulos and Matsudaira (A16) and

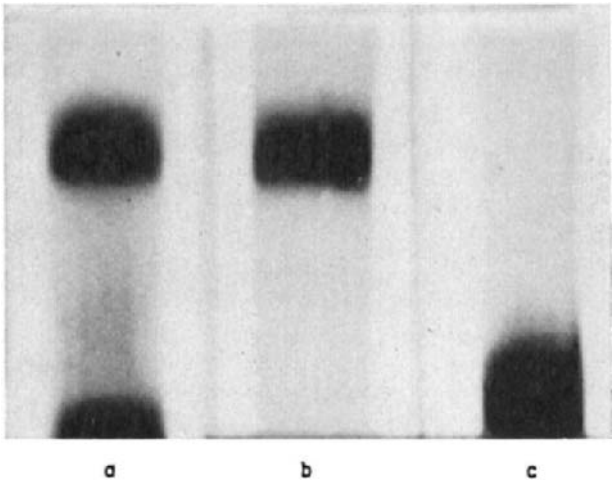


FIG. 18. Starch-gel pattern of placental alkaline phosphatase isozymes in the final steps of the purification: (a) variant A and B in the best alcohol fractions before Sephadex-gel filtration, (b) variant A after removing B in the Sephadex-gel column, and (c) crystalline alkaline phosphatase (variant B).

Ahmed and King (A4) or by investigators who used other mammalian or bacterial sources (M4, P7).

Variant B of the human placenta may represent a mixture of less soluble multimers and their aggregates. Thus, studies on Sephadex-gel filtration, sucrose density-gradient centrifugation, and ultracentrifugation suggest that the B-form (MW, 200,000 plus) represents heavy molecular aggregates of the A-form. The slow-moving variant B of human placental alkaline phosphatase has been most readily separated from the fast-moving variant A by molecular sieve chromatography, the former coming off first from a Sephadex G-200 column during gel filtration (Fig. 20). Finally, variant B has been prepared in crystalline form

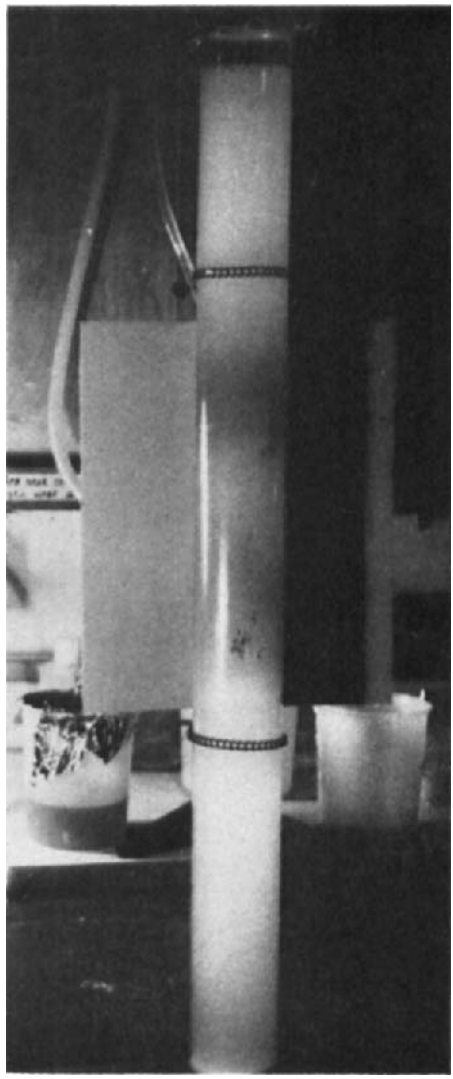


FIG. 19. A photograph of the Sephadex G-200 column showing two zones. Variant A is recovered from the slower band, and variant B from the faster band (closest to the bottom of the column). Compare with Fig. 17.

(Fig. 21) and is slowly transformed into variant A on prolonged storage (4 months) in the cold room at pH 9.2. Variant A has not yet been prepared in the crystalline state. Its molecular weight is 70,000 and the sedimentation constant is 4.2. The details of the purification and the properties of variants A and B will appear elsewhere.

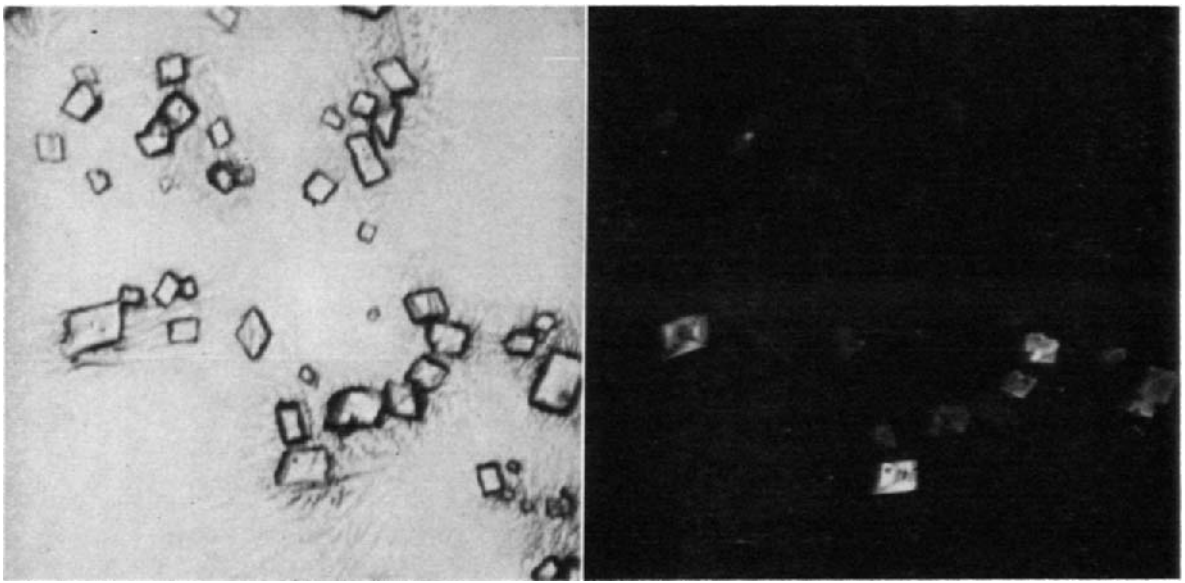


FIG. 20. Appearance of crystalline alkaline phosphatase (variant B) in bright light (*left*) and under polarized light (*right*), ×600.

Attempts were made to purify brain alkaline phosphatase (S5).

Heterogeneity of alkaline phosphatase in human and mouse intestine (M34, M35), calf intestine (B13), liver (M33), and kidney (B46) has been reported in the literature, but the separation of the individual components, especially the slow- and fast-moving isozymes completely free from each other has not been achieved. It may be reasonable to suggest that in each organ several variants may be expected, but their biochemical significance is basically the same.

Techniques of eluting proteins from starch gel (G14, J1) may find application for isolation and quantitation (J4) of variant forms of isoenzymes.

3.3. Immunochemical Properties

3.3.1. *Fundamental Approach*

Theoretically, since immunological specificity is extremely precise, an antibody to a pure antigen will react exclusively with this antigen. If alkaline phosphatase proteins differ according to the tissue from which they have been prepared, then specific antibodies for each can be produced by animals being immunized with the individual organ protein. A desirable condition is that the antigen–antibody complex be insoluble. An undesirable circumstance would be the presence of cross-reactions between the antibody of one organ with the antigen of several organs. Also the possibility exists that the purified antigen may represent only one variant form of the particular organ isoenzyme.

3.3.2. *Observations*

Schlamowitz (S10) was the first to undertake studies designed to test the immunological approach. Sera were collected from rabbits which had previously received alum-adsorbed suspensions of purified dog intestinal enzyme. The resulting antibodies were specific, precipitating intestinal enzyme completely from a mixture with liver or kidney enzyme. It was noted, however, that the complex remained soluble with quantities of antigen which were either smaller or greater than the amount that gave complete precipitation. To accomplish precipitation under all conditions of antigen (alkaline phosphatase) concentration, resort was made to the addition of horse antirabbit α-globulin antibody, which combines with the antigen–antibody complexes. Also, Schlamowitz (L1, S11–S14) employed inactivated intestinal alkaline phosphatase (cysteine-treated) as carrier in the system to increase the concentration of antigenic protein. However, antisera prepared against human bone alkaline phosphatase precipitated human liver and kidney phosphatase in addition to the bone

enzyme in the presence of horse antibodies. A 38-year-old male exhibited an intestinal component, based on the specific antibody test, that constituted 28% of the total serum alkaline phosphatase.

In 1961 Nisselbaum *et al.* (N22) concluded that the major portion of serum alkaline phosphatase was osteogenic in origin because approximately 66% of the alkaline phosphatase in the sera of three normal patients was precipitated by antihuman bone phosphatase sera. This percentage was appreciably higher in five cancer patients (two with liver metastases, two with bone metastases, and one with metastases to both liver and bone). However, the antibone phosphatase serum precipitated liver and kidney alkaline phosphatase as effectively as it did bone. The authors were not willing for other reasons to attribute to kidney and liver a role as tissue sources of serum alkaline phosphatase, but favored bone as the major source. Pelichova *et al.* have extended the work on intestine (P3).

Boyer's studies (B39) appear to indicate a lower degree of organ enzyme specificity than that observed by Nisselbaum *et al.*, in that antihuman intestine alkaline phosphatase sera cross-reacted with kidney and placenta. Also, the antihuman bone preparation precipitated alkaline phosphatase from spleen, liver, kidney, and *intestine*. Boyer considers liver, bone, spleen, and kidney 3-phosphatases to be closely related proteins, followed by intestine and placenta, the enzyme from these latter tissues representing a second and third class of alkaline phosphatase proteins. Intestine and placenta partially cross-react with one another and with a minor kidney component. Three genetic loci are considered by Boyer, therefore, to control the synthesis of alkaline phosphatase. The most recent study in this area is reported by Birkett *et al.* (B19).

At the present time, therefore, one cannot assume that, even if each organ possesses an antigenically different alkaline phosphatase, it could be isolated without change. The variation in results may result from the use as antigens of enzyme preparations in various degrees of purity, homogeneity, and molecular size. It would be hazardous to interpret without qualification or without other evidence the medical significance of immunochemical data with individual organ alkaline phosphatase antisera.

3.4. Electrophoresis

Significant advances have been made during the last decade in the development of new techniques of paper, starch-, and agar-gel electrophoresis. Soon after the introduction by Smithies in 1955 of the technique of starch-gel electrophoresis as an important analytical tool for the fractionation of serum proteins (P21, R8), this procedure proved val-

uable in the discovery of multiple molecular forms of enzymes, beginning with lactic dehydrogenase (F28, H9, H11, H14, H15, M7, M8, V9, W8, Z1). Then there followed the demonstration of multiple-molecular forms of a number of enzymes such as esterase (A11, H23, H24), ribonuclease (M11), acid phosphatase (A9, E9, H18), and alkaline phosphatase (A10, C22, E8, K23, K24, M37, P13, R24, S2).

We will now deal separately with the electrophoresis on paper, starch gel, agar gel, and Sephadex gel of alkaline phosphatase.

3.4.1. *Paper Electrophoresis*

Baker and Pellegrino (B3) carried out paper electrophoresis of serum alkaline phosphatase and identified the enzyme by cutting out the appropriate areas of the paper, eluting the enzyme, and performing the enzyme assay, or by incubating the whole strip with buffered α-naphthyl phosphate and coupling the liberated α-naphthol with diazotized *o*-dianisidine. The enzyme band coincided with the α_2-globulin zone. The identity of the enzyme in the paper strip was established by determining its optimum pH. Wolfson (W12) and Ahmed *et al.* (A4) were also able to visualize the alkaline phosphatase after paper electrophoresis by incubating with *p*-nitrophenyl phosphate and then exposing the paper to ammonia vapor. Similar attempts were made by Taleisnik *et al.* (T1) and Keiding (K5).

In Ghosh and Nath's experiments with paper electrophoresis (barbitone buffer, pH 8.6), rachitic serum showed an alkaline phosphatase mobility close to the mobility of β-globulin (N11). Sera from infective hepatitis and obstructive biliary cirrhosis, however, showed maximum alkaline phosphatase activity in the α_2-globulin zone (G5).

3.4.2. *Starch-Gel Electrophoresis*

The findings made in healthy individuals will be reviewed before the results in pathological tissues and disease.

The resolution of the isozymes of human alkaline phosphatase in normal individuals by starch-gel electrophoresis was systematically studied in 1961 by Boyer, who observed a characteristic alkaline phosphatase pattern similar in pregnancy sera and in placenta (B38). With regard to placenta, recent work (H5, R15, R16) has indicated genetic variation of placental alkaline phosphatase in human placenta when the starch-gel electrophoresis is carried out at two different pH's (8.6 and 6.0). Other tissues could not be differentiated by their starch-gel patterns by Boyer (B38, B39).

The combination of immunochemical and starch-gel techniques for identifying isoenzymes was applied by Boyer, who studied the supernatant fractions of centrifuged mixtures of enzyme plus antihuman

alkaline phosphatase sera for the particular tissue source and also for other tissue sources. The results have been referred to earlier and must be regarded as disappointing from the point of view of organ identification of serum alkaline phosphatase.

Much of our information on the behavior of purified human tissue alkaline phosphatases on starch gel has come from Dr. Moss' laboratory. Thus, studies on normal human liver alkaline phosphatase (M33) in

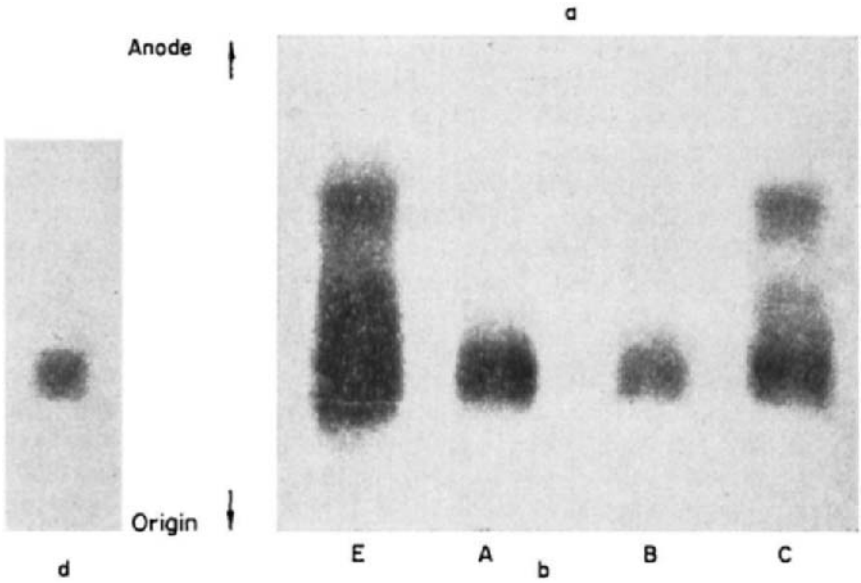


FIG. 21. Alkaline phosphatase zones after starch-gel electrophoresis of human intestinal extracts. The single pattern on the left labeled *d* is that of fresh butanol extract of human intestine [from Fig. 1 in Moss' report (M34)]. The pattern labeled E is the same intestinal extract stored for 4 months at −20°C (before chromatography). A, B, and C are different fractions obtained by DEAE-cellulose chromatography of E (M34).

autolyzed and butanol-extracted preparations in 1962 demonstrated the existence of four different variants in this tissue; the relative amount of the different fractions, however, was found to be dependent on the methods of preparation of the enzyme. In the case of human intestinal alkaline phosphatase, several zones were produced by aged butanol extracts (M34). [A similar suggestion of the alteration in properties of the enzyme as a result of the change in the active center of nonspecific alkaline phosphatase during the course of extraction and purification was made by Nath and Ghosh (N5), Vecerek *et al.* (V6, V7), and Sandler and

Bourne (S3).] Moss and King (M36) separated different zones of human alkaline phosphatase by starch-gel electrophoresis and determined their Michaelis constants. They also found a high degree of overlap of the various bands on starch-gel electrophoresis of purified human tissue alkaline phosphatases. In another study, purified intestinal alkaline phosphatase (M34, M35) was found to travel to several positions including the typical slow zone. More recently Moss *et al.* (M38) reported the neuraminidase sensitivity of human liver alkaline phosphatase. Butterworth and Moss (B45) showed that purified human renal alkaline phosphatase is also neuraminidase-sensitive, as the electrophoretic mobility of the enzyme in the starch gel was considerably reduced after neuraminidase treatment.

Studies on starch-gel electrophoresis of normal human urinary alkaline phosphatase (B44) revealed the presence of a urinary phosphatase that moved faster than any seen in serum or in any of the human-tissue extracts. This zone was also present in increased amounts in the urine of patients with renal disease (B44, B47). Sephadex G-200 gel filtration demonstrated urinary alkaline phosphatase to have a lower molecular weight than that of the renal enzyme.

Also, Hodson *et al.* (H17) applied Smithies' technique for the characterization of alkaline phosphatases from different tissues (intestine, liver, bone, etc.). In their report they questioned the contention that the osseous system is the main contributing source of human plasma alkaline phosphatase (H17), and preferred liver alkaline phosphatase as the main fraction of serum alkaline phosphatase. Hodson *et al.* (H17) observed the occasional presence of alkaline phosphatase at the intestinal region also in sera after electrophoresis.

Starch-gel electrophoresis of the alkaline phosphatase in the butanol extracts of leukocytes revealed three variants of the enzyme. Peacock *et al.* (P1) have devised a method for leukocyte alkaline phosphatase assay. An additional variant was detected in blood leukocytes of leukemia patients treated with 6-mercaptopurine (R10). Robinson and Pierce (R7) indicated that there might be a fundamental difference in molecular structure of the human serum alkaline phosphatase proteins because serum alkaline phosphatase, when incubated with neuraminidase prior to electrophoresis, demonstrated reduced anodal migration of those isoenzymes that are not L-phenylalanine-sensitive. L-Phenylalanine-sensitive enzyme of intestinal origin was found to be neuraminidase-resistant.

Much attention has been devoted in this laboratory to the starch-gel electrophoretic studies of various human tissue alkaline phosphatases, especially those intestinal and placental alkaline phosphatase isoenzymes that undergo stereospecific inhibition by L-phenylalanine and not by its

D-isomer. It has been possible to separate and identify the different variants of alkaline phosphatases in human placenta by Sephadex-gel filtration, sucrose density-gradient centrifugation, and ultracentrifugation, and in these studies starch-gel electrophoresis has proved to be unique in characterization of different isoenzyme fractions. Figure 17

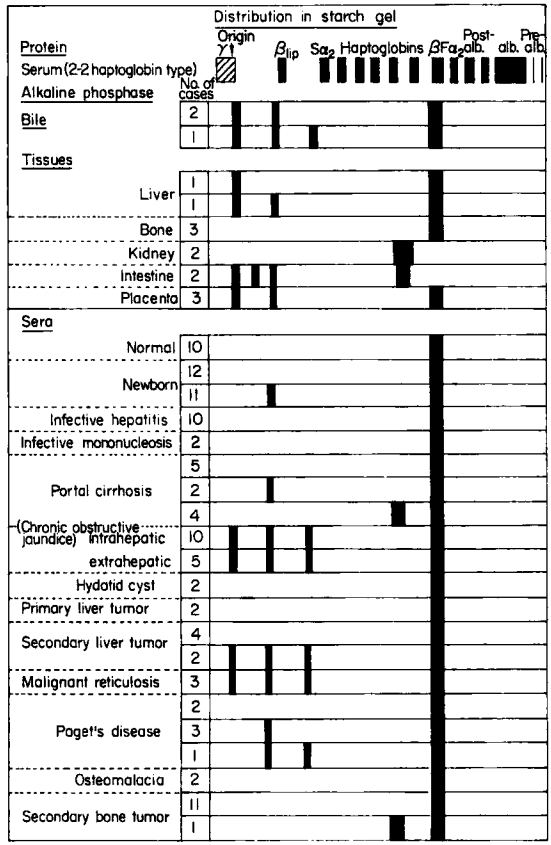


FIG. 22. Starch-gel patterns in the bile, tissues, and sera [according to Chiandussi *et al.* (C7)].

shows typical starch-gel zymograms showing the progress of the filtration of slow- and fast-moving species of placental alkaline phosphatase, which is explained by the difference in molecular weights of the variants (G6).

Starch-gel electrophoresis of pathological sera led to the indication that there might be different alkaline phosphatase enzyme patterns in sera of patients with bone and hepatobiliary disease (C7). Taswell and

Jeffers (T2) did electrophoretic studies on serum alkaline phosphatase in hepatobiliary and skeletal disease (Fig. 1). They found eight separate zones of alkaline phosphatase activity arranged in three distinct patterns, and suggested the use of the starch-gel zymogram in the differentiation between obstructive, metastatic, or infiltrative hepatobiliary disease, parenchymal hepatic disease, and osteoblastic disorders.

Most of the studies before 1965 were unable to make a clear-cut distinction between liver and bone components, since they frequently overlap with each other because of a subtle difference in the rate of their

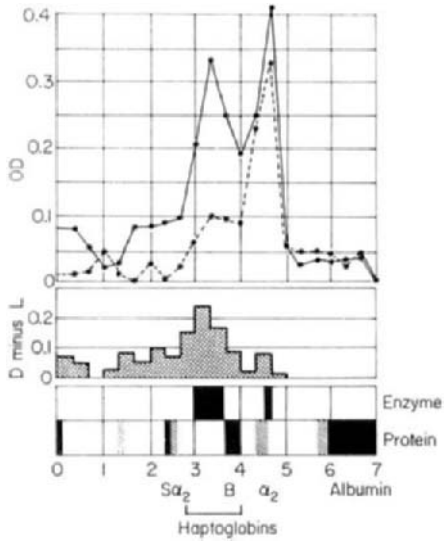


FIG. 23. Demonstration of heterogeneity of L-phenylalanine-sensitive serum alkaline phosphatase in a patient with alcoholic cirrhosis [according to Kreisher *et al.* (K25)].

migrations in the electric field. In addition, in these previous studies interpretation was lacking for a multitude of slow-moving components (T2) (Fig. 22).

Attention has been directed recently to the slow band that appears in the serum pattern of many normal subjects as well as of ill patients. Its discoverers (A17, B10, S32) and others did not identify the tissue source of this band, although it was in the intestinal position. By employing L-phenylalanine sensitivity as a criterion, the slow band was identified as intestinal by Fishman and Kreisher (F9), Kreisher *et al.* (K25) (Fig. 23), and Robinson and Pierce (R7). The band can be demonstrated in all individuals who are "secretors" of ABH substance in the saliva

after ingestion of a fat-enriched breakfast, but is normally not seen in individuals of blood type A. L-Phenylalanine-sensitive alkaline phosphatase has been found to be elevated in a number of normal subjects ingesting a fat-enriched breakfast (I2, L2).

It is now possible to demonstrate [after electrophoresis for 18 hours at 5°C (B6)] the location of L-phenylalanine-sensitive alkaline phosphatase in gels directly by a postcoupling technique developed by Inglis and Fishman (Fig. 24); the simultaneous coupling reaction in the

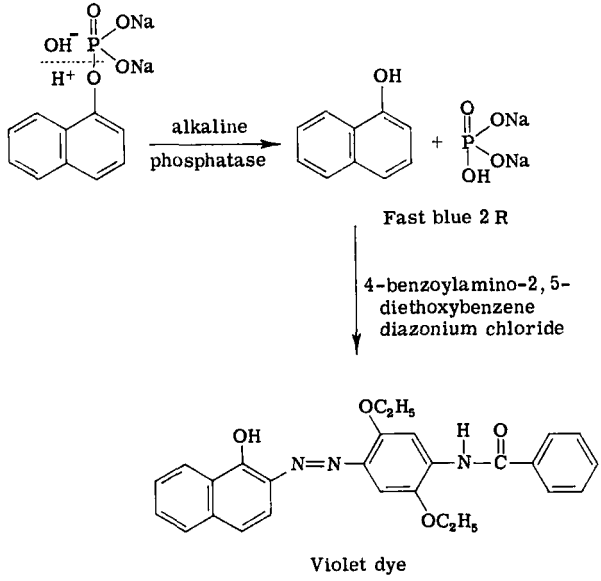


FIG. 24. Postcoupling azo dye reaction for naphthol as employed in developing starch-gel zymograms of alkaline phosphatase.

presence of L-phenylalanine leads to decomposition and the appearance of dye precipitate that makes the gels uninterpretable. The gels are now overlaid with filter paper, one segment in contact with paper wet with buffered α-naphthyl phosphate and 0.005 M D-phenylalanine, and a second segment with buffered substrate plus 0.005 M L-phenylalanine. After incubation for 2 hours at 37°C, the strips are removed and the gels flooded with 4-benzylamino-2,5-diethoxybenzene diazonium chloride solution and the mixture is kept in the refrigerator until the intensity of the bands is fully developed (5–60 minutes, depending on the enzyme concentration). The same staining technique is applied to gels in which one is comparing the results before and after heating the serum for 15 minutes at 56°C and before or after neuraminidase treatment (*vide infra*).

The sensitivity of the technique is satisfactory, since sera with only a modest amount of serum alkaline phosphatase (1.0 Bodansky-Shinowara unit) can be successfully electrophoresed without resorting to prior concentration of the serum.

The gels are cleared in hot gelatin, mounted on glass slides, and protected with a glass coverslip. In this condition, clearly interpretable photographs are taken and scanning in a densitometer (Photovolt) becomes practical and reproducible (Fig. 25).

3.4.3. *Agar-Gel Electrophoresis*

Stevenson (S47) and Port and Van Venrooy (P14) were able to demonstrate alkaline phosphatase activity following agar-gel electrophoresis. This technique was further developed by Haije and DeJong (H1), who obtained characteristic separate bands for liver and bone alkaline phosphatases. Dymling (D24) applied the technique to pregnancy serum and placenta.

Electrophoresis on cellulose acetate paper has also been employed for the characterization of human alkaline phosphatase isoenzymes by Korner (K20, K21) and Posen *et al.* (P19). A modification of the Gomori technique (G11–G13) for the histochemical localization of alkaline phosphatase was made by Allen and Hyncik (A10) to visualize the enzyme zones in the starch and agar gels.

3.4.4. *Sephadex-Gel Electrophoresis*

Although starch-gel electrophoresis has proved to be uniquely useful in the fractionation of different isoenzymes and in the separation of serum proteins (S38–S41), it also has its limitations. The development of serum protein patterns on the starch gel after electrophoresis presents no problem at all, presumably because the concentration of protein is very high (70 mg/ml). But when the starch-gel technique for the identification of protein was applied to purified preparations of tissue alkaline phosphatases, we did not succeed in visualizing the protein bands of the very low amounts of enzyme. With a view to solving the problem of resolving weak protein solutions (0.05–1.0 mg/ml), we have developed techniques of Sephadex-gel electrophoresis (I3) for the fractionation and characterization of human tissue alkaline phosphatase preparations. The patterns (Fig. 26) show the Sephadex-gel electrophoresis zymograms of an alkaline phosphatase specimen prepared from human placenta along with a variety of sera. The fractionation of human alkaline phosphatases in pregnancy serum is somewhat sharper than that obtained with starch gel.

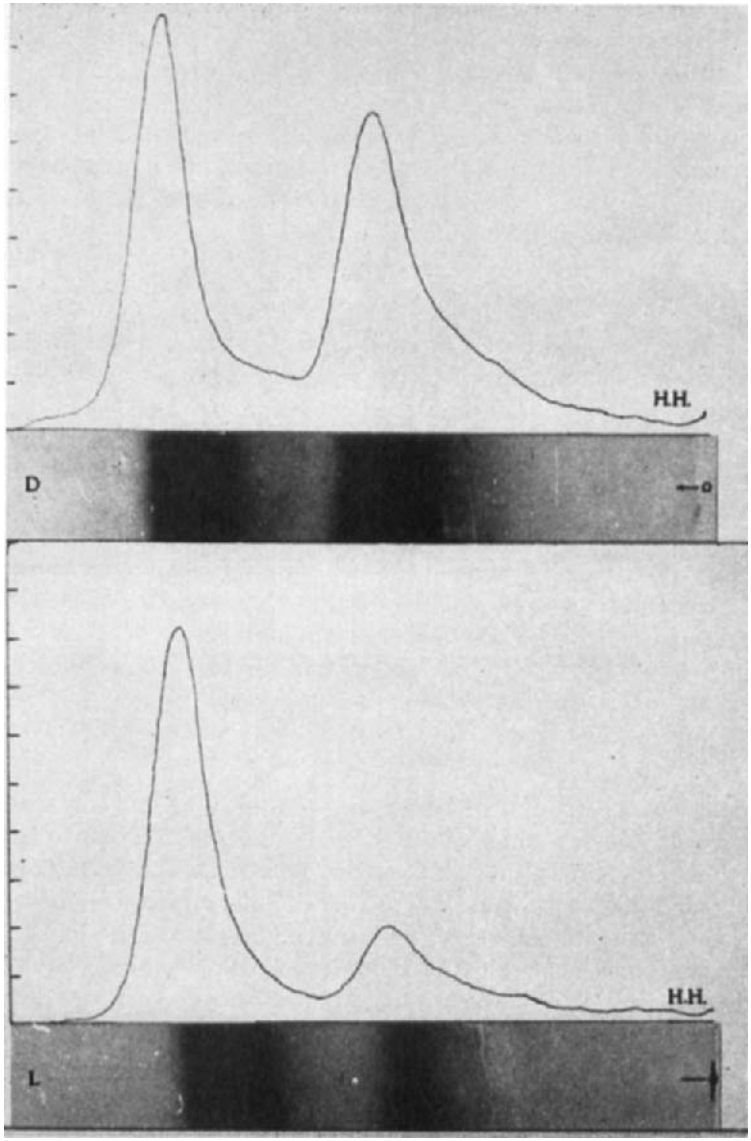


FIG. 25. Densitometer tracings and starch-gel pattern of a patient with cirrhosis of the liver. Upper gel and tracings were obtained in the presence of D-phenylalanine applied with substrate to the gel; lower gel and tracings were obtained in the presence of L-phenylalanine plus substrate. The direction of migration is from right to left. Note that the intensity of the slow-moving bands is reduced both visibly and by densitometry in the presence of L-phenylalanine. [Reproduced through the courtesy of Stolbach *et al.* (S49)].

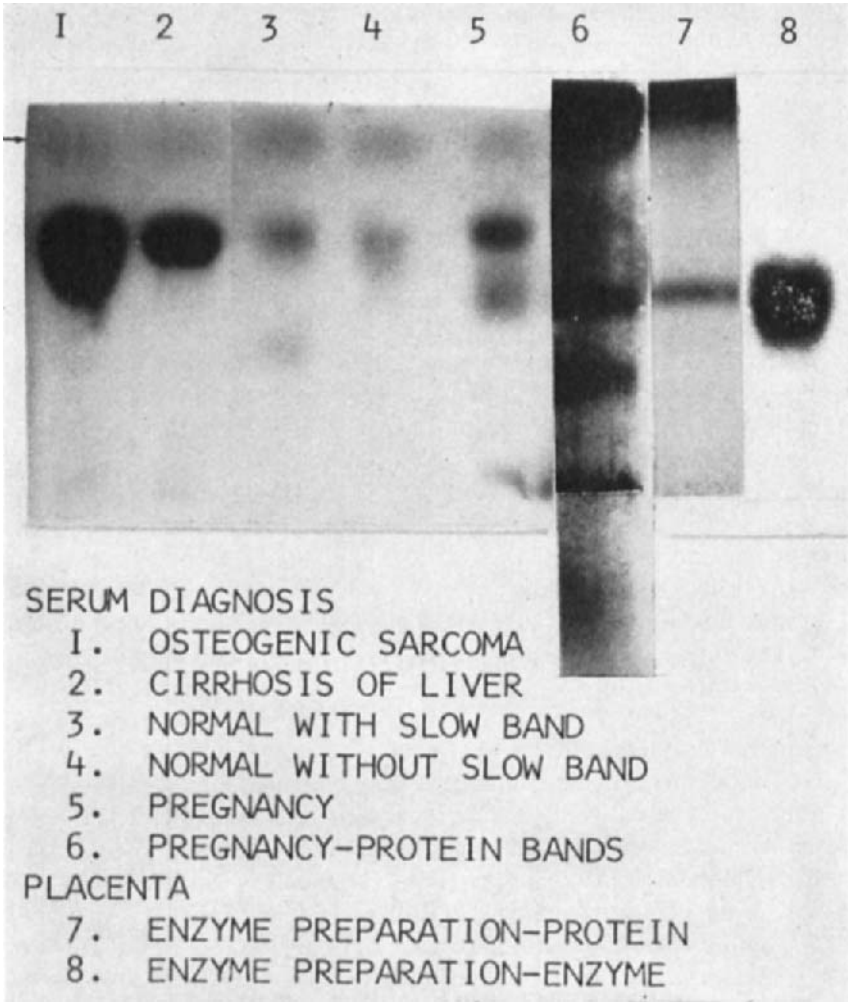


FIG. 26. Sephadex-gel electrophoresis of sera and of placenta as indicated. Albumin (*arrow*) bands are enzyme-negative. Thick gel technique (I3).

Disc electrophoresis with acrylamide gel (S35) has been developed (D7, O2) and is now frequently used for enzyme separations.

3.5. HEAT SENSITIVITY

The greater heat sensitivity of bone alkaline phosphatase as compared to that of liver, kidney, and intestine was first reported without comment by Moss and King (M36). The incubation periods (at 55°C and pH 7.0)

required to reduce activity to one-half the initial value were 8, 18, 12, and 15 minutes for bone, liver, kidney, and intestine, respectively.

By contrast, the placental enzyme is distinguished by great heat stability, its activity unchanged after 30 minutes at 70°C in the presence of Mg^{2+}, according to Neale *et al.* (N18). It is noteworthy that pregnancy serum, especially in the third trimester, possesses a preponderance of heat-stable alkaline phosphatase (M15, N18).

The Mg requirement (10^{-2} *M*) was necessary to preserve the heat stability (75°C) of alcohol-fractionated and freeze-dried placental homogenate, but not of fresh placental homogenate and serum enriched with placental enzyme (N18).

Attempts to employ the heat instability of bone to interpret the tissue origin of serum alkaline phosphatase were made by Posen *et al.*, who stated (P19), without presenting an analysis of their data, that mixtures of "osteoblastic" and "hepatic" sera exhibited the heat inactivation to be expected from their individual heat inactivation values. Magnesium ion was absent from the sera during heating. It was clearly shown that a significant degree of variation could be expected in the heat sensitivity of eight different bile specimens and five individual bone homogenates.

In this laboratory, partially purified enzyme preparations from bone, liver, and intestine have been employed in experiments designed to test the quantitative utility of the heat sensitivity. A typical experiment is reproduced in Table 9, and demonstrates the recovery in mixtures of the three organ enzymes.

Our data support the statement that the heat sensitivity of each enzyme source remains characteristic and independent of the influence of the others in the mixture, and that the resultant heat inactivation is an additive function of the heat-sensitivities of members of the mixture. Bone enzyme from different sources is very consistently heat-sensitive (85–90%), unlike intestinal (50–65%), and liver enzyme (50–75%). However, the heat sensitivity of the LPSAP of normal serum can vary from 33 to 85% and of the non-LPSAP fraction from 50 to 95%. Therefore one cannot determine the identity of the organ sources of serum alkaline phosphatase with a knowledge of only the heat sensitivity and the total alkaline phosphatase. However, by correcting the heat-inactivation of serum by that contributed by intestinal component, one obtains the heat-inactivation of non-intestinal sources of alkaline phosphatase. If this value is 90% or more, the non-intestinal component could be presumed to be of osseous origin; if 60% or less, of hepatic origin.

The L-phenylalanine-sensitive component of serum alkaline phosphatase exhibits heat sensitivity that on occasion is far below or far above the expected heat sensitivity for intestine. For this reason, it is

unjustified to employ an average tissue value for computing the heat sensitivity of intestinal and nonintestinal moieties from a knowledge of the total, LPSAP, and heat inactivation values.

The extent of heat inactivation of the LPSAP and non-LPSAP moieties of serum alkaline phosphatase is a measurement which is an important feature of the biochemical pattern of serum alkaline phosphatases to be described in Section 7.

3.6. Alkaline Phosphatase as a Substrate for Neuraminidase

Neuraminidase is an accepted reagent for identifying terminal sialic acid residues in protein (S21), which in turn permits one to consider such a hydrolyzable protein as a glycoprotein (sialoprotein). The loss of the

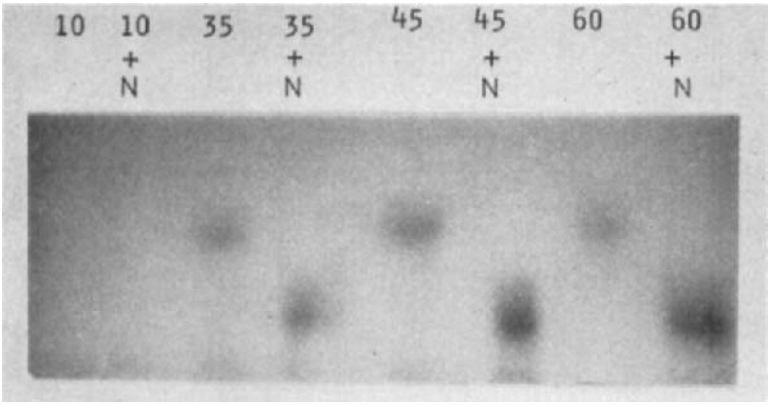


Fig. 27. Action of neuraminidase on Sephadex-gel filtrates (Fig. 18) containing placental alkaline phosphatase. Four experiments with $(\overset{+}{N})$ and without neuraminidase are illustrated. Note that the electrophoretic mobility (toward anode) of the fast-moving band is reduced by neuraminidase treatment.

strongly acidic sialic acid residue lowers the anodic mobility of the sialic acid-free protein remnant (Fig. 27).

Does the failure of a protein, following neuraminidase treatment, to undergo change in electrophoretic migration mean that it is not a sialoprotein? Also, does the change in migration represent the release of one, two, or more sialic acid molecules per mole of enzyme? If alkaline phosphatase is a substrate for neuraminidase, what are the pH and substrate requirements for optimal hydrolysis? What is the evidence for the identity of the product of neuraminidase hydrolysis?

The experimental answers to these questions have been sought in our laboratory (G6), using placental alkaline phosphatase. Applying acid hydrolysis to highly purified placental alkaline phosphatase and then the

TABLE 9

MEASUREMENTS OF BONE AND LIVER ALKALINE PHOSPHATASE BASED ON HEAT INACTIVATION[a]

Time at 55°C	Alkaline phosphatase activity	Heat-inactivation (%)		Intestine[b]	Bone		Liver	
		Found	Expected	Found	Found	Expected	Found	Expected
Bone (B)								
0	9.16							
16′	0.80	91.2	—	—	—	—	—	—
Liver (L)								
0	8.68							
16′	4.22	51.4	—	—	—	—	—	—
Intestine (I)								
0	10.19							
16′	4.50	55.8	—	—	—	—	—	—
Proportions of B/L/I								
1:1:1								
0	9.40							
16′	3.12	66.9	66.1	3.40	3.25	3.05	2.75	2.89

2:1:1								
0	9.81							
16′	2.55	74.1	72.4	2.55	5.37	4.58	1.89	2.17
1:2:1								
0	9.67							
16′	3.52	63.6	62.6	2.56	2.73	2.29	4.38	4.34
1:1:2								
0	9.94							
16′	3.65	63.3	63.4	5.12	2.40	2.29	2.42	2.17

[a] The tissue preparations were diluted (1:12) in heat-inactivated sera (1 hour at 55°C), and incubated in 0.02 *M* Veronal buffer (pH 9.8) containing 0.018 *M* disodium phenyl phosphate for 2 hours at 37°C. Phenol was measured via a diazo coupling procedure. Conditions for heat-inactivating the tissue enzymes were 16 minutes at 55°C. By subtracting from the total activity the intestinal component, which is measured by L-phenylalanine sensitivity, one obtains the sum of the activities of liver and bone. The ratio of the two was computed from the heat inactivation minus that attributed to intestine, employing 91.2% heat inactivation to represent 100% bone and 51.4% heat inactivation indicating all liver. In this way one arrives at values for bone, liver, and intestinal alkaline phosphatase.

[b] This was computed by multiplying the D minus L value by 100/77.

thiobarbituric acid color reaction, not less than 1.5% of the enzyme is sialic acid. Of this amount, the major part (75%) is released by prolonged incubation with neuraminidase. The absorption maximum of the hydrolyzate following the thiobarbituric acid reaction matched the one for pure sialic acid (Fig. 28), permitting the statement that placental alkaline phosphatase is sialoprotein. Although our supply of pure placental enzyme was inadequate for completing a kinetic study based on substrate concentration, it was possible to measure the pH optimum

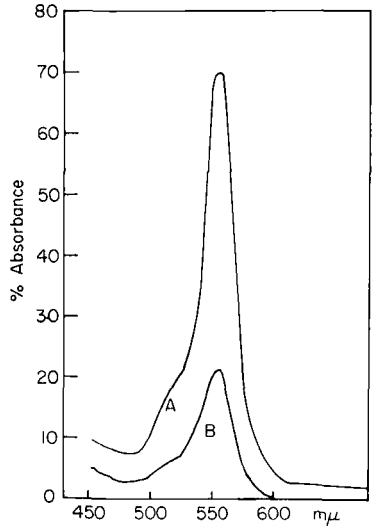


FIG. 28. Light absorption spectrum of the pigment in the sialic acid color reaction (Warren-thiobarbiturate technique): (A) pure sialic acid, and (B) the product of hydrolysis of placental alkaline phosphatase (G6a).

(Fig. 29) for the liberation of sialic acid from purified placental alkaline phosphatase.

It is reasonable to assume that all human alkaline phosphatases are sialoproteins, but the distinction in the case of intestine is that the sialic acid residues may not be terminal or that the terminal residues are "buried" in the three-dimensional structure of the enzyme.

Of interest are recent observations from Harris' laboratory demonstrating that graded neuraminidase concentrations in placental alkaline phosphatase digests yielded up to eight bands (R16). Participation of neuraminidase-sensitive sialic acid in the K^+-dependent nitrophenyl phosphatase in isolated rat liver plasma membranes (E1) has been reported.

4. Relation of Alkaline Phosphatase to Ultrastructure

4.1. Examples of Localization on Absorptive Membranes

The location of alkaline phosphatase in the cell and tissue is information basic to a full explanation of an increase in serum alkaline phosphatase. Modern techniques of enzyme histochemistry and of electron microscopy (R2) have succeeded in providing a clearer picture (G10, M22). The cell wall is the location at the electron microscope level of

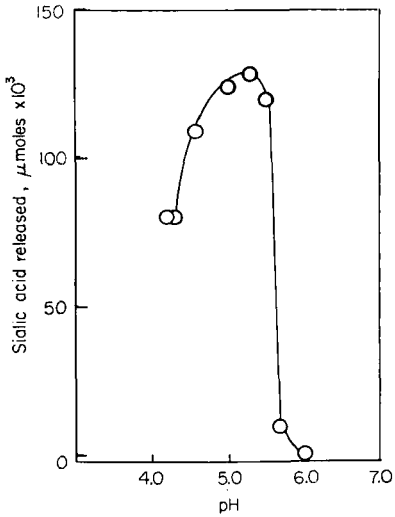


Fig. 29. Optimum pH of hydrolysis of human placental alkaline phosphatase by neuraminidase. The liberated sialic acid was measured by the Warren-thiobarbiturate procedure, using *N*-acetylneuraminic acid as the standard (G6a).

alkaline phosphatase in *E. coli* (D20, G2, G3, M31). The enzyme is also present in isolated rat liver cell-wall membranes studied biochemically (E1).

Sites of alkaline phosphatase activity are frequently in endothelial cells of blood capillaries, mucous glandular cells (F3), microvilli of intestine (C6, C10, D2, H21, P8, W7), bile canaliculi (D21, F27, W2), and placenta (W3), as well as in the brush border of the lumenal surface of epithelial cells of the proximal convoluted renal tubules (M22, W1). The location of L-phenylalanine-sensitive alkaline phosphatase in human intestine and placenta is illustrated in Fig. 30. Electron micrographs (Fig. 31) show the details of the alkaline phosphatase, and illustrate the

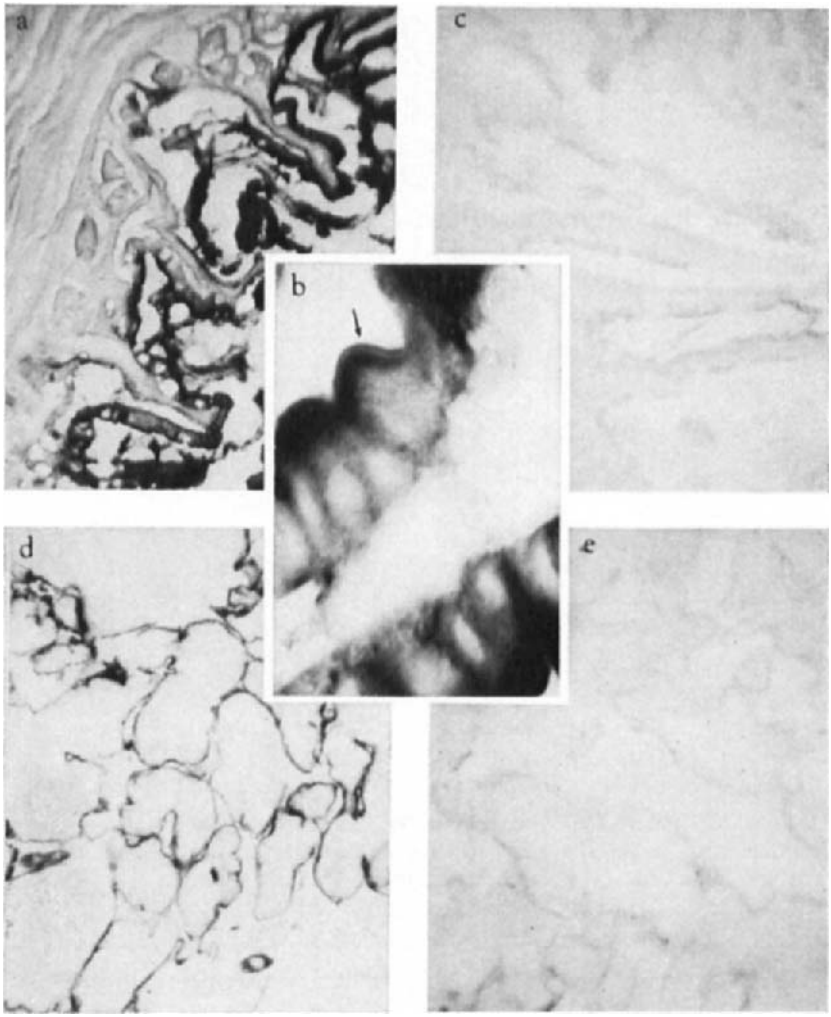


FIG. 30. Enzyme staining reactions for L-phenylalanine-sensitive alkaline phosphatase in human placenta and intestine [conditions were those of Watanabe and Fishman (W7)]: (a) human intestine in the presence of D-phenylalanine, ×400; (b) high power view of human intestine showing brush border (*arrow*), terminal web, and apical concentration of alkaline phosphatase, ×1200; (c) human intestine in the presence of L-phenylalanine, ×400; (d) human placenta in the presence of D-phenylalanine, ×400; (e) human placenta in the presence of L-phenylalanine, ×400. Note that the enzyme location is on the peripheral absorptive surfaces of the intestine and placenta.

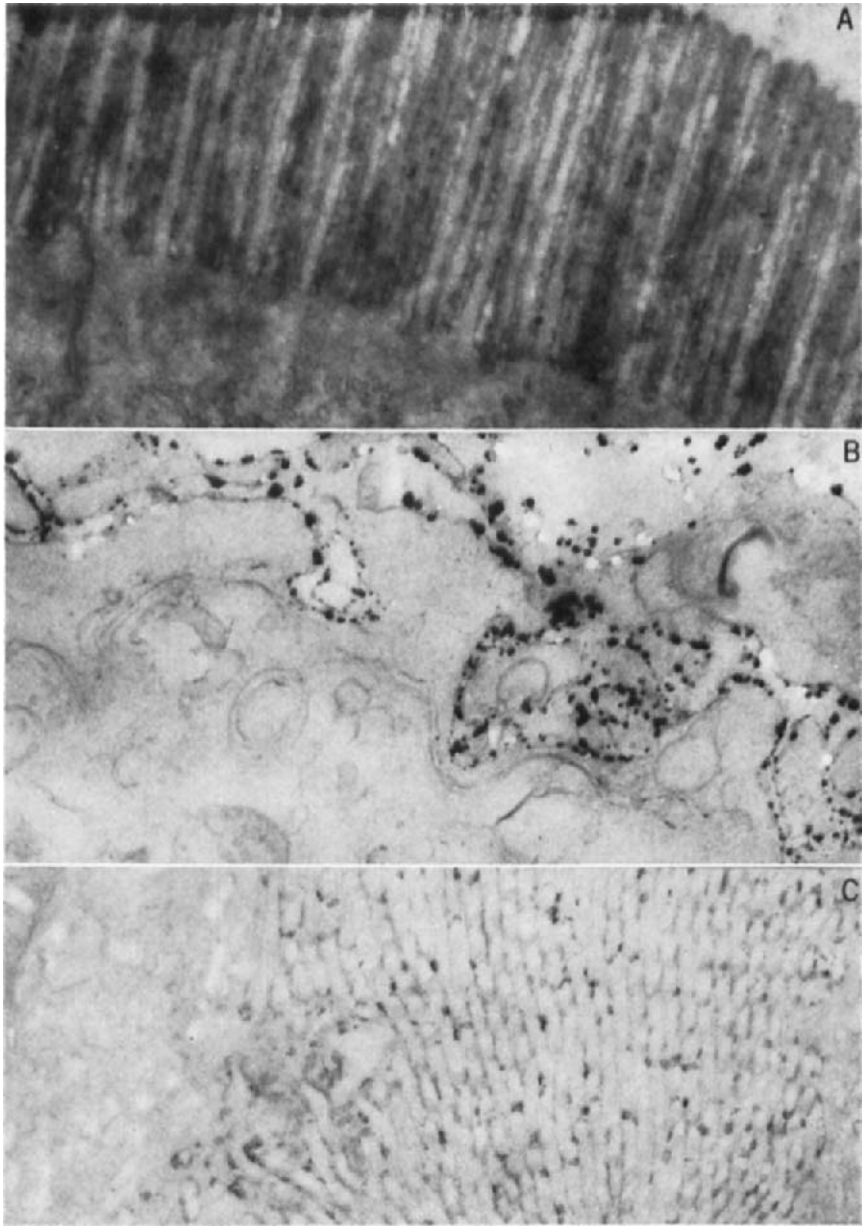


FIG. 31. (A) Rat duodenum stained for alkaline phosphatase with azo dye method (B5a). Semidense particles of azo dye are deposited in the plasma membranes of the microvilli; ×70,000. (B) Placental labyrinth cell reacted for alkaline phosphatase with the cadmium method (M22). Deposits are related only to the cell surface; ×55,000. (C) Brush border of proximal tubule cell of the kidney showing deposits of final product related to the surface of the microvilli. Cadmium method of Mizutani and Barrnett (M22); ×35,000. Electron micrographs, courtesy of R. J. Barrnett.

characteristic location of alkaline phosphatase on the outermost surfaces of membranes whose function is their participation in active transport and absorption.

4.2. Site of Production of Alkaline Phosphatase in Absorptive Cells

There appears to be no research that traces the path of synthesis of alkaline phosphatase to its final location on the absorptive surfaces of cell-wall membranes. The usual sequence of specific enzyme synthesis envisions the collection of newly synthesized protein in secretion vacuoles that eventually leave the cell of origin as part of a secretion process. The sites of synthesis are the ribosomes of the rough-surfaced endoplasmic reticulum.

On the other hand, the Golgi apparatus is implicated in the synthesis of cell-wall membranes. It is possible that the alkaline phosphatase is a catalytically inactive, structural protein of such cell membranes when they are still in the cytoplasm, which may explain many failures to demonstrate its presence in the Golgi apparatus. Upon becoming part of the outer surface, say of the brush border of the intestinal villus, the enzyme may be activated by the alkaline contents of the duodenal lumen. The concept of catalytically inactive membrane enzyme proteins is developed in a recent publication with regard to β-glucuronidase (F10).

5. Alkaline Phosphatase Isoenzymes as Related to Physiological Processes

5.1. Induction by Steroid Hormones

Many studies in the realm of microbiology (B36, M6, N26, R25) have emphasized the role of substrate and hormone induction of enzymes. It is not surprising, therefore, that similar mechanisms have been searched for, have been found, and are being studied in mammalian systems.

Beginning with Nitowsky and Herz's observations of quantitative differences between established and recently isolated cell lines (K4) of diverse origins, demonstrations have been published of substrate induction (N23), of induction by osmolarity of the nutrient medium (N25), and of induction by adrenal glucocorticoids (C19, C20, N24).

Interesting indeed are the recent studies of Griffin and Cox (G20, G21) on the mechanism of adrenocorticoid induction of alkaline phosphatase in human cell cultures. Two temporal phases in the induction of enzyme in HeLa cells are reported: a first 12-hour period exhibiting a slow rise in activity, followed by a sudden marked linear increase extending from 15 to 80 hours. The elevation of activity is marked (around 10-fold). The fact that puromycin blocked induction immediately, and actinomycin D

after the first phase, was explained by *de novo* synthesis of alkaline phosphatase and a relatively stable mRNA. This opinion has been revised in that *de novo* synthesis is not implicated, but the enhanced activity is attributed to steroid-induced change in the conformational state of the enzyme during its synthesis.

Human leukocytes possess alkaline phosphatase (L13, O3, R10, T6, V3, V4) whose activity parallels the concentration of certain circulating steroids. Thus, during the menstrual cycle, the leukocyte alkaline phosphatase evidences a peak at midcycle corresponding to the higher estrogen level. In the later stages of pregnancy the enzyme level increases. Corticoid hormones also exert an effect on leukocyte alkaline phosphatase.

Recently Watson *et al.* have demonstrated intestinal alkaline phosphatase level is normally under adrenal control (W7a). Hydrocortisone administration produced large increases in intestinal alkaline phosphatase activity.

There is sufficient evidence to consider enzyme induction as a process which could explain elevations in tissue (V8) and in serum alkaline phosphatase levels.

5.2. Relation to Fat Absorption

When rats are placed on a high-fat diet, the intestinal mucosa and the blood become enriched with alkaline phosphatase (F19, F20, M1, M2). That the striated borders of the absorptive epithelial cells in rats possess the L-phenylalanine-sensitive alkaline phosphatase was proven by Watanabe and Fishman (W7). Human intestinal cells grown in tissue culture (W7) and human intestine exhibit L-phenylalanine sensitivity. Phosphatase has long been found to be present in feces (L8).

More recently, studies in man have demonstrated the enrichment of the thoracic lymph with alkaline phosphatase during fat absorption (K6, K7). This phosphatase was L-phenylalanine-sensitive and was located as a slow-moving band on starch-gel electrophoresis, proving it to be intestinal in origin.

In this laboratory, subjects ingesting a high-fat breakfast evidence an increase in L-phenylalanine-sensitive serum alkaline phosphatase, which shows wide individual differences (I2). Harris and co-workers (L2), in an interesting genetic study, have demonstrated a similar increase that correlates well with the slow-moving intestinal band in the starch-gel electrophoresis. Most interesting is the observation that the slow-moving band is missing from the serum of individuals lacking the ability to secrete ABH substance in the saliva ("secretors"). Persons possessing blood types B and O exhibited the intestinal component most frequently.

In the studies of the London group, there appeared to be no association

between the turbidity of the serum in the postabsorption state and the blood type or secretor status. Among the possibilities considered to explain the effects of blood type and secretor status were the following: differences in the processes of fat absorption, differences in the alkaline phosphatase content of the intestinal mucosa, and variations in the permeability of absorptive cells. The lipoprotein particles in cow and human milk are rich in alkaline phosphatase (H10, M27), as are β-lipoproteins of human serum (L7). Studies of the effect of vitamin A on phosphatase in various tissues have appeared (M19).

5.3. Active Transport of Amino Acids and Sugars

Force-feeding of L-amino acids to rats resulted in an increase in both intestinal and serum alkaline phosphatase (T5). It is reasonable to assume that the association is not merely circumstantial, because intestine (S31) possesses the enzyme systems necessary for activating (H16) the carboxyl group of L-amino acids via adenosine triphosphate or its generating system (K8). Examples can be found in the recent literature in which intestinal absorption of amino acids can be reduced by the presence of glucose, and vice versa (F2, H13, S8). An association of the absorption of carbohydrates (glucose, dextrin) with a rise in serum alkaline phosphatase was reported over 30 years ago by A. Bodansky (B25, B26), who first raised the question of nonosseous origin of serum alkaline phosphatase (B27). That the intestine contributed alkaline phosphatase to the circulation was inferred from the fact that fasting reduced serum alkaline phosphatase.

A systematic study with modern methods has been undertaken by Lincheer *et al.* (L14) of the relationship of intestinal alkaline phosphatase and glucose absorption. Apparently glucose is capable of stimulating the intestinal mucosa to secrete an L-phenylalanine-sensitive alkaline phosphatase.

5.4. Osteogenesis

The first physiological function attributed to alkaline phosphatase (R11–R14) was that of liberating inorganic phosphorus from organic phosphates and the consequent deposition of calcium phosphate at the site of osteogenesis. This concept has been accepted for many years and a number of clinical observations are consistent with it (C9). Thus in situations of bone growth or repair such as healing fractures, metastatic bone lesions, rickets, or Paget's disease, the serum becomes enriched with alkaline phosphatase.

In analogy with the intestinal and kidney alkaline phosphatase that is

associated with absorption, active transport, etc., one should look for a similar physical location and physiological function for osteoblasts. It may turn out that the cell has a restricted permeability for divalent cations such as Ca^{2+} and that these deposit in the extracellular space along with phosphate. It must be pointed out, however, that no modern studies give any indication of the rate of production of bone alkaline phosphatase and its access to the circulation.

5.5. Pregnancy and Placental Physiology

French workers (C4, C18) discovered in 1934 that pregnancy serum exhibits elevations in alkaline phosphatase. The origin of the enzyme has been attributed variously to maternal osteoblastic activity, to fetal osteoblastic activity, and to placenta. Following Boyer's electrophoretic studies (B38), which showed that serum exhibits a strong enzyme band in the placental region, and Posen's demonstration of heat-stable placenta-type alkaline phosphatase in serum (C13), the reasonable view now is that the placenta supplies alkaline phosphatase to the circulation.

Although few studies (M5) have been made on the physiological role of placental alkaline phosphatase, it is reasonable to assume that it is concerned with absorption and active transport of maternal nutrients destined for the maintenance of fetal life.

Donayre and Pincus have studied serum alkaline phosphatase in the menstrual cycle (D19), and Climie *et al.* (C11) have related neutrophile alkaline phosphatase and pregnancy.

Kerr and Waisman (H19, K9) suggested that L-phenylalanine is actively transported across the placenta from the maternal blood to the fetus. Thus, a pregnant female who is a phenylketonuric may exert a profound effect on the fetus by her abnormally high serum L-phenylalanine levels, leading to the birth of mentally retarded children. It is tempting to suggest that alkaline phosphatase may be involved in this important physiological process since, in fact, brain damage and the high blood L-phenylalanine in phenylketonuric patients are associated with a reduced level of brain alkaline phosphatase. However, more detailed investigation (C3) would be necessary to arrive at a definite conclusion with respect to the precise role played in brain metabolism by L-phenylalanine. The studies on enzyme inhibition by phenylalanine and the related investigations are reported by other workers (B1, B4, R26, S9).

5.6. Development

Extensive studies by Moog (E11, G19, N16, M23–M26) have described the nature of the alkaline phosphatase in the microvilli of the duodenum during development in the mouse, rat, and chick. There is a rise in

activity that parallels development, high concentrations accumulating at critical periods that just precede the onset of absorptive capacity. Cortisol accelerates the differentiation of the duodenum with a consequent earlier appearance of high duodenal alkaline phosphatase. Also, evidence has been reported here for a strain-specific factor in mouse milk that influences duodenal phosphatase activity.

The increase in enzyme activity is observed to correlate with a rise in the phenylphosphate-preferring moiety as compared to the moiety with an ability to hydrolyze β-glycerophosphate. The elevation is enhanced, not prevented, by actinomycin D administered to mice, suggesting conformational alterations rather than *de novo* synthesis. The phenylphosphate-preferring enzyme was located in the proximal part of the duodenum and little was present in the distal part of the duodenum and the jejunum. Proof of the isoenzyme nature of the two substrate-preferring activities was recently published. These activities were separated by DEAE-cellulose chromatography and by starch-gel electrophoresis (M26). A growing literature is appearing in the field of development (D3, D14, M39).

5.7. Homeostasis

It is a fact that, in a normal adult healthy individual, the serum alkaline phosphatase remains relatively constant. This event is indistinguishable from the constancy of other serum proteins such as albumin, glycoproteins, etc. Therefore the considerations that apply to the homeostasis of serum proteins logically apply to serum enzymes. These have been discussed elsewhere (F5).

An interesting study on the fate of injected placental alkaline phosphatase (N17) has been reported by Posen *et al.* (P18), who measured heat-stable alkaline phosphatase. Following injection, there was an initial rapid fall in enzyme activity lasting 3–5 hours and then a less rapid disappearance. After 4 hours, two thirds of the initial activity remained, but it took several weeks for complete disappearance. These kinetics resembled those known for serum albumin labeled with iodine-131.

Because of the similar behavior of injected alkaline phosphatase and albumin, it becomes unnecessary to consider the liver as an excretory organ for serum alkaline phosphatase to any greater extent than it is required to excrete albumin, which has never been considered a significant process.

A consideration of importance relates to the various routes and physical states by which alkaline phosphatase enters the circulation. Thus, during fat absorption the intestinal alkaline phosphatase is incorporated into

chylomicrons, which travel in the intestinal lymph to the jugular vein (B22, K6, K7). The fate of this form of alkaline phosphatase should reasonably be expected to be that of the chylomicrons. These particulates become substrates for lipoprotein lipase in endothelial cells and may be taken into Kupffer cells intact. On the other hand, alkaline phosphatase being absorbed with carbohydrates and with amino acids would enter the venules draining into the portal vein, and enter the liver sinusoids without mixing with the systemic circulation. Accordingly, the intestinal alkaline phosphatase contribution to the circulation represents a mixture that has traveled two independent routes, and has been subjected to an entirely different set of experiences on each route.

The liver must be producing alkaline phosphatase to renew that on the outer surfaces of the bile canaliculi whose contents empty not into venules but into the common bile duct. The amount of alkaline phosphatase in bile is of a low order, and quantitatively can bear little comparison to that produced by the intestinal mucosa. There is the possibility, however, that the biliary alkaline phosphatase can be reabsorbed in an enterohepatic circulation.

Homeostasis of serum alkaline phosphatase must therefore necessarily involve diverse processes proceeding at different rates and by various routes, in which the liver must necessarily play several roles. Accordingly, hyperphosphatasemia in liver disease requires for its understanding a knowledge primarily of how the damaged liver handles intestinal alkaline phosphatase.

6. Genetics

6.1. Lower Forms of Life

In developing *Drosophila,* Schneiderman *et al.* (S20) observed that development is characterized by the presence of organ-specific bands visible on starch-gel electrophoresis. Moreover, two separate chromosomal loci have been demonstrated: one designated *Aph* (larval) is on chromosome III (B12), and the other locus, *Aph*-2, is on chromosome II. It is highly significant that the type of alkaline phosphatase in an organ is under genetic control.

Genetic studies on the domestic fowl (K28, L6) have demonstrated that a fast-moving form of alkaline phosphatase is determined by a simple autosomal dominant gene (Ap^2) that is allelic to the slow-moving Ap^4 allele. The organ source of the plasma enzyme was not identified. Among species lower than man, genetic control of alkaline phosphatase has been observed in sheep (R1, R4) and cattle (G1).

6.2. Blood Group Association

The physiological genetics of human blood factors (C5) now includes an association between ABO blood groups, secretor status, and the slow-moving LPSAP band in the starch-gel zymogram for serum alkaline phosphatase.

The earliest reports of an association between human serum phosphatases and blood groups did not clearly relate to alkaline phosphatase (A17), as did the later ones from Sweden and England (B5, B10, B11). In addition, the relevance of secretor status was first assigned to the Lewis factor (A18, B10) and later changed to the ABH substance in saliva (B11, S32). Shreffler's (S33) and Beckman's analyses carried out in twins and families show that factors, in addition to blood groups B and O and ABH secretor factors, influence the variation in activity of a slow-moving alkaline phosphatase band. Conceivably, however, single gene control may have been obscured by age, temporal effects, diet, and inadequacies of visual grading of gel bands. Shreffler designates bone as the source of the fast-moving band and probably intestine as the organ derivation of the slow-moving B band.

Arfors *et al.* (A17) suggested that there was an actual chemical linkage between alkaline phosphatase protein and blood group H substance. However, Aalund *et al.* (A1), working with the (sheep sera) slow-moving band, were able to separate the enzyme protein from blood group substance by chromatography. The association is probably physical in nature. Confirmatory studies (E12, P23) have appeared in relation to ABO blood, secretor, and alkaline phosphatase.

The identity of the slow-moving band observed in all of these studies is the intestine, based on its L-phenylalanine sensitivity (F9, K25, R7, S49, N20, W6) and resistance to neuraminidase (R7). This is even more certain following the demonstration of an elevation in thoracic lymph and in blood serum of L-phenylalanine-sensitive alkaline phosphatase in subjects absorbing fat. The slow-moving band becomes intensified in sera of individuals who are secretors (K7, L2).

6.3. Placental Studies

Harris and his group (R15, R16) in London recently initiated genetic studies on human placental alkaline phosphatase, and demonstrated phenotypic differences in this enzyme. In this study, butanol extracts of alkaline phosphatase from each of 338 placentas were prepared, and the enzyme preparations were subjected to starch-gel electrophoresis at two different pH's (8.6 and 6.0). The electrophoretic patterns obtained were classified in six different groups representing six distinct phenotypes. The

studies were extended to the pairs of placentas derived from dizygotic twins, and these results suggested that probably three autosomal allelic genes are controlling and determining the polymorphism of placental alkaline phosphatase. In earlier studies, Boyer (B39) also put forward the similar suggestion that the electrophoretic difference of human placental alkaline phosphatase might be under genetic control. These studies have certainly great potentiality in unraveling the roles that the chromosomal genes play in regulating alkaline phosphatase synthesis, and hence may throw much light in the near future on the factors related to the interpretation of human disease involving alkaline phosphatase isoenzymes.

6.4. Chromosomal Studies

Polymorphonuclear leukocyte alkaline phosphatase (R10, T6, V3, V4) has attracted attention with regard to its elevation in mongoloids exhibiting trisomy 21 and its deficiency in patients with chronic myelogenous leukemia in whom one of the short acrocentric chromosomes is missing.[3] Accordingly, there is reason to implicate chromosomes whose genes determine the extent of leukocyte alkaline phosphatase activity.

A particularly relevant study by De Carli *et al.* (D9) is described. A karyotype analysis was made of both a very high alkaline phosphatase strain of an embryonic clone of strain EUE cells and two sublines (1 and 2) of cells exhibiting very low activity. Cells of lines Sub 1 and Sub 2 show a consistent reduction of one or two units in long acrocentric and a much more pronounced reduction in short acrocentric chromosomes. The high EUE cells accordingly have twice as many short acrocentric chromosomes. Finally, there was no simple relationship between chromosome dose and levels of enzyme activity in these cell lines, although this relationship has been seriously considered by Alter *et al.* (A12, A13) for the leukocytes.

7. Distinctive Biochemical Properties in Relation to Isoenzymes

7.1. Experimental Approach to Measurements

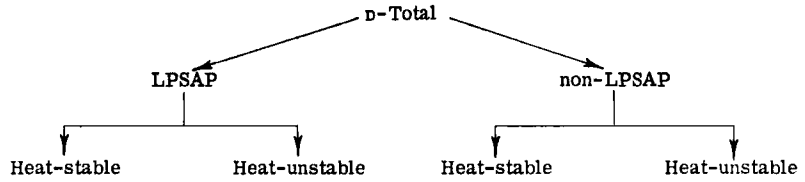
In our opinion, it is unwise to base a classification of isoenzymes solely on starch-gel electrophoresis data. For example, the work of Boyer, Chiandussi, and many others (B39, C7, M33–M36) shows a great complexity in the location of bands derived from individual organs. True, one can identify the heat-stable or -unstable zones and even those

[3] Conference on "Leukocyte Chemistry and Morphology Correlated with Chromosome Anomalies," N. Y. Academy of Sciences, November 3–5, 1966.

bands sensitive to L-phenylalanine (S49, W6). However, reliance on studies of this type provides only qualitative information, in which the variability of staining intensity of bands and of the visual acuity of the observer introduces a considerable amount of subjectivity. On the other hand, biochemical properties of isoenzymes offer opportunity for quantitative measurements of LPSAP, non-LPSAP, and their respective heat-sensitive fractions. Moreover, to this information can be added observations on starch gel, in which the results of neuraminidase treatment, L-phenylalanine inhibition, and heat inactivation of the serum alkaline phosphatase are visible. Next, with the knowledge of the blood type and secretor status, a further means of classification is introduced.

7.2. Biochemical Patterns of Serum Alkaline Phosphatases

The pattern requires the division of alkaline phosphatase activity into two main parts (LPSAP and non-LPSAP) and each of these into two subgroups (heat-stable and heat-unstable moieties):



The analysis of such data obtained on *normal* subjects is considered basic to attempts to interpret hyperphosphatasemia in patients.

Data to be presented in Sections 7.2, 8, and 9.2 were all obtained with the manual method (S49, F14). It is to be expected that these results may differ numerically to some extent from those now being collected in greater numbers with the AutoAnalyzer. Accordingly, Tables 10, 11, 12, and 14 are significant in that they provide an experimental basis for developing the concept of a biochemical pattern for serum alkaline phosphatase activity. The figures (mean $\pm$ SD) will no doubt require revision with the availability of AutoAnalyzer data by the method of Fishman and Green (F8).

Individuals characterized by the slow-moving band in the intestinal position are invariably secretors of ABH substance according to Langman *et al.* (L2). This is the basis for dividing the normal subjects into two groups, one with the slow-moving band and the other without the slow-moving band. Some secretors with A blood type fail to exhibit the band (L2).

The faster-moving components in subjects irrespective of blood groups

TABLE 10

DIFFERENT MOIETIES OF ALKALINE PHOSPHATASE (KA UNITS/100 ML) IN NORMAL SUBJECTS

PART I: SLOW INTESTINAL BAND ABSENT

No.	Blood Type	Sex	D-total Value	LPSAP (D − L) Value	LPSAP (D − L) % Heat sensitive	Non-LPSAP (L) Value	Non-LPSAP (L) % Heat sensitive	Ratio of heat-stable non-LPSAP/LPSAP
(1)	A	M	5.0	1.0	49.0	4.0	72.8	2.10
(2)	A	M	5.8	1.2	71.0	4.6	89.9	1.43
(3)	A	F	5.7	1.3	72.0	4.4	85.8	1.80
(4)	A	M	7.1	1.9	72.0	5.2	64.3	3.44
(5)	A	M	9.4	1.5	63.4	7.9	76.3	3.44
(6)	A	M	9.1	1.6	61.4	7.5	62.1	4.70
(7)	A	M	8.2	2.0	77.3	6.2	62.1	4.90
(8)	A	M	6.3	1.5	56.0	4.8	61.2	2.81
(9)	A	M	7.6	1.0	0.0	6.6	63.3	2.36
(10)	A	M	17.4	2.5	50.0	14.9	52.0	7.00
(11)	A	F	7.9	2.0	78.6	5.9	73.4	1.23
(12)	AB	F	3.4	0.6	49.5	2.8	80.9	2.00
(13)	AB	M	16.2	2.5	52.6	13.7	65.9	5.20
(14)	AB	M	13.7	2.6	80.0	11.1	75.8	5.60
(15)	AB	F	5.8	1.7	81.0	4.1	76.1	3.20
(16)	O	F	6.6	1.0	48.0	5.6	34.9	7.60
(17)	O	F	11.7	2.1	53.5	9.6	74.3	2.40
Mean ± SD			8.6	1.6	59.7	6.9	68.9	3.55
			±3.9	±0.6	±20.2	±3.5	±8.8	±1.93

PART II: SLOW INTESTINAL BAND PRESENT

No.	Blood Type	Sex	D-total Value	LPSAP (D − L) Value	LPSAP (D − L) % Heat sensitive	non-LPSAP(L) Value	non-LPSAP(L) % Heat sensitive	Ratio of heat-stable non-LPSAP/LPSAP
(18)	A	M	7.6	1.9	71.8	5.7	79.2	2.26
(19)	A	M	5.7	1.7	58.5	4.0	72.8	1.47
(20)	B	M	10.5	1.8	50.0	8.7	79.9	1.95
(21)	B	F	11.0	2.5	52.5	8.5	88.3	0.83
(22)	B	M	7.4	2.2	61.9	5.2	79.2	1.30
(23)	B	M	5.9	2.0	34.0	3.9	81.8	0.53
(24)	B	F	6.5	1.4	58.0	5.1	73.2	1.03
(25)	B	M	10.5	2.8	55.0	7.7	75.0	1.53
(26)	B	F	8.6	2.6	66.0	6.0	79.8	1.33
(27)	AB	M	8.1	2.6	70.7	5.5	85.9	1.00
(28)	AB	M	11.3	2.6	34.0	8.7	64.1	1.81
(29)	O	F	4.6	1.7	78.0	2.9	74.2	2.10
(30)	O	M	7.8	3.1	58.0	4.7	84.1	0.57
(31)	O	M	7.0	2.2	36.0	4.8	70.0	1.04
(32)	O	M	9.7	1.9	34.0	7.8	68.0	2.05
(33)	O	F	14.0	2.4	41.0	11.6	68.9	2.50
Mean ± SD			8.5	2.2	53.7	6.3	76.5	1.45
			±2.4	±0.5	±14.5	±2.3	±6.9	±0.72

or secretor status undoubtedly include some LPSAP activity and a mixture of heat-stable and heat-unstable components.

This order of evaluation (biochemical, first; starch gel, second) is amply justified from our own experiences and the consensus of opinion at a recent symposium on multiple molecular forms of enzymes.[1] In particular, heterogeneity of human intestinal alkaline phosphatase on starch gel was reported by Moss (M34) and Fishman and Kreisher (F9, K25). A certain amount of LPSAP intestinal enzyme may occupy the same position that liver alkaline phosphatase migrates to. On the other hand, more than one band in nonliver positions can be produced by intestine. Another similar situation is seen with placental alkaline phosphatase, which may show three bands. Consequently, the starch-gel data can be correlated with biochemical studies only if the nature and organ source of the preparation are known in advance.

The biochemical partition data on serum alkaline phosphatase do not in themselves justify inferences regarding the number of individual forms of the enzyme present in the serum. However, they provide a profile for that individual, which may be of significance in quite a different fashion (*vide infra*).

8. Normal Subjects

In Table 10 (Parts I and II) are listed the data on the partition of serum alkaline phosphatase for 33 normal subjects according to blood type and status of the slow intestinal band. A statistical analysis appears in Table 11.

TABLE 11
SUMMARY OF STATISTICALLY SIGNIFICANT DIFFERENCES IN NORMAL SUBJECTS WITH AND WITHOUT THE SLOW BAND

No.	Moiety	Slow Intestinal Band: Present (n = 16)	Slow Intestinal Band: Absent (n = 17)	t	P
(1)	Total LPSAP	2.2 ± 0.5	1.6 ± 0.6	10.4	<0.001
(2)	Heat-stable LPSAP	1.0 ± 0.4	0.6 ± 0.3	3.4	<0.01
(3)	% Heat-sensitive non-LPSAP	76.5 ± 6.9	68.9 ± 8.8	2.7	<0.02
(4)	Ratio heat-stable non-LPSAP/LPSAP	1.5 ± 0.7	3.6 ± 1.9	4.2	<0.001

Those individuals whose sera on starch gel exhibited a slow band in the intestinal location had a greater amount of LPSAP that was heat-stable compared to those persons lacking the slow bond. In the latter, however,

the non-LPSAP was more heat-stable, and contributed to a higher ratio of this component to the heat-stable LPSAP (line 4, Table 11).

In the comparison of individuals possessing blood type A versus blood type B (Table 12), the significant differences are the percent heat-sensitive non-LPSAP and the ratio of the heat-stable non-LPSAP to LPSAP (lines 8 and 10). Blood type B exhibits more total and heat-stable LPSAP than blood type A, a pattern that is expected in a group showing the slow intestinal bond compared to a group lacking it. This in fact is the case for blood type B individuals (Table 10).

TABLE 12
STATISTICAL COMPARISON OF MEAN VALUES OF DIFFERENT MOIETIES OF SERUM ALKALINE PHOSPHATASE IN BLOOD GROUP A AND B SUBJECTS

No.	Moiety	Blood type A (n = 11)	Blood type B (n = 7)	t	P
(1)	D-Total	8.6 ± 3.6	8.6 ± 2.1	0	—
(2)	Total LPSAP	1.6 ± 0.5	2.2 ± 0.5	2.6	<0.05
(3)	Heat-sensitive LPSAP	1.0 ± 0.5	1.2 ± 0.4	1.0	0.40
(4)	Heat-stable LPSAP	0.6 ± 0.3	1.0 ± 0.3	3.0	<0.02
(5)	% Heat-stable LPSAP/ total LPSAP	36.7 ± 22.6	46.1 ± 10.3	1.2	<0.20
(6)	Total non-LPSAP	6.5 ± 3.1	7.8 ± 2.2	1.0	0.40
(7)	Heat-sensitive non-LPSAP	4.3 ± 1.4	6.1 ± 2.0	2.1	<0.10
(8)	% Heat-sensitive non-LPSAP	69.3 ± 11.4	79.6 ± 4.9	2.8	0.01
(9)	Heat-stable non-LPSAP	2.2 ± 1.8	1.6 ± 0.6	1.0	0.40
(10)	Ratio heat-stable non-LPSAP/LPSAP	3.2 ± 1.8	1.2 ± 0.5	4.2	<0.01

It would appear likely that a sex difference possibly exists in alkaline phosphatase among individuals lacking the slow intestinal bands. Female subjects exhibit an appreciably lower ratio of the heat-stable fractions for non-LPSAP and LPSAP.

Starch-gel patterns representative of normal subjects (ABO blood groups) appear in Fig. 32, and can be related to the biochemical data (Table 10) for the individual sera.

We assume that normal individuals have the same organ sources of serum alkaline phosphatase whether or not the slow intestinal band is present. Thus, persons with the band have more total and heat-stable LPSAP and those lacking the band, more heat-stable non-LPSAP. This can be explained if some of the intestinal alkaline phosphatase, before, during, or after absorption loses its sensitivity to L-phenylalanine but

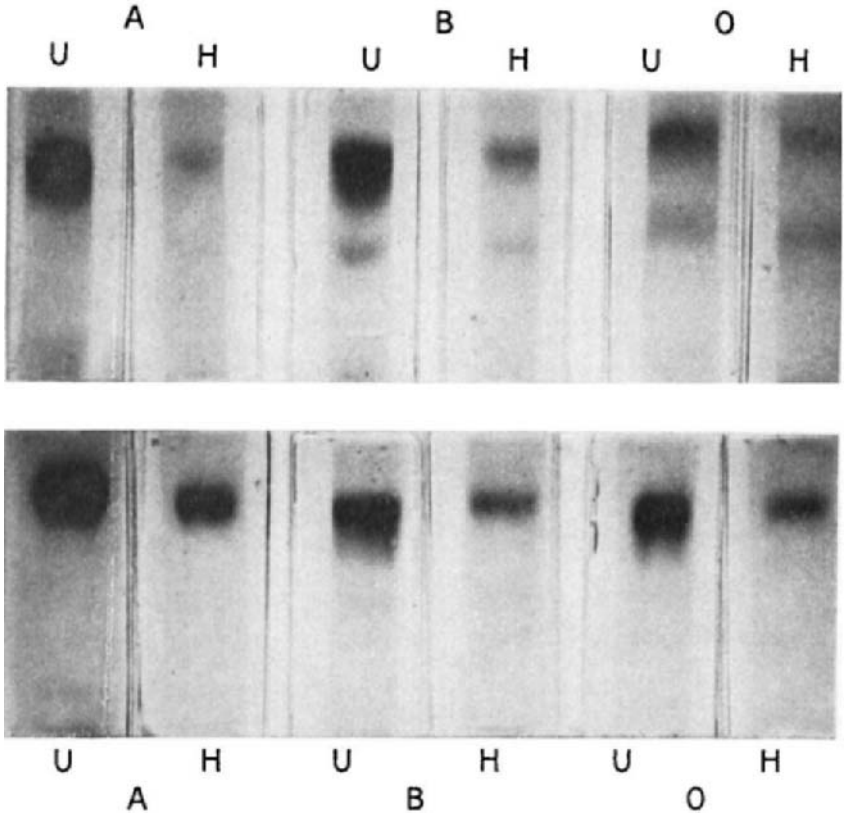


FIG. 32. Starch-gel zymograms of alkaline phosphatase in 6 normal subjects. A, B, and O represent the blood types; U = unheated, and H = heated serum (16 minutes at 55°C). The upper 3 pairs from left to right belong to subjects 2, 22, and 30 listed in Table 10 (Parts I and II); the lower 3 subjects from left to right are 10, 24, and 17.

still retains its relative heat-stability. In this case, the heat-stable non-LPSAP would be a metabolic variant of intestinal alkaline phosphatase.

8.1. VIEWS ON THE NATURE OF SERUM ALKALINE PHOSPHATASE IN NORMAL ADULT SUBJECTS

The serum of the normal subject is essentially a mixture of alkaline phosphatase activities, whose total activity is stable but which includes moieties that fluctuate in a manner related to the genetic constitution of the individual. What is the origin of these moieties?

In relation to the data presented in Table 10 (Parts I and II) and Fig.

32, almost all the facts *in the normal subject* can be explained on the basis of intestinal origin of serum alkaline phosphatase.

Since there is no difference in the amount of alkaline phosphatase in the intestinal mucosa of subjects of various blood types and secretor status (L2) and since this enzyme is presumed to be L-phenylalanine-sensitive (L14), then the difference in the relative amount of heat-stable LPSAP in persons with or without the slow band may relate to events

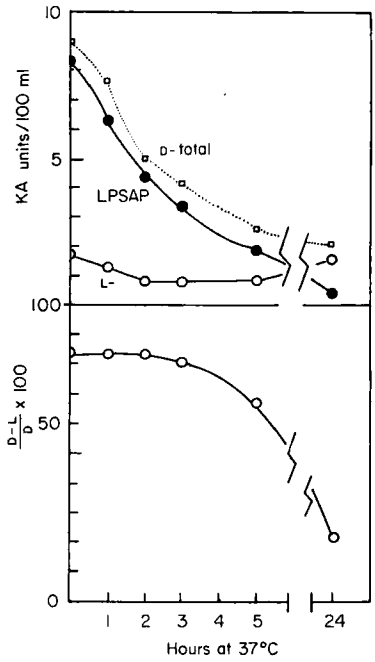


FIG. 33. Effect of maintaining gastrointestinal secretion at 37°C as a function of time. Dotted line is the total alkaline phosphatase activity, (L) the activity in the presence of L-phenylalanine, and LPSAP the difference between D and L (D — L = LPSAP).

prior to absorption. Here, recent evidence (Figs. 33 and 34) demonstrates that the enzyme (which is almost all LPSAP) in duodenal and gastric juice exhibits a very heat-sensitive fraction that travels to the actual position of bone alkaline phosphatase. The slow LPSAP band is generally heat-stable, as is alkaline phosphatase of the supernatant of a centrifuged Tris homogenate of intestine. Moreover, at 37°C with time the intestinal enzyme loses its heat-stability and sensitivity to L-phenylalanine (Fig. 33). This may explain why not all the circulating phos-

phatase in normal subjects is sensitive to L-phenylalanine and why the heat-sensitive moieties vary.

Therefore the length of time the intestinal juice remains in the intestine until it enters the circulation is a single factor which alone could determine the properties of the serum enzyme. In figure 32, subject 22 with blood type B showing the slow band might have a rapid rate of intestinal absorption which would enrich the blood with relatively more

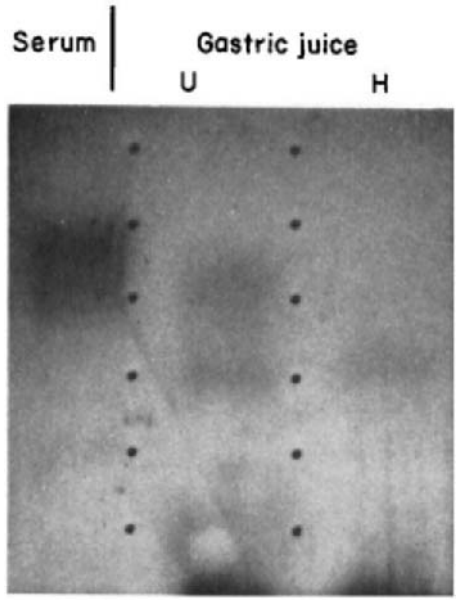


FIG. 34. Starch-gel pattern of serum and gastric juice of normal subject. U = unheated, H = heated (16 minutes at 55°C). Note absence of fast-moving band in the heated gastric juice found in the unheated juice between 4 and 5 cm.

heat-stable LPSAP, which represents the properties of the freshly secreted intestinal alkaline phosphatase. On the other hand, subject 2 with blood type A lacking the slow band may be absorbing intestinal alkaline phosphatase that had remained much longer in the intestine, the absorbed material being fast-moving (bone position) and completely heat-sensitive. Since both individuals are adults of the same age (30), it would be unjustifiable to invoke an intestinal source to explain the facts in subject 22 but not in subject 2.

Fast-moving bands of serum alkaline phosphatase essentially would represent metabolically altered intestinal alkaline phosphatase (analogous to the enzyme products in aging butanol extracts; Fig. 21).

In addition, the interplay of factors other than rate and extent of

absorption may be important in determining the relative amounts of LPSAP, non-LPSAP, their respective heat sensitivities, and subsequent positions on starch-gel electrophoresis. Thus, the excessive functioning of lipoprotein lipase and of the reticuloendothelial system could reduce the chylomicron population with its LPSAP activity to low levels, and vice versa. The role, as yet unproved, of substrate and/or hormonal induction could be important in influencing the level as well as the type of alkaline phosphatase in the intestine and elsewhere (G22).

The genetic factor recognized by blood type and secretor status could be operating at one or more of the following loci: intestinal mucosa, rate of absorption, rate of secretion, function and level of lipoprotein lipase and the reticuloendothelial system, neuraminidase level in intestinal contents and other tissues. In this latter connection, blood group carbohydrates are found on the cell walls of the epithelium of the microvilli of the intestine.

A number of other circumstances favor the supposition that the intestine must be a major source of serum alkaline phosphatase in the normal. Thus, a consideration of the relative concentration of alkaline phosphatase in tissues of the body and the total enzyme contributed by a single organ leaves no doubt that the intestine is by far the most important in this respect (B32a). Our own many experiences in preparing alkaline phosphatase from human liver, bone, and intestine have convinced us that intestine is many orders higher in its alkaline phosphatase activity. When one takes into account the tremendous absorptive surface area of the mucosa of the duodenum, jejunum, and ileum, the fact that alkaline phosphatase is secreted (L14) and absorbed into lymph (K7) and blood circulation (B25, K7), the intestine must reasonably be expected to be the major source of serum alkaline phosphatase. Now in the case of the rat, all the serum alkaline phosphatase has properties indistinguishable from those of the intestinal alkaline phosphatase (F11, F19, M1). A number of physiological experiments, such as fat feeding, have resulted in an enrichment of the intestinal mucosa and a subsequent elevation in serum alkaline phosphatase. Starvation markedly reduces serum alkaline phosphatase (B25). Bile duct ligation (F12) reduces the intestinal contribution to the circulation and increases the liver source. Normal individuals depending on blood type and secretor status have exhibited as much as 66% intestinal enzyme, based on LPSAP measurements. In the case of cirrhosis of the liver, individuals have been found in which 90% of the circulating alkaline phosphatase has the properties of intestinal mucosal alkaline phosphatase. In such patients an extraordinarily rapid and copious secretion of intestinal juice occurs, which, coupled with defective liver function, may readily lead to the accumulation in the blood of the unmodified intestinal enzyme. Where is the so-called major

"bone" component of the serum in these individuals which should have accumulated owing to the failure of the liver to excrete phosphatase?

The major part of the non-LPSAP is now considered to be intestinal enzyme that has undergone modification prior to or after absorption.

The above considerations are not incompatible with the likelihood that liver and bone may be sources of alkaline phosphatase in the *normal* individual, but make it very probable that their contributions are relatively minor indeed compared to intestine. Moreover, since the varieties of unmodified and modified intestinal alkaline phosphatases have among them all the properties that have been employed to identify liver and bone components of serum alkaline phosphatase in biochemical and starch-gel experiments, the interpretation of enzyme bands in the nonpregnant individual must be stated as "intestine plus liver" (H17) or "intestine plus bone" (R20) and not "liver" or "bone."

Therefore, we are adopting as a working hypothesis that the main origin of serum alkaline phosphatase is the intestinal mucosa in nonpregnant normal adults. This hypothesis requires only one assumption, namely, that the intestinal alkaline phosphatase undergoes alteration *in vivo* to forms indistinguishable from the alkaline phosphatases in liver and bone preparations. The hypothesis enables one to interpret the biochemical and starch-gel data in a fashion that admits the fewest assumptions.

In the pregnant subject, the placenta and intestine become the two major sources of serum alkaline phosphatase. Like intestine, placenta is a rich source of alkaline phosphatase and it enriches the outermost cell membranes of the microvilli of the syncytiotrophoblast, which is bathed by the maternal circulation. The properties of the placental enzyme differ sufficiently from those of the intestinal isozyme to permit their differentiation.[4]

[4] The alkaline phosphatase of both human intestine and placenta are L-phenylalanine-sensitive and undergo uncompetitive inhibition to the same extent (nearly 80%) by 0.005 *M* L-phenylalanine. However, we have been able to find several distinguishing biochemical characteristics of the two enzymes: (1) the anodic mobility of intestinal alkaline phosphatase remains unchanged after neuraminidase treatment, whereas the placental enzyme is sialidase-sensitive and hence the electrophoretic mobility on starch gel is considerably reduced by such treatment, (2) the Michaelis constant of placental alkaline phosphatase at a definite pH is appreciably higher than that of the intestinal enzyme (at pH 9.3 the K_m values of placenta and intestine are 316 and 160 μM, respectively), and (3) the pH optima (with 0.018 *M* phenyl phosphate as substrate) of the two enzymes are different; the values for intestinal and placental enzymes with 0.005 *M* D-phenylalanine are 9.9 and 10.6, respectively, and the respective values in the presence of 0.005 *M* L-phenylalanine are 10.2 and 11.1. Finally, contrary to the behavior of intestinal alkaline phosphatase, placental enzyme is completely heat stable (P19).

9. Clinical Application

The hypothesis of the intestinal and placental origin of normal serum alkaline phosphatase does not exclude the possibility that alkaline phosphatase from other organs may, under pharmacological or pathological circumstances, enter the circulation in significant amounts.

It was possible, in the preceding section, to arrive at a biochemical classification in normal healthy subjects. The moieties measured are simply the alkaline phosphatase activity of unheated and heated serum in the presence of D- and L-phenylalanine, respectively. Depending on the individual's blood type, secretor status, and sex, the mean values and relative proportions of each may differ.

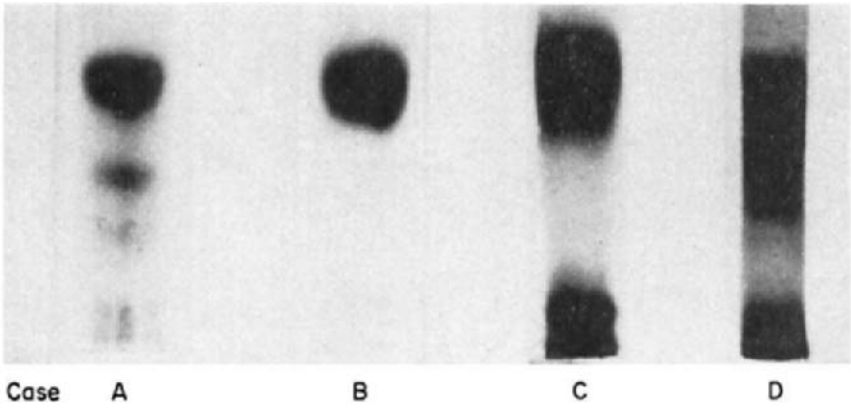


FIG. 35. Starch-gel patterns of Cases A, B, C, and D described in text.

It is reasonable to examine similar data obtained in patients exhibiting hyperphosphatasemia and to develop interpretations. Although one can collect many such cases, it must be evident after reflection that such populations are statistically and medically heterogeneous. The intrusion of uncertainties can be expected, especially in cancer patients. These may be the presence or absence of metastases, their quiescence or activity, and relative activity of liver, bone, and intestinal sources under the influence of the disease and its therapy. Accordingly, until adequate numbers of statistically and medically *homogeneous* cases become available, it is proposed to evaluate each case individually against the proper corresponding control values based on blood type, sex, and secretor status and the starch-gel patterns available for reference. The analysis is focused on a *change* in the level of serum alkaline phosphatase in which the various parameters examined permit a partial interpretation.

In the following four examples (starch gel, Fig. 35; biochemistry, Figs. 36–39) the emphasis is on the manner in which the hyperphosphatasemias have been analyzed from the biochemical data and the inferences which may be reasonably drawn. These cases belong to a larger series undergoing analysis at the Lemuel Shattuck Hospital (to be reported in detail elsewhere by Stolbach *et al.*).

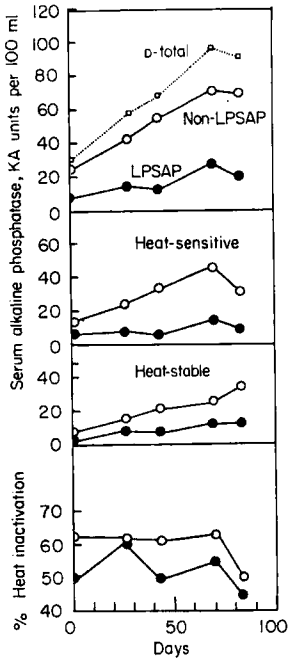


FIG. 36. Case A. Hyperphosphatasemia during androgen therapy in cancer of the endometrium. D-total = total alkaline phosphatase activity, non-LPSAP = activity in the presence of L-phenylalanine open circles, and D — L = LPSAP, closed circles. The same symbols apply to Figures 37, 38, and 39.

Examples

Case A (Fig. 36). This female patient (blood type B) with cancer of the endometrium metastatic to liver and lung showed no change in alkaline phosphatase on the first 60 days of Delalutin (progesterone) therapy, and then showed a rise for the next 80 days. The elevated values over this period showed a reasonably close proportionate rise in the LPSAP and non-LPSAP. This circumstance applies also to the heat-sensitive and -insensitive fractions. The percent heat inactivation remains constant for both LPSAP and non-LPSAP moieties at 62 and 50%

approximately. Normal individuals with blood type B and the slow-moving band exhibit 53.7 and 76.5%, respectively. From this it may be stated that only the heat sensitivity of the non-LPSAP band is abnormally low. It may also be surmised that no new source of enzyme, or a source showing different properties from those described, is appearing during the 80 days. In view of the hyperbilirubinemia, progressive obstruction of the biliary tree could account for the accumulation in the circulation of alkaline phosphatase isoenzymes, whose pattern, however, is normal in most respects and the intestine would be considered as the

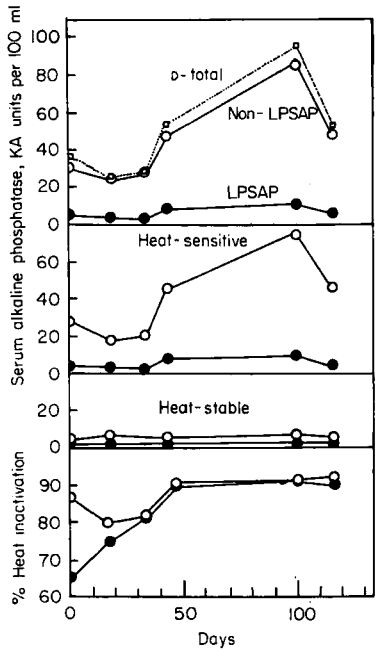


FIG. 37. Case B. Hyperphosphatasemia in androgen therapy of cancer of the breast. See Fig. 36.

major source. The possibility also exists of hormone induction of alkaline phosphatase owing to impaired liver function, resulting in the accumulation of unconjugated steroid hormones, and to the administered Delalutin.

Case B (Fig. 37). This female patient (blood type A) with cancer of the breast metastatic to bone showed a rise in alkaline phosphatase while on androgen (Halotestin) therapy. There was a proportionate increase in both LPSAP and non-LPSAP moieties, which could be attributed to the alteration in the heat-sensitive portions; the heat-insensitive fractions showed no appreciable alteration. With the increase, the per-

centage of heat inactivation of both LPSAP and non-LPSAP steadied at 90% approximately, which was far above the expected value for a blood group A slow band absent 70%. The sites most likely to deliver a high heat-sensitive alkaline phosphatase to the circulation are bone and intestine. The response may simply owe to androgenic hormone induction of bone and intestinal alkaline phosphatase or to a healing process.

Case C (Fig. 38). A female patient with cancer of the breast metastatic to bone exhibited a drop in serum alkaline phosphatase while on androgen therapy. This diminution can be attributed to the loss of

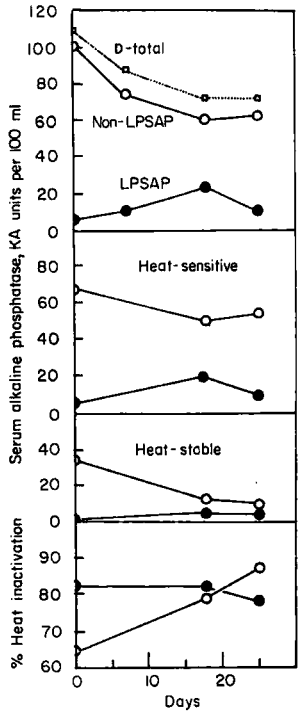


FIG. 38. Case C. Alteration in serum alkaline phosphatase in a patient with cancer of the breast.

heat-stable non-LPSAP. However, there is a coincident rise in heat-sensitive LPSAP. The percent heat inactivations were around 80% for both; these are higher than that (69%) expected for a female secretor. As in Case B, bone or intestine could be considered a source of the increased heat-sensitive fraction that persisted in this patient. The inverse relation between heat-stable non-LPSAP and heat-sensitive LPSAP

would appear to reflect an entirely different phenomenon from that noted in Case B.

Case D (Fig. 39). This patient (blood group O, secretor) with cancer of the lung metastatic to lymph nodes and other organs showed an atypical alkaline phosphatase picture. The rise in alkaline phosphatase resulted from an increase in a very heat-stable LPSAP fraction that accounted for 50% of the total. The non-LPSAP percent heat inactivation is also lower than expected (76.5%). The interpretation is that a new tissue source of serum alkaline phosphatase with altogether unusual

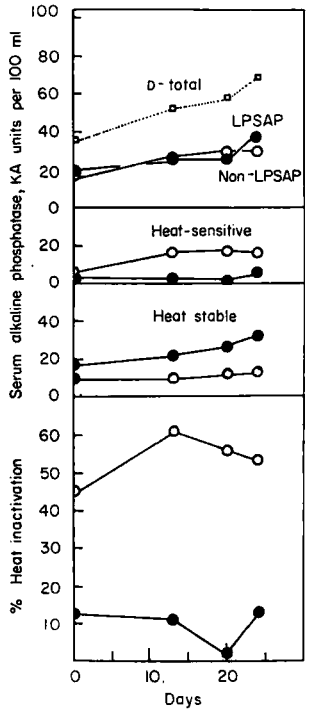


FIG. 39. Case D. Presence of abnormal amounts of heat-stable LPSAP in hyperphosphatasemia.

properties explains the increase. [Evidence to be presented elsewhere in detail (F15) establishes that this patient's cancer tissue contains large amounts of heat-stable LPSAP resembling placenta.]

These few examples illustrate the means by which one can evaluate the biochemical isoenzyme information in patients stressing the impor-

tance of employing a control isoenzyme pattern based on blood type and the presence or absence of the slow intestinal band.

This section has been organized around the findings in the normal followed by examination of the same type of information in disease. The following and last section of this chapter concerns clinical entities (pregnancy and disease) in which the current status of our understanding of the significance of the serum alkaline phosphatase is described, as well as the inferences which may now be permitted from the foregoing biochemical approach.

The more clinical aspects of serum alkaline phosphatase have been widely discussed (B28, F6, G18, G22, H7, I1, L13, M10, M14, O3, P2, P10, R5, R17, S15, W11).

9.1. Pregnancy

The elevation of serum alkaline phosphatase in pregnancy has been investigated by many workers (B28, K14, L3, L12, L17, L18, M16, M17, R9) during the last 30 years, and until recently it was believed that the enhanced level of this enzyme resulted from the contribution of growing fetal bone. In 1950 Beck and Clark (B8) reported that the increase in serum alkaline phosphatase during the last several weeks of pregnancy was taurocholate-resistant. The fact, however, that taurocholate was not an inhibitor of intestinal and placental alkaline phosphatases, although it inhibited alkaline phosphatase of other tissues, suggested that possibly placenta and to a lesser extent perhaps intestine were the main contributors of serum alkaline phosphatase in pregnancy.

A number of different studies have made it certain that placenta is the main contributor to serum alkaline phosphatase in late pregnancy. Thus, McMaster *et al.* (M15) and Zuckerman (Z2) noted the heat-stable enzyme appearing in the maternal circulation might be of placental origin. There was, however, practically no heat-stable enzyme in the umbilical cord blood. The immunological studies supported the theory of placental contribution in pregnancy, as the anti-enzyme (prepared by injecting human placental alkaline phosphatase into rabbits) inactivated the heat-stable fraction of alkaline phosphatase in the serum of pregnant women. Also, on the basis of their results on EDTA inactivation and electrophoresis on cellulose acetate, Kitchener *et al.* (K13) suggested that half the maternal serum alkaline phosphatase in pregnancy and puerperium was of placental origin. Latner (L4) also proposed that the onset of placental degeneration during the last weeks of pregnancy leads to the enhancement of alkaline phosphatase level in pregnancy serum. Beckman and Grivea (B11) recently reported their results on the variations of

serum alkaline phosphatase in a series of pregnant women and newborn children. The alkaline phosphatase in the maternal serum indicated two electrophoretic variants. The pregnancy enzyme was found in cord sera in a few exceptional cases. They noted a significant reduction of the P_p^2 "antigen-associated" alkaline phosphatase in pregnant women and newborn children.

Boyer (B38) was the first to indicate the polymorphism of human placental alkaline phosphatase by starch-gel electrophoresis. He suggested also the existence of genetic variants of the placental isoenzymes, which were investigated in greater detail by Harris and his collaborators (R15, R16).

With the aid of photomicrographs of the L-phenylalanine-sensitive placental alkaline phosphatase, one can picture the placental villi in contact with the maternal circulation (H3) and so enriching it with alkaline phosphatase. In support of this view, we have demonstrated a progressive rise in pregnancy of heat-stable LPSAP with an optimum pH of 10.7.

9.2. Liver Disease

This organ plays a central role in the physiopathology of alkaline phosphatase (B9, C2, D4, D5, M21, N1, P2, R6, S7, S26, S28, S29). Over the years the prevailing concept has been that the liver's function is an excretory one insofar as serum alkaline phosphatase coming to the liver is concerned, the source of the enzyme being osseous. Now, one part of this view has been challenged by Posen, who states that the liver does not excrete alkaline phosphatase and that hyperphosphatasemia owes to "regurgitation" of bile ducts (B20).

From physiological and ultrastructural considerations, it would appear that the alkaline phosphatase of the bile canaliculi (the only liver structures that are histochemically positive) would have the same function as that assigned to the brush border of intestine and placenta. It may serve in membranes participating in the active transport of metabolites and ions into and out of the bile duct epithelium. The bile normally contains relatively low alkaline phosphatase activity, which can be considered enzyme produced by the bile canaliculi only.

The portal blood-borne alkaline phosphatase from the intestine undergoes mixing in the liver with the enzyme that has traveled the lymphatic route. The proportions of these two populations of enzyme molecules, and their heat lability and sensitivity to L-phenylalanine are under the control of a number of physiological variables already discussed.

Cirrhosis of the Liver. This entity is given detailed attention here, since

it appears to illustrate well some of the factors to be considered in interpreting the hyperphosphatasemia of liver disease.

Fishman *et al.* (F14) reported that cirrhotics with hyperphosphatasemia can be divided into two categories: one in which the L-phenylalanine-sensitive moiety is increased, and one in which it is not. Careful clinical studies then and later (S49) have failed to reveal a correlation of the LPSAP with any one feature of the disease. On the other hand, a positive correlation was discovered between LPSAP and the ABO blood groups, subjects with blood types B and O exhibiting elevated LPSAP. On this evidence, our viewpoint now is that the genetic constitution of the individual is the factor that determines in hyperphosphatasemia the proportions of LPSAP of the total alkaline phosphatase.

Why then is the alkaline phosphatase elevated? It is known that cirrhotics exhibit hypersecretion of gastrointestinal juices. Such a hypersecretion with an accompanying rapid absorption into the lymph and portal blood would enrich the blood with intestinal alkaline phosphatase. Those individuals of blood types B and O would show high LPSAP levels and a slow band on starch gel [and those of type A (nonsecretors) high non-LPSAP levels] not only because of increased supply of intestinal enzyme, but perhaps also because of the malfunction of the liver mechanism for handling chylomicron intestinal alkaline phosphatase. Also, since in chronic cirrhosis increased levels of free estrogen accumulate in the body, it is conceivable that estrogen induction of alkaline phosphatase of liver, intestine, and other sites of origin of the enzyme may occur. Very probably all these factors may be operating to a lesser or greater extent simultaneously.

The biochemical fractions of the serum alkaline phosphatase in cirrhosis are listed in Table 13 according to blood types A and O. It may be advantageous to compare the various moieties in these patients with the data on normal subjects of corresponding blood type (Table 10). In some cases (4, 5, 14) the elevation is proportionate in the LPSAP and non-LPSAP moieties, whereas in others (1, 2, 3, 9, 10, 12, 13, 15) there is a disproportionate elevation in the LPSAP fraction. Subject 7 exhibits the largest contribution of LPSAP (60%). There appears to be no similarity of the heat sensitivities of LPSAP and non-LPSAP in subjects 4, 5, 7, 14, and 15 with total LPSAP hyperphosphatasemia. In fact, the relatively high heat inactivation of sera 5, 6, 7, and 12 would register negatively for liver in a heat-inactivation test of total alkaline phosphatase (P19).

Other Liver Diseases. Studies on liver diseases other than cirrhosis of the liver are in progress. However, elevations in LPSAP as an explanation of hyperphosphatasemia are much rarer.

It must be pointed out that the connection between abnormal liver function tests and serum alkaline phosphatase is often not clear. It then becomes important to devise means of identifying the particular mechanism or mechanisms that have been damaged in the liver, in order to interpret precisely the significance of hyperphosphatasemia in liver disease.

TABLE 13
DIFFERENT MOIETIES OF SERUM ALKALINE PHOSPHATASE IN CIRRHOTIC PATIENTS[a]

			LPSAP (D − L)		Non-LPSAP (L)		
Patient	Blood type	D-total Value	Value	% Heat sensitive	Value	% Heat sensitive	Ratio of heat-stable non-LPSAP/LPSAP
(1) Bi	O	22.4	3.4	53.1	19.0	69.4	3.57
(2) Ma	O	27.5	6.5	78.3	21.0	68.3	4.77
(3) Hi	O	30.0	4.6	61.0	25.4	61.9	5.39
(4) Ho	O	48.0	18.5	87.7	29.5	68.9	4.02
(5) Mi	O	26.6	12.6	75.8	14.0	82.1	0.82
(6) Cu	O	25.4	9.2	70.8	16.2	80.3	1.19
(7) Mu	O	34.7	20.9	66.7	13.8	81.0	0.38
(8) Si	O	34.4	8.3	59.2	26.1	68.4	2.42
(9) Sv	A	16.3	2.9	59.2	13.4	71.6	3.24
(10) Fa	A	60.0	7.1	41.7	52.9	59.0	5.21
(11) Dw	A	39.1	10.7	64.1	28.4	59.5	3.00
(12) Am	A	25.9	3.3	54.8	22.6	85.3	2.21
(13) Ca	A	27.0	5.9	89.8	21.1	74.5	9.00
(14) Br	A	25.9	10.1	66.3	15.8	76.7	1.08
(15) Lo	A	44.8	12.5	79.4	32.3	61.2	4.84

[a] KA units/100 ml.

9.3. BONE DISORDERS

The earliest literature on alkaline phosphatase (F21, R11–R14) has emphasized the osteoblastic source of serum alkaline phosphatase in growing children and in patients with bone disease, such as hyperparathyroidism (D12), Paget's disease, etc. A voluminous literature has followed (J2, K2, K17, M12, M31, N1, S45, T3, V1, V2).

Starch-gel migration of bone alkaline phosphatase yields bands in locations that can be occupied by gastrointestinal juice alkaline phosphatase as well as by the enzyme of kidney, lung, and spleen. High heat sensitivity is a property of the alkaline phosphatase from these various sources. Hence findings based on starch-gel electrophoresis and heat sensitivity in themselves are not diagnostic of a bone source.

The following question must also be considered. Is bone disease pos-

sibly coincident with an associated intestinal disorder, and is a greater than normal contribution of intestinal enzyme made in bone disease? This question is stimulated by the presence in sera of such patients of a major heat-labile component, in the bone-plus-intestine position in starch gel.

In fact in experimental animals (rat), rickets produced by avitaminosis D results in hyperphosphatasemia (F12) [normal 6.19 units/100 ml (F11), rickets 17.9 units/100 ml], that results from *both "intestinal"* and "bone" components [see also (D21) and R20)].

In general, it would seem that osteoblasts could not compare as a source of alkaline phosphatase with intestine or placenta. In pregnancy the level of alkaline phosphatase is rarely increased above 10 Bodansky or Shinowara units, and yet the placenta's microvilli, which are extremely rich in alkaline phosphatase, are directly immersed in the ample and efficient maternal blood supply (H3). In the nonpregnant individual, therefore, before hyperphosphatasemia can be attributed to bone it would appear necessary to evaluate the intestinal contribution that is evident as heat-sensitive non-LPSAP protein.

Another consideration is that properties of sera from individuals of a particular blood type and secretor status may resemble those of bone disease. If such individuals were blood type A nonsecretors with predominantly heat-sensitive non-LPSAP in the bone position and underwent substrate or hormonal induction, their hyperphosphatasemic sera would be indistinguishable in starch-gel patterns, LPSAP, and heat sensitivity from sera in Paget's disease.

One may ask, therefore, if the properties of hyperphosphatasemic sera from patients with bone disease are similar only because such patients represent a population that is either particularly susceptible to bone disease, or who survive long enough to become patients in a chronic disease hospital where clinical investigations are frequently conducted.

9.4. Skeletal and Hepatic Disorders

It must be stressed that hyperphosphatasemia in uncomplicated liver or bone disease, although of academic interest, has never posed a mystery of interpretation to the clinician nor has it confused the grounds for his decision making. The problem has arisen principally in dealing with laboratory findings in coexisting liver and bone disease, especially where a sudden elevation has occurred in the serum phosphatase level, as in hormonal therapy of cancer of the breast or of the prostate.

A variety of approaches require the application of starch-gel electrophoresis, physical conditions, and biochemical reagents. The majority

opinion among various workers is that liver and bone alkaline phosphatases are not readily separable by starch-gel electrophoresis, although a degree of success is claimed by some (C7, T2). The question of interpreting the pattern in coincident liver and bone disorders is not considered experimentally in the literature.

The application of the heat-sensitive measurement by Posen *et al.* (P19, P20) has been restricted to sera of patients with uncomplicated liver *or bone disease.* Here, sera with high inactivation of alkaline phosphatase are interpreted as bone and those that are relatively heat-stable as liver. Particularly disturbing to us were sera from patients with cirrhosis of the liver (patients 5, 6, and 12, Table 13) exhibiting heat sensitivity of non-LPSAP in the "bone" range. The measurement of heat sensitivity of serum alkaline phosphatase alone cannot be used for the certain identification of liver or bone sources or their mixtures.

The use of the L-phenylalanine inhibition and heat sensitivity does serve (as illustrated in Cases A, B, C, and D) to separate into a separate category those sera whose biochemical patterns are abnormal in type compared to the pattern of a normal individual of the same blood type, sex, and secretor status. If the heat sensitivity of the non-LPSAP is higher than expected, one has grounds to consider the presence of a bone phosphatase. Its amount can be quantitated readily.

In our opinion, a single set of this laboratory's partition values on serum is not diagnostic in itself of the relative degree of liver and bone involvement. Additional parameters need to be evaluated such as starch gel, neuraminidase treatment, clinical status (Y_1), etc.

9.5. Hormone-Related Hyperphosphatasemias

Wray (W16) found that the prostatic cancer patients with normal levels of serum alkaline phosphatase evidenced a rise immediately after the start of stilbestrol therapy. After the peak the value fell to normal more slowly. In those cases in which serum alkaline phosphatase was markedly raised to begin with, there was an initial fall in its value coincident with the start of stilbestrol therapy, but after a few days this downward trend reversed and the subsequent increase went beyond its initial level to attain a maximum followed by a gradual decline as in the former case. It was shown that when bone metastases were seen radiographically but the primary source was not evident, the presence of an alkaline phosphatase "kick" following administration of stilbestrol would indicate that the metastases were of prostatic origin, whereas its absence would be some evidence against such a source. Woodward *et al.* (W13–W15) have come to the conclusion that the hyperphosphatasemias in

hormone-treated subjects were the result of two basic phenomena: one related to hormone action, and the other related to the disease.

9.6. Renal Disease

The measurement of alkaline phosphatase was also employed in elucidating the pathogenesis and prognosis of renal disease (B43). Spink and Landeryou (S42) observed that kidneys from patients dying from shock and renal failure as a result of hemorrhage, myocardial infarction, or sepsis contained reduced amounts of phosphatase. A quantitative decrease in the alkaline phosphatase content of human kidneys was reported by Brain and Kay (B40) in severe renal disease. The investigations carried out by Latner *et al.* (L4) on the isoenzyme pattern in serum resulting from the rejection of a kidney homograft are interesting. Although most of the work was done with the dog, isoenzyme patterns on starch gel of a patient who received a kidney from his father were studied. After the operation an altered serum isoenzyme pattern was noted and was clearly evident when the graft was rejected 7 weeks later, the starch-gel patterns containing bands very similar to those of kidney extracts. These techniques of analyzing alkaline phosphatase isoenzymes, therefore, could be developed into a useful tool for detecting complete or partial rejection of the grafted organ.

Butterworth and Moss (B44, B47) reported the presence of a fast-moving isoenzyme of alkaline phosphatase in human urine. The normal level of alkaline phosphatase in human urine (around 0.2 KA unit/100 ml) was low, but the greatest quantities were found in relation to the onset of diuresis in patients suffering from acute renal tubular necrosis. In patients with nephrotic syndrome a similar phenomenon is found in the "nephritic" exacerbations. The urinary alkaline phosphatase had a molecular weight lower than that in the kidney extracts. It could be contended that the lower molecular weight enzyme is a product of dissociation occurring in the patient's urine, which contains urea in very high concentrations, and urea is well known for its capability of dissociating heavy molecular enzymes into their subunits. In diuresis the elevation of urinary alkaline phosphatase tended to occur in sharp peaks of short duration separated by troughs of lower levels (B46).

Other studies have reported raised urinary levels of alkaline phosphatase in a wide variety of renal disease (A2, A14, R22, W4). The patterns of distribution of alkaline phosphatase isozymes in the urines of patients having higher blood levels of this enzyme closely resemble those in the circulation. Urine enzyme may also originate from the prostate (S25).

9.7. Other Diseases

An increased serum alkaline phosphatase activity was reported in pulmonary infarction first by Nikkila (N21) and recently confirmed by Dijkman and Kloppenberg (D15). Nath and Ghosh (N6, N13) suggested a method for the differential diagnosis of Hb-E-thalassemia, in which determination of serum alkaline phosphatase activity simultaneously against phenyl phosphate as well as fructose 1,6-diphosphate was recommended. The ratio of the activity of fructose-1,6-diphosphatase to phenyl phosphatase was measured in a variety of pathological cases, and it was observed that the mean value of this ratio was 3 times higher in Hb-E-thalassemia than those in normal and nonthalassemic cases. Latner (L4) studied extracts of dental pulp by starch-gel electrophoresis, and observed that the band of activity traveled with exactly the same velocity as that of the bone enzyme. It was suggested, therefore, that odontoblasts possess the same alkaline phosphatase as osteoblasts. Hypophosphatasemia is one of the characteristic features of a genetic disease, as observed by Harris and Robson (H6), evidencing an increased excretion of ethanolamine phosphate in the urine. Premature shedding of the anterior deciduous teeth was found by Pimstone *et al.* (P5) in hypophosphatasemia.

9.8. Tumors as Tissue Sources for Serum Alkaline Phosphatase

Osteogenic sarcoma has been the classical example of a tumor that produces alkaline phosphatase in amounts sufficient to produce hyperphosphatasemia. Here the usual explanation is that the tumor has retained a biochemical characteristic of the tissue of origin (bone).

The association of hyperphosphatasemia with tumors of nonbone origins has been reported, as in the case of pancreatic cancer (S15), but the source of the serum enzyme has not been clearly traced back to the tumor.

Hitherto unreported are examples of patients such as Case D (Fig. 39) whose cancer of the lung and its metastases exhibited a placenta-like alkaline phosphatase that enriched the serum. This case may well be classified with those neoplasms that have apparently undergone derepression and produce proteins normally unrelated to the tissue of origin. The study of this interesting tumor is continuing (F15).

The point of referring to this case is to alert investigators to the possibility that the neoplasm itself may be the source of an elevation in serum alkaline phosphatase. Such cases may prove to be more common than hitherto suspected and represent a new and interesting field of oncologic significance.

Finally, mention should be made of the association of ABO substances in gastrointestinal carcinoma (D6).

10. Summary of Present Status of the Problem

Although the terms isoenzymes and variants have been clearly defined in this chapter, particularly for human intestinal and placental alkaline phosphatases, they may be found inadequate to describe phenomena presently being investigated. For example, how is one to deal with a metabolically altered isoenzyme which may have a variety of physical properties depending on the extent of its metabolic alteration, as, for example, in gastrointestinal juice prior to absorption? Although the properties of isoenzymes in individual tissues such as intestine and placenta can be systematically studied and interpreted, what happens to these components when they have been circulating in the blood for a period of time? Also, what transformations may take place in these enzymes attached to lipid in the population of small and large chylomicrons present in the circulation?

When one takes into consideration the physiological factors which can operate to modify enzyme proteins, one must also include as a fundamental basis of departure the genetic constitution of the individual. At present the traits are associated with ABO blood groups and secretor status. But who knows when the genetic factor may directly explain the individual serum alkaline phosphatase distribution in terms of specific enzymes?

It is against this background of genetic predetermination and physiological factors such as absorption and pregnancy, that the biochemical partitioning of alkaline phosphatases has been presented in this chapter. It is provided to the investigators in the field as a working arrangement that enables one to evaluate the normality or abnormality of the biochemical properties of the serum alkaline phosphatase for a given individual of known blood type and secretor status. From the biochemical study of normal individuals has evolved a picture of a predominantly intestinal contribution of alkaline phosphatase to the circulation, which undergoes modification in the body to an extent dependent on the individual. All the positions on starch gel are known positions of modified and unmodified intestinal alkaline phosphatase and the evidence is strongest that intestine does supply alkaline phosphatase to the circulation.

The nature of the stereospecific inhibition of alkaline phosphatase by L-phenylalanine has been presented insofar as it is known. Hopefully, this knowledge may be helpful in the design or detection of better stereospecific inhibitors, which may have utility in developing the biochemical approach to isoenzymes.

Information resulting from starch-gel and other types of electrophoresis is desirable, but in itself cannot be interpreted with the precision that is possible in combination with the biochemical information.

In the present state of knowledge and in the absence of information of the patient's diagnosis, one cannot yet expect to correctly identify a serum as "mostly bone" or "mostly liver" in origin with respect to its alkaline phosphatase by heat inactivation or L-phenylalanine inhibition. With the inclusion of additional studies, such as starch-gel electrophoresis, neuraminidase treatment, and continued study over a period of time, one can increase the certainty of the interpretation of the origin of the serum alkaline phosphatase in patients.

Information in the field of the isoenzymes of alkaline phosphatase may then be regarded as part of the evolving understanding of isoenzymes in general, which at the present time is rather confused with respect to its biological and clinical significance.

Dedication

This chapter is dedicated to Dr. Oscar Bodansky who has pioneered in the subject of alkaline phosphatase.

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The data on human patients are part of a collaborative study in progress with Drs. M. J. Krant and L. L. Stolbach at the Lemuel A. Shattuck Hospital and will be published in detail.

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References

A1. Aalund, O., Rendel, J., and Freedland, R. A., Isolation and characterization of ovine serum alkaline phosphatases. *Biochim. Biophys. Acta* **110,** 113–123 (1965).

A2. Abul-Fadl, M. A. M., and Metwalli, O. M., Studies on certain urinary and blood serum enzymes in bilharziasis and their possible relation to bladder cancer in Egypt. *Brit J. Cancer* **17,** 137–141 (1963).

A3. Agus, S. G., Cox, R. P., and Griffin, M. J., Inhibition of alkaline phosphatase by cysteine and its analogues. *Biochim. Biophys. Acta* **118,** 363–370 (1966).

A4. Ahmed, Z., and King, E. J., Purification of placental alkaline phosphatase. *Biochim. Biophys. Acta* **40,** 320–328 (1960).

A5. Ahmed, Z., and King, E. J., Kinetics of placental alkaline phosphatase. *Biochim. Biophys. Acta* **45,** 581–592 (1960).

A6. Ahmed, Z., Abul-Fadl, M. A. M., and King, E. J., The co-enzyme factor of alkaline phosphatase. *Biochim. Biophys. Acta* **36,** 228–240 (1959).

A7. Akamatsu, S., and Kobayashi, K., Chemical nature of the co-phosphomonoesterase. *Enzymologia* **15,** 154–157 (1951).

A8. Alberty, R. A., Enzyme Kinetics. In "Currents in Biochemical Research" (D. E. Green, ed.), p. 582. Wiley (Interscience), New York, 1956.

A9. Allen, J. M., and Gockerman, J., Electrophoretic separation of multiple forms of particle associated acid phosphatase. *Ann. N. Y. Acad. Sci.* **121,** 616–633 (1964).

A10. Allen, J. M., and Hyncik, G., Localization of alkaline phosphatases in gel matrices following electrophoresis. *J. Histochem. Cytochem.* **11,** 169–175 (1963).

A11. Allen, S. L., Genetic control of the esterases in the protozoan tetrahymena pyriformis. *Ann. N. Y. Acad. Aci.* **94,** 753–773 (1961).

A12. Alter, A. A., Lee, S. L., Pourfar, M., and Dobkin, G., Leukocyte alkaline phosphatase in mongolism; a possible chromosome marker. *J. Clin. Invest.* **41,** 1341 (1962).

A13. Alter, A. A., Lee, S. L., Pourfar, M., and Dobkin, G., Studies of leukocyte alkaline phosphatase in mongolism. A possible chromosome marker. *Blood* **22,** 165–177 (1963).

A14. Amador, E., Zimmerman, T. S., and Wacker, W. E. C., Urinary alkaline phosphatase activity. I. Elevated urinary LDH and alkaline phosphatase activities for the diagnosis of renal adenocarcinomas. *J. Am. Med. Assoc.* **185,** 769–775 (1963).

A15. Anagnostopoulos, C., Action comparée de quelques réactifs des fonctions amines sur diverses phosphatases acides et alcalines. *Biochim. Biophys. Acta* **4,** 584–595 (1950).

A16. Anagnostopoulos, C., and Matsudaira, H., Purification and kinetic studies of the alkaline phosphatase of human placenta. *Proc. Intern. Symp. Enzyme Chem., Tokyo Kyoto,* 1957 (K. Ichihara, ed.), p. 166. Maruzen, Tokyo, 1958.

A17. Arfors, K. E., Beckman, L., and Lundin, L. G., Genetic variations of human serum phosphatases. *Acta Genet. (Statist. Med.)* **13,** 89–94 (1963).

A18. Arfors, K. E., Beckman, L., and Lundin, L. G., Further studies on the association between human serum phosphatases and blood groups. *Acta Genet. (Statist. Med.)* **13,** 366-368 (1963).

A19. Arion, E. J., and Nordlie, R. C., Liver microsomal glucose-6-phosphatase, inorganic pyrophosphatase, and pyrophosphate-glucose-phosphotransferase. II. Kinetic studies. *J. Biol. Chem.* **239,** 2752–2757 (1964).

A20. Awad, E. S., Neurath, H., and Hartley, B. S., An interpretation of the apparent

inversion of antipodal specificity of α-chymotrypsin. *J. Biol. Chem.* **235,** PC35 (1960).

B1. Baar, H. S., Grümer, H. D., Beverage, E., Gordon, M., and Lee, S. Q., Enzyme inhibition by phenylalanine and its derivatives. *Proc. 2nd. Intern. Congr. Mental Retardation, Vienna,* Part I, pp. 62–69 (1963).

B2. Babson, A. L., Greeley, S. J., Coleman, C. M., and Phillips, G. E., The use of phenolphthalein monophosphate as a substrate for serum alkaline phosphatase. *Clin. Chem.* **12,** 482–490 (1966).

B3. Baker, R. W. R., and Pellegrino, C., The separation and detection of serum enzymes by paper electrophoresis. *Scand. J. Clin. Lab. Invest.* **6,** 94–101 (1954).

B4. Baldridge, R. C., The molecular biology of disease and inborn errors of metabolism. *Exptl. Med. Surg.* **23,** 165–171 (1965).

B5. Bamford, K. F., Harris, H., Luffman, J. E., Robson, E. B., and Cleghorn, T. E., Serum alkaline phosphatase and the ABO blood groups. *Lancet* **I,** 530–531 (1965).

B5a. Barrnett, R. J., The demonstration with the electron microscope of the end-products of histochemical reactions in relation to the fine structure of cells. *Exptl. Cell Res.* Suppl. 7, 65–89 (1959).

B6. Barrett, R. J., Friesen, H., and Astwood, E. B., Characterization of pituitary and peptide hormones by electrophoresis in starch gel. *J. Biol. Chem.* **237,** 432–439 (1962).

B7. Barron, E. S. G., and Singer, T. P., Studies on biological oxidations. XIX. Sulfhydryl enzymes in carbohydrate metabolism. *J. Biol. Chem.* **157,** 221–240 (1945).

B8. Beck, E., and Clark, L. C., Plasma alkaline phosphatase. II. Normative data for pregnancy. *Am. J. Obstet. Gynecol.* **60,** 731–740 (1950).

B9. Becker, K. L., and Stauffer, M. H., Evaluation of concentrations of serum alkaline phosphatase in hepatitis and obstructive jaundice. *Am. J. Med. Sci.* **243,** 222–227 (1962).

B10. Beckman, L., Associations between human serum alkaline phosphatase and blood groups. *Acta Genet. Statist. Med.* **14,** 286–297 (1964).

B11. Beckman, L., and Grivea, M., Serum alkaline phosphatase variations in pregnant women and new-born children. *Acta Genet. Statist. Med.* **15,** 218–223 (1965).

B12. Beckman, L., and Johnson, F. M., Variations in larval alkaline phosphatase controlled by Aph Alleles in drosophilia melanogaster. *Genetics* **49,** 829–835 (1964).

B13. Behal, F. J., and Center, M., Heterogeneity of calf intestinal alkaline phosphatase. *Arch. Biochem. Biophys.* **110,** 500–505 (1965).

B14. Belfanti, S., Contardi, A., and Ercoli, A., Researches on the phosphatases. II. Inactivation and reactivation of the phosphatases of animal organs. *Biochem. J.* **29,** 842–846 (1935).

B15. Bender, M. L., and Nakamara, K., The effect of structure on the rates of some α-chymotrypsin-catalysed reactions. *J. Am. Chem. Soc.* **84,** 2577–2582 (1962).

B16. Bessey, O. A., Lowry, O. H., and Brock, M., A method for the rapid determination of alkaline phosphatase with five cubic millimeters of serum. *J. Biol. Chem.* **164,** 321–329 (1946).

B17. Binkley, F., Comparison of the alkaline phosphatases of E. Coli and renal tissue. *Biochem. Biophys. Res. Commun.* **6,** 67–70 (1961).

B18. Binkley, F., Alexander, V., Bell, F. E., and Lea, C., Peptidases and alkaline phosphatases of swine kidney. *J. Biol. Chem.* **228,** 559–567 (1957).

B19. Birkett, D. J., Done, J., and Neale, F. C., Serum alkaline phosphatase in pregnancy: An immunological study. *Brit. Med. J.* **1,** 1210–1212 (1966).

B20. Birns, M., Masek, B., and Auerbach, O., The effects of experimental acute biliary obstruction and release on the rat liver. *Am. J. Pathol.* **40,** 95-111 (1962).

B21. Bjerrum, J., Schwarzenbach, G., and Sillen, L. G., "Stability Constants of Metal Ion Complexes," Parts 1 and 2. The Chemical Society, London, 1957 and 1958.
B22. Blomstrand, R., and Werner, B., Alkaline phosphatase activity in human thoracic duct lymph. *Acta Chir. Scand.* **129,** 177–191 (1965).
B23. Bodansky, A., Phosphatase studies. I. Determination of inorganic phosphate, Beer's Law and interfering substances in the Kuttner-Lichtenstein Method. *J. Biol. Chem.* **99,** 197–206 (1932).
B24. Bodansky, A., Phosphatase studies. II. Determination of serum phosphatase factors influencing the accuracy of the determination. *J. Biol. Chem.* **101,** 93–104 (1933).
B25. Bodansky, A., Phosphatase studies. VI. Non osseous origins of serum phosphatase. Its increase after ingestion of carbohydrates. *J. Biol. Chem.* **104,** 473–482 (1934).
B26. Bodansky, A., Non-osseous origins of serum phosphatase. *Proc. Soc. Exptl. Biol. Med.* **42,** 800–804 (1939).
B27. Bodansky, A., and Jaffe, H. L., Phosphatase studies. IV. Serum phosphatase of non-osseous origin; significance of the variations of serum phosphatase in jaundice *Proc. Soc. Exptl. Biol. Med.* **31,** 107–109 (1933).
B28. Bodansky, M., Campbell, K., and Ball, E., Changes in serum calcium, inorganic phosphate and phosphatase activity in the pregnant woman. *Am. J. Clin. Pathol.* **9,** 35–51 (1939).
B29. Bodansky, O., Are the phosphatases of bone, kidney, intestine and serum identical? The use of bile acids in their differentiation. *J. Biol. Chem.* **118,** 341–362 (1937).
B30. Bodansky, O., The inhibitory effects of dl-alanine, l-glutamic acid, l-lysine and l-histidine on the activity of intestinal, bone and kidney phosphatases. *J. Biol. Chem.* **174,** 465–476 (1948).
B31. Bodansky, O., Influence of magnesium and cobalt on inhibition of phosphatases of bone, intestine and osteogenic sarcoma by amino acids. *J. Biol. Chem.* **179,** 81–102 (1949).
B32. Bodansky, O., "Biochemistry of Disease," pp. 419 and 761. Macmillan, New York, 1952.
B32a. Bodansky, O., Blood enzymes in cancer and other diseases *in Advan. Cancer Res.* **6,** 34 (1961).
B33. Bodansky, O., and Schwartz, M. K., Comparative effects of L-histidine on the activities of 5′-nucleotidase and alkaline phosphatase. *J. Biol. Chem.* **238,** 3420–3427 (1963).
B34. Boman, H. G., and Westlund, L. E., Protein chromatography on an anion-exchange resin. *Arch. Biochem. Biophys.* **64,** 217–239 (1956).
B35. Bowers, G. N., and McComb, R. B., A continuous spectrophotometric method for measuring the activity of serum alkaline phosphatase. *Clin. Chem.* **12,** 70–89 (1966).
B36. Bowne, S. W., Jr., Ondrey, J. A., and Wise, J. L., Intracellular repressor of alkaline phosphatase. *Nature* **211,** 980 (1966).
B37. Boyer, P. D., and Harrison, W. H., On the mechanism of enzymic transfer of phosphate and other groups. *In* "A Symposium on the Mechanism of Enzyme Action" (W. D. McElroy and B. Glass, eds.), p. 658. Johns Hopkins Press, Baltimore, Maryland, 1954.
B38. Boyer, S. H., Alkaline phosphatase in human sera and placentae. *Science* **134,** 1002–1004 (1961).
B39. Boyer, S. H., Human organ alkaline phosphatases: Discrimination by several means including starch-gel electrophoresis of antienzyme-enzyme supernatant fluids. *Ann. N.Y. Acad. Sci.* **103,** 938–951 (1963).

B40. Brain, R. T., and Kay, H. D., Kidney phosphatase. II. The enzyme in disease. *Biochem. J.* **21,** 1104–1108 (1927).
B41. Brestkin, A. P., and Rzhekhina, N. I., On the effect of concentration and nature of the buffer solution upon the catalytic properties of alkaline phosphatase. *Biokhimiya* **30,** 471–476 (1965).
B42. Buice, T. C., Fife, T. H., Bruno, J. J., and Brandon, N. E., Hydroxyl group catalysis. II. The reactivity of the hydroxyl group of serine. The nucleophilicity of alcohols and the ease of hydrolysis of their esters as related to their PK'a. *Biochemistry* **1,** 7–12 (1962).
B43. Burgen, A. S. V., Urinary excretion of phosphatases in man. *Lancet* **252,** 329–331 (1947).
B44. Butterworth, P. J., and Moss, D. W., Starch-gel electrophoresis of urinary alkaline phosphatase. *Biochem. J.* **88,** 19P–20P (1963).
B45. Butterworth, P. J., and Moss, D. W., Action of neuraminidase on human kidney alkaline phosphatase. *Nature* **209,** 805–806 (1966).
B46. Butterworth, P. J., Moss, D. W., Pitkanen, E., and Pringle, A., Patterns of urinary excretion of alkaline phosphatase in acute renal failure. *Clin. Chim. Acta* **11,** 212–219 (1965).
B47. Butterworth, P. J., Moss, D. W., Pitkanen, E., and Pringle, A., Some characteristics of alkaline phosphatase in human urine. *Clin. Chim. Acta* **11,** 220–226 (1965).
C1. Caplow, M., and Jencks, W. P., The effect of substituents on the deacylation of of benzoylchymotrypsins. *Biochemistry* **1,** 883–893 (1962).
C2. Carlsten, A., Edlund, Y., and Thulesius, O., Bilirubin, alkaline phosphatase and transaminases in blood and lymph during biliary obstruction in the cat. *Acta Physiol. Scand.* **53,** 58–67 (1961).
C3. Carver, M. J., Schain, R. J., and Copenhaver, J. H., Entry of L-phenylalanine-C^{14} into brain and cerebrospinal fluid. *Proc. Soc. Exptl. Biol. Med.* **122,** 75-78 (1966).
C4. Cayla, J., and Fabre, P., La phosphatase serique pendant la gestation. *Compt. Rend. Soc. Biol.* **120,** 748–750 (1935).
C5. Ceppellini, R., Physiological genetics of human blood factors. *Ciba Found. Symp. Biochem. Human Genet., 1960* p. 242.
C6. Chase, W. H., The demonstration of alkaline phosphatase activity in frozen-dried mouse gut in the electron microscope. *J. Histochem. Cytochem.* **11,** 96–101 (1963).
C7. Chiandussi, L., Greene, S. F., and Sherlock, S., Serum alkaline phosphatase fractions in hepatobiliary and bone diseases. *Clin. Sci.* **22,** 425–434 (1962).
C8. Clark, B., and Porteous, J. W., The metal ion activation of the alkaline beta glycerophosphatase of rabbit small intestine. *Biochem. J.* **95,** 475–482 (1965).
C9. Clark, L. C., and Beck, E., Plasma "alkaline" phosphatase activity, I. Normative data for growing children. *J. Pediat.* **36,** 335–341 (1950).
C10. Clark, S. L., The localization of alkaline phosphatase in tissues of mice using the electron microscope *Am. J. Anat.* **109,** 57–83 (1961).
C11. Climie, A. R. W., Heinrichs, W. L., and Foster, I. J., Neutrophilic alkaline phosphatase test. A review with emphasis on findings in pregnancy. *Am. J. Clin Pathol.* **38,** 95–103 (1962).
C12. Cloetens, R., Identification de deux phosphatases "alcalines" dans les organes animaux. *Enzymologia* **6,** 46–56 (1939).
C13. Clubb, J. S., Neale, F. C., and Posen, S., The behavior of infused placental alkaline phosphatase in human subjects. *J. Lab. Clin. Med.* **66,** 493–507 (1965).
C14. Cohn, E. J., and Edsall, J. T., "Proteins, Amino Acids and Peptides," p. 445. Reinhold, New York, 1943.

C15. Cohn, M., Mechanisms of cleavage of glucose-1-phosphate. *J. Biol. Chem.* **180,** 771–781 (1949).
C16. Comfort, D., and Campbell, D. J., One-step automated alkaline phosphatase analysis. *Clin. Chim. Acta* **14,** 263–269 (1966).
C17. Cooke, K. B., and Silva, J. F., Serum alkaline phosphatase fractionation as an aid to diagnosis. *J. Clin. Pathol.* **14,** 500–501 (1961).
C18. Coryn, G., Elevation of serum alkaline phosphatase in pregnancy. *J. Chir. (Paris)* **33,** 213 (1934).
C19. Cox, R. P., and MacLeod, C. M., Hormonal induction of alkaline phosphatase in human cells in tissue culture. *Nature* **190,** 85–87 (1961).
C20. Cox, R. P., and MacLeod, C. M., Alkaline phosphatase content and the effects of prednisolone on mammalian cells in culture. *J. Gen. Physiol.* **45,** 439–485 (1962).
C21. Cunningham, L. W., Proposed mechanism of action of hydrolytic enzymes. *Science* **125,** 1145–1146 (1957).
C22. Cunningham, V. R., and Rimer, J. G., Isoenzymes of alkaline phosphatase of human serum. *Biochem. J.* **89,** 50P (1963).
D1. Dabich, D., and Neuhas, O. W., Purification and properties of bovine synovial fluid alkaline phosphatase. *J. Biol. Chem.* **241,** 415–420 (1966).
D2. Daems, W. T., and Persijn, J. P., Enzyme histochemistry in electron microscopy. In "Enzymes in Clinical Chemistry" R. Ruyssen and K. Vandendriessche, eds., pp. 75–104. Elsevier, Amsetrdam, 1965.
D3. Dahlqvist, A., and Lindberg, T., Development of the intestinal disaccharidase and alkaline phosphatase activities in the human fetus. *Clin. Sci.* **30,** 517–528 (1966).
D4. Dalgaard, J. B., Serum phosphatase after hepatectomy in dogs. *Acta Physiol. Scand.* **15,** 308–317 (1949).
D5. Dalgaard, J. B., On the cause of increase in serum phosphatase in obstructive jaundice. *Acta Physiol. Scand.* **22,** 200–210 (1951).
D6. Davidsohn, I., Kovarik, S., and Lee, C., A, B, and O substances in gastrointestinal carcinoma. *Arch. Pathol.* **81,** 381–390 (1966).
D7. Davis, B. J., Disc electrophoresis. II. Method and application to human serum proteins. *Ann. N.Y. Acad. Sci.* **121,** 404–427 (1964).
D8. Dayan, J., and Wilson, I. B., The phosphorylation of Tris by alkaline phosphatase. *Biochim. Biophys. Acta* **81,** 620–623 (1964).
D9. De Carli, L., Maio, J. J., and Nuzzo, F., Alkaline phosphatase activity and chromosome variations in human cells in culture. *J. Natl. Cancer Inst.* **31,** 1501–1509 (1963).
D10. Deitrich, R. A., Hellerman, L., and Wein, J., Diphosphopyridine nucleotide-linked aldehyde dehydrogenase I. Specificity and Sigma-Rho gunction. *J. Biol. Chem.* **237,** 560–564 (1962).
D11. Demuth, F., Uber phosphatstoffwechsel. I. Mitteilung. Uber hexosephosphatasen in menschlichen organen und koerperfluessigkeiten. *Biochem. Z.* **159,** 415–423 (1925).
D12. Dent, C. E., and Harper, C. M., Plasma alkaline phosphatase in normal adults and in patients with primary hyperparathyroidism. *Lancet* **I,** 559 (1962).
D13. Deren, J. J., Williams, L. A., Muench, H., Chalmers, T., and Zamcheck, N., Comparative study of four methods of determining alkaline phosphatase. *New Engl. J. Med.* **270,** 1277–1283 (1964).
D14. Desalu, A. B. O., Correlation of localization of alkaline and acid phosphatases with morphological development of the rat kidney. *Anat. Record* **154,** 253–259 (1966).

D15. Dijkman, J. H., and Kloppenborg, P. W. C., Increased serum alkaline phosphatase activity in pulmonary infarction. *Acta Med. Scand.* **180,** 273–281 (1966).
D16. Dixon, M., The effect of pH on the affinities of enzymes for substrates and inhibitors. *Biochem. J.* **55,** 161–170 (1953).
D17. Dixon, M., and Webb, E. C., "Enzymes," pp. 142–149, 172, and 176. Longmans, Green, New York, 1958.
D18. Dodgson, K. S., Spencer, B., and Williams, K., The hydrolysis of substituted phenyl sulfates by the arylsulphatase of *Alcaligenes metalcaligenes*. *Biochem. J.* **64,** 216–221 (1956).
D19. Donayre, J., and Pincus, G., Serum enzymes in the menstrual cycle. *J. Clin. Endocrinol. Metab.* **25,** 432–433 (1965).
D20. Done, J., Shorey, C. D., Loke, J. P., and Pollak, J. K., The cytochemical localization of alkaline phosphatase in Escherichia coli at the electron-miscroscope level. *Biochem. J.* **96,** 27C–28C (1965).
D21. Doose, H., Studies uber die alkalische serum phosphatase der Ratte. D. Mitteilung: Das Verhalten der alkalischen serumphosphatase der rachitischen Ratte unter Vitamin D. *Z. Exptl. Med.* **134,** 73–81 (1960).
D22. Drill, V. A., and Riggs, D. S., Inhibition of alkaline serum phosphatase activity during liver disease. *J. Biol. Chem.* **162,** 21–25 (1946).
D23. Dryer, R., Tammes, A., and Routh, J., The determination of phosphorus and phosphatase with *N*-phenyl-*p*-phenylenediamine. *J. Biol. Chem.* **225,** 177–183 (1957).
D24. Dymling, J. F., Separation of serum and placental alkaline phosphatase by agarose gel electrophoresis and Sephadex chromatography. *Scand. J. Clin. Lab. Invest.* **18,** 130–133 (1966).
E1. Emmelot, P., and Bos, C. J., On the participation of neuraminidase-sensitive sialic acid in the K^+-dependent phosphohydrolysis of *p*-nitrophenylphosphate by isolated rat-liver plasma membranes. *Biochim. Biophys. Acta* **115,** 244–247 (1966).
E2. Engstrom, L., Studies on calf intestinal alkaline phosphatase. I. Chromatographic purification, microheterogeneity and some other properties of the purified enzyme. *Biochim. Biophys. Acta* **52,** 36–48 (1961).
E3. Engstrom, L., Studies on calf-intestinal alkaline phosphatase. II. Incorporation of inorganic phosphate into a highly purified enzyme preparation. *Biochim. Biophys. Acta* **52,** 49–59 (1961).
E4. Engstrom, L., Further studies on the incorporation of inorganic phosphate into calf-intestinal alkaline phosphatase. *Biochim. Biophys. Acta* **54,** 179–185 (1961).
E5. Engstrom, L., Incorporation of inorganic phosphate into alkaline phosphatase from E. coli. *Biochim. Biophys. Acta* **52,** 606–609 (1962).
E6. Engstrom, L., Studies on bovine-liver alkaline phosphatase, purification, phosphate incorporation. *Biochim. Biophys. Acta* **92,** 71–78 (1964).
E7. Engstrom, L., The amino acid sequence around the reactive serine in calf intestinal alkaline phosphatase. *Biochim. Biophys. Acta* **92,** 78–84 (1964).
E8. Estborn, B., Visualization of acid and alkaline phosphatase after starch-gel electrophoresis of seminal plasma, serum and bile. *Nature* **184,** Suppl. 21, 1636-1637 (1959).
E9. Estborn, B., Localization of prostatic serum acid phosphatase by starch-gel electrophoresis. *Clin. Chim. Acta* **6,** 22–24 (1961).
E10. Estborn, B., Separation of phosphatase iso-enzymes by gel filtration. *Z. Klin. Chem.* **2,** 53–54 (1964).
E11. Etzler, M. E., and Moog, F., Inactive alkaline phosphatase in duodenum of nursling mouse: Immunological evidence. *Science* **154,** 1037–1038 (1966).

E12. Evans, D. A. P., Confirmation of association between ABO blood groups and salivary ABH secretor phenotypes and electrophoretic patterns of serum alkaline phosphatase. *J. Med. Genet.* **2,** 126–127 (1965).

E13. Evered, D. F., and Steenson, T. I., Citrate inhibition of alkaline phosphatase. *Nature* **202,** 491–492 (1964).

F1. Fernley, H. N., and Walker, P. G., Kinetic behavior of calf-intestinal alkaline phosphatase with 4-methylumbelliferyl phosphate. *Biochem. J.* **97,** 95–103 (1965).

F2. Finch, L. R., and Hird, F. J. R., The uptake of amino acids by isolated segments of rat intestine. II. A survey of affinity for uptake from rates of uptake and competition for uptake. *Biochim. Biophys. Acta* **43,** 278–287 (1960).

F3. Fischer, E., Alkaline inorganic pyrophosphatase in the mucous glandular cells. *Acta Biol. Acad. Sci. Hung.* **17,** 159–165 (1966).

F4. Fischer, E., Effect of inhibitors on the histochemical reaction of alkaline α-naphthyl and naphthol-AS-phosphomonoesterase in the kidney of edible-frog (Rana Esculenta L.) *Acta Biol. Acad. Sci. Hung.* **16,** 383–388 (1966).

F5. Fishman, W. H., Plasma enzymes. *Plasma Proteins* **2,** 59–103 (1960).

F6. Fishman, W. H., and Davidson, H. M., Determination of serum acid phosphatases. *Methods Biochem. Anal.* **4,** 257–284 (1957).

F7. Fishman, W. H., and Ghosh, N. K., Influence of reagents reacting with metal, thiol and amino sites on catalytic activity and L-phenylalanine inhibition of rat intestinal alkaline phosphatase. *Biochem. J.* (1967) (in press).

F8. Fishman, W. H., and Green, S., Automated differential isoenzyme analysis. I. L-phenylalanine-sensitive isoenzymes of human serum alkaline phosphatase. *Enzymologia,* **33,** 89–99 (1967).

F9. Fishman, W. H., and Kreisher, J. H., Stereospecific, organ-specific inhibition of intestinal alkaline phosphatase. *Ann. N.Y. Acad. Sci.* **103,** 951–955 (1963).

F10. Fishman, W. H., Goldman, S. S., and Delellis, R., Dual localization of β-glucuronidase in the endoplasmic reticulum and in lysosomes. *Nature 213,* 457–460 (1967).

F11. Fishman, W. H., Green, S., and Inglis, N. I., Organ-specific behavior exhibited by rat intestine and liver alkaline phosphatase. *Biochim. Biophys. Acta* **62,** 363–375 (1962).

F12. Fishman, W. H., Green, S., and Inglis, N. I., Decline in rat-serum alkaline phosphatase following bile-duct ligation. *Biochim. Biophys. Acta* **62,** 429–431 (1962).

F13. Fishman, W. H., Green, S., and Inglis, N. I., L-phenylalanine: An organ specific, stereospecific inhibitor of human intestinal alkaline phosphatase. *Nature* **198,** 685–686 (1963).

F14. Fishman, W. H., Inglis, N. I., and Krant, M. J., Serum alkaline phosphatase of intestinal origin in patients with cancer and with cirrhosis of the liver. *Clin. Chim. Acta* **12,** 298–303 (1965).

F15. Fishman, W. H., Inglis, N. I., Krant, M. J., and Stolbach, L. L., A serum alkaline phosphatase isoenzyme of neoplastic tissue origin. *Cancer Res.* (1967) (in press).

F16. Fishman, W. H., Inglis, N. I., Sarke, F., and Ghosh, N. K., Distinguishing characteristics of serum alkaline phosphatase isoenzymes. *Federation Proc.* **25,** 748 (1966).

F17. Fishman, W. H., Kato, K., Anstiss, C. L., and Green, S., Human serum β-glucuronidase; its measurement and some of its properties. *Clin. Chim. Acta* **15,** 435–447 (1967).

F18. Fiske, C. H., and Subbarow, Y., The colorimetric determination of phosphorus. *J. Biol. Chem.* **66,** 375–400 (1925).

F19. Flock, E. V., and Bollman, J. L., Alkaline phosphatase in the intestinal lymph of the rat. *J. Biol. Chem.* **175,** 439–449 (1948).

F20. Flock, E. V., and Bollman, J. L., The influence of bile on the alkaline phosphatase activity of intestinal lymph. *J. Biol. Chem.* **184,** 523–528 (1950).

F21. Folley, S. J., and Kay, H. D., The phosphatases. *Ergeb. Enzymforsch.* **5,** 159–212 (1936).

F22. Fraenkel-Conrat, H., Methods for investigating the essential groups for enzyme activity. *Methods Enzymol.* **4,** 256–259 and 263 (1957).

F23. Fraenkel-Conrat, H., Other reactive groups of enzymes. *In* "The Enzymes" (P. D. Boyer, H. Lardy, and K. Myrbäck, eds.), 2nd ed., Vol. 1, pp. 589–618. Academic Press, New York, 1959.

F24. Fraenkel-Conrat, H., and Mecham, D. K., The reaction of formaldehyde with proteins. VI. Demonstration of intermolecular cross-linking by means of osmotic pressure measurements. *J. Biol. Chem.* **177,** 477–486 (1949).

F25. Fraenkel-Conrat, H., and Olcott, H. S., Reaction of formaldehyde with proteins. VI. Cross-linking of amino groups with phenol, imidazole or indole groups. *J. Biol. Chem.* **174,** 827–843 (1948).

F26. Frajola, W. J., Williams, R. D., and Austad, R. A., The kinetic spectrophotometric assay for serum alkaline phosphatase. *Am. J. Clin. Pathol.* **43,** 261–264 (1965).

F27. Fraenkel, H. H., and Peters, R. L., A modified calcium-cobalt method for the demonstration of alkaline phosphatase. The use of para-nitrophenyl phosphate as a substrate. *Am. J. Clin. Pathol.* **42,** 324–327 (1964).

F28. Freier, E. F., Genetic and conformational heterogeneity of lactate dehydrogenase enzymes. *Texas Rept. Biol. Med.* **24,** 375–385 (1966).

F29. Frieden, C., Treatment of enzyme kinetic data. I. The effect of modifiers on the kinetic parameters of single substrate enzymes. *J. Biol. Chem.* **239,** 3522–3531 (1964).

F30. Friedenwald, J. S., and Maengwyn-Davies, G. D., Elementary kinetic theory of enzymatic activity; first order theory. *In* "The Mechanism of Enzyme Action" (W. D. McElroy and B. Glass, eds.), p. 163. Johns Hopkins Press, Baltimore, Maryland, 1954.

G1. Gahne, B., Genetic variation of phosphatase in cattle serum. *Nature* **199,** 305–306 (1963).

G2. Garen, A., Genetic control of the bacterial enzyme, alkaline phosphatase. *In* "Microbial Genetics" (W. Hayes and R. C. Clowes, eds.), pp. 239–247. Cambridge Univ. Press, London and New York, 1960.

G3. Garen, A., and Levinthal, C., A fine-structure genetic and chemical study of the enzyme alkaline phosphatase of E. coli. *Biochim. Biophys. Acta* **38,** 470–483 (1960).

G4. Gelb, D., West, M., and Zimmerman, H. J., The relation of the Bodansky to the King-Armstrong method for the determination of the serum alkaline phosphatase. *Am. J. Clin. Pathol.* **38,** 198–202 (1962).

G5. Ghosh, N. K., and Fishman, W. H., On the mechanism of inhibition of intestinal alkaline phosphatase by L-phenylalanine. I. Kinetic studies. *J. Biol. Chem.* **241,** 2516–2522 (1966).

G6. Ghosh, N. K., and Fishman, W. H., Characterization of human placental alkaline phosphatase isoenzymes. *Federation Proc.* **26,** 558 (1967).

G6a. Ghosh, N. K., Goldman, S. S., and Fishman, W. H., Human placental alkaline phosphatase; a sialoprotein *Enzymologia,* **33,** 113–124 (1967).

G7. Ghosh, N. K., and Nath, R. L., Paper electrophoresis of human serum alkaline phosphatase in hepatic disorders. *Proc. Combined 51st and 52nd Indian Sci. Congr., Calcutta*, Part III, Sect. XI (Physiology), pp. 595–596 (1964–1965).

G8. Ghosh, N. K., Inglis, N. I., and Fishman, W. H., Properties of purified and crystalline human placental alkaline phosphatase. *Abstr. Papers, 152nd Meeting Am. Chem. Soc., New York*, p. 281c (1966).

G9. Glasstone, S., "Textbook of Physical Chemistry," p. 1090. Macmillan, New York, 1956.

G10. Goldfischer, S., Essner, E., and Novikoff, A. B., The localization of phosphatase activities at the level of ultrastructure. *J. Histochem. Cytochem.* **12,** 72–95 (1964).

G11. Gomori, G., The distribution of phosphatase in normal organs and tissues. *J. Cellular Comp. Physiol.* **17,** 71–84 (1941).

G12. Gomori, G., Microtechnical demonstration of phosphatase in tissue sections. *Proc. Soc. Exptl. Biol. Med.* **42,** 23–26 (1939).

G13. Gomori, G., Sources of error in enzymatic histochemistry. *J. Lab. Clin. Med.* **35,** 802–809 (1950).

G14. Gordon, A. H., A technique for elution of proteins from starch gel. *Biochim. Biophys. Acta* **42,** 23–27 (1960).

G15. Gould, B. S., The action of "alkaline" phosphatase. *J. Biol. Chem.* **156,** 365–371 (1944).

G16. Green, S., Giovanniello, T. J., and Fishman, W. H., Automated differential analysis of several serum phosphatase isoenzymes. *Proc. Technicon Symp. Automation Anal. Chem.*, New York (in press).

G17. Greenberg, L. S., Fluorometric measurement of alkaline phosphatase and aminopeptidase activities in the order 10^{-14} mole. *Biochem. Biophys. Res. Commun.* **9,** 430–435 (1962).

G18. Greene, C. H., Shattuck, H. F., and Kaplowitz, L., Phosphatase content of the blood serum in jaundice. *J. Clin. Invest.* **13,** 1079–1087 (1934).

G19. Grey, R. D., and Moog, F., Elevation of alkaline phosphatase activity in the intestine of the chick embryo by Actinomycin D. *Nature* **211,** 418–419 (1966).

G20. Griffin, M. J., and Cox, R. P., Studies on the mechanism of hormonal induction of alkaline phosphatase in human cell cultures. I. Effects of Puromycin and Actinomycin D. *J. Cell Biol.* **29,** 1–9 (1966).

G21. Griffin, M. J., and Cox, R. P., Studies on the mechanism of hormone induction of alkaline phosphatase in human cell cultures. II. Rate of enzyme synthesis and properties of base level and induced enzymes. *Proc. Natl. Acad. Sci. U.S.* **56,** 946–953 (1966).

G22. Gutman, A. B., Serum alkaline phosphatase activity in diseases of the skeletal and hepatobiliary systems. *Am. J. Med.* **27,** 875–901 (1959).

H1. Haije, W. H., and DeJong, M., Iso-enzyme patterns of serum alkaline phosphatase in agar-gel electrophoresis and their clinical significance. *Clin. Chim. Acta* **8,** 620–623 (1963).

H2. Hall, A. N., Hollingshead, S., and Rydon, H. N., The influence of structure on the hydrolysis of substituted phenyl-α-D-glucosides by α-glucosidase. *Biochem. J.* **84,** 390–394 (1962).

H3. Hamilton, W. J., and Boyd, J. D., Specializations of the syncytium of the human chorion. *Brit. Med. J.* **I,** 1501–1506 (1966).

H4. Hammett, L. P., "Physical Organic Chemistry," p. 184. McGraw-Hill, New York, 1940.

H5. Harris, H., Genes and enzymes in man. *Cancer Res.* **26,** 2054–2063 (1966).

H6. Harris, H., and Robson, E. B., A genetical study of ethanolamine phosphate excretion in hypophosphatasia. *Ann. Human Genet.* **23,** 421–433 (1959).
H7. Heino, A. E., and Jokipii, S. G., Serum alkaline phosphatase in the aged. *Ann. Med. Internae Fenniae* **51,** 105–109 (1962).
H8. Henneman, P. H., Rourke, G. M., and Jackson, W. P. U., Depression of serum alkaline phosphatase activity by human serum albumin. *J. Biol. Chem.* **213,** 19–25 (1955).
H9. Hess, B., DPN-dependent enzymes in serum. *Ann. N.Y. Acad. Sci.* **75,** 292–303 (1958).
H10. Heyndricks, G. U., Investigations on the enzymes in human milk. *Ann. Paediat.* **198,** 356–362 (1962).
H11. Hill, B. R., Further studies of the fractionation of lactic dehydrogenase of blood. *Ann. N.Y. Acad. Sci.* **75,** 304–310 (1958).
H12. Hilliard, S., O'Donnell, J. F., and Schenker, S., On the nature of the inhibitor of urinary alkaline phosphatase. *Clin. Chem.* **11,** 570–574 (1965).
H13. Hindmarsh, J. T., Kilby, D., and Wiseman, G., The effect of amino acids on sugar absorption. *J. Physiol. (London)* **186,** 166–174 (1966).
H14. Hirs, C. H. W., Moore, S., and Stein, W. H., A chromatographic investigation of pancreatic ribonuclease. *J. Biol. Chem.* **200,** 493–506 (1953).
H15. Hirs, C. H. W., Stein, W. H., and Moore, S., Chromatography of proteins. Ribonuclease *J. Am. Chem. Soc.* **73,** 1893 (1951).
H16. Hoagland, M. G., Keller, E. B., and Zamecnik, P. C., Enzymatic carboxyl activation of amino acids. *J. Biol. Chem.* **218,** 345–348 (1956).
H17. Hodson, A. W., Latner, A. L., and Raine, L., Isoenzymes of alkaline phosphatase. *Clin. Chim. Acta* **7,** 255–261 (1962).
H18. Hopkinson, D. A., Spencer, N., and Harris, H., Red cell acid phosphatase variants. A new human polymorphism. *Nature* **199,** 969–971 (1963).
H19. Horwitz, I., and Waisman, H. A., Some biochemical changes in hamsters fed excess phenylalanine diets. *Proc. Soc. Exptl. Biol. Med.* **122,** 750–754 (1966).
H20. Hsu, R. Y., Cleland, W. W., and Anderson, L., Mechanism of action of the nonspecific phosphomonoesterase from potatoes. *Biochemistry* **5,** 799–807 (1966).
H21. Hübscher, G., and West, G. R., Specific assays of some phosphatases in subcellular fractions of small intestinal mucosa. *Nature* **205,** 799–800 (1965).
H22. Huggins, C., and Talalay, P., Sodium phenolphthalein phosphate as a substrate for phosphatase tests. *J. Biol. Chem.* **159,** 399–410 (1945).
H23. Hunter, R. L., and Markert, C. L., Histochemical demonstration of enzymes separated by zone electrophoresis in starch gels. *Science* **125,** 1294–1295 (1957).
H24. Hunter, R. L., and Strachan, D. S., The esterases of mouse blood. *Ann. N.Y. Acad. Sci.* **94,** 861–867 (1961).
I1. Induye, M., and Higashi, K., Properties of acetone insensitive phosphatase from ascites hepatoma of rat. *J. Biochem.* **54,** 130–137 (1963).
I2. Inglis, N. I., Krant, M. J., and Fishman, W. H., Influence of a fat-enriched meal on human serum (L-phenylalanine-sensitive) "intestinal" alkaline phosphatase. *Proc. Soc. Exptl. Biol. Med.* **124,** 699–702 (1967).
I3. Inglis, N. I., Ghosh, N. K., and Fishman, W. H., Sephadex G-200 gel electrophoresis of human serum alkaline phosphatase. *Anal. Biochem.* (in press).
J1. Jarrige, P., and Lafoscade, P., Elution of the proteins after separation by electrophoresis through a starch-gel. *Bull. Soc. Chim. Biol.* **40,** 1197 (1959).
J2. Jeffree, G. M., Phosphatase activity in the limb bones of monkeys (Lagothrix humboldti) with hyperparathyroidism. *J. Clin. Pathol.* **15,** 99–111 (1962).
J3. Jenkins, W. T., and D'Ari, L., The kinetics of alkaline phosphatase. *J. Biol. Chem.* **241,** 295–296 (1966).

J4. Johnson, E. M., Quantification of enzyme activity subsequent to zone electrophoresis in starch gel. *Anal. Biochem.* **8,** 210–216 (1964).

J5. Judd, R., Elinor, H., and Kwok, G., A rapid ultramicro technique for alkaline phosphatase. *Am. J. Med. Technol.* **00,** 87–93 (1964).

K1. Kay, H. D., Plasma phosphatase. I. Method of determination. Some properties of the enzyme. *J. Biol. Chem.* **89,** 235–247 (1930).

K2. Kay, H. D., Phosphatase in growth and disease of bone. *Physiol. Rev.* **12,** 384–422 (1932).

K3. Keay, H., and Trew, J. A., Automated determination of serum alkaline phosphatase using a modified Bodansky technic. *Clin. Chem.* **10,** 75–82 (1964).

K4. Keefe, S. A., Marchant, D. J., and Kelsey, W. H., Alkaline phosphatase activity of L-M mouse cells and variant. *Proc. Soc. Exptl. Biol. Med.* **118,** 1031–1037 (1965).

K5. Keiding, N. R., Differentiation into three fractions of serum alkaline phosphatase and the behaviour of the fractions in diseases of bone and liver. *Scand. J. Clin. Lab. Invest.* **11,** 106–112 (1959).

K6. Keiding, N. R., The alkaline phosphatase fractions of human lymph. *Clin. Sci.* **26,** 291–297 (1964).

K7. Keiding, N. R., Intestinal alkaline phosphatase in human lymph and serum. *Scand. J. Clin. Lab. Invest.* **18,** 134–140 (1966).

K8. Keller, E.B., and Zamecnik, P. C., The effect of guanosine diphosphate and triphosphate on the incorporation of labelled amino acids into proteins. *J. Biol. Chem.* **221,** 45–59 (1956).

K9. Kerr, G. R., and Waisman, H. A., Phenylalanine: Transplacental concentrations in Rhesus monkeys. *Science* **151,** 824–825 (1966).

K10. Kind, P. R. N., and King, E. J., Estimation of plasma phosphatase by determination of hydrolysed phenol with amino-antipyrine. *J. Clin. Pathol.* **7,** 322–326 (1954).

K11. King, E. J., and Armstrong, A. R., A convenient method for determining serum and bile phosphatase activity. *Can. Med. Assoc. J.* **31,** 376–381 (1934).

K12. King, E. J., and Delory, G. E., A carbonate veronal buffer solution covering pH 7.5 to 10.7. *Enzymologia* **8,** 278–279 (1940).

K13. Kitchener, P. N., Neale, F. C., Posen, S., and Brudenell-Woods, J., Alkaline phosphatase in maternal and fetal sera at term and during the puerperium. *Am. J. Clin. Pathol.* **44,** 654–661 (1965).

K14. Klees, E. and Frenzel, G., Zur problematik der erhohung der alkalischen serum phosphatase Activitat unter der Geburt und im Wechenbett. *Klin. Wochschr.* **38,** 540–543 (1960).

K15. Klein, B., Auerbach, J., and Morganstern, S. W., Automated determination of acid phosphatase. *Clin. Chem.* **11,** 998–1008 (1965).

K16. Klein, B., Read, P. A., and Babson, A. L., Rapid method for the quantitative determination of serum alkaline phosphatase. *Clin. Chem.* **6,** 269–275 (1960).

K17. Klein, L., Lafferty, F. W., Pearson, O. H., and Curtiss, P. H., Jr., Correlation of urinary hydroxyproline, serum alkaline phosphatase and skeletal calcium turnover. *Metab. Clin. Exptl.* **13,** 272–284 (1964).

K18. Klotz, I. M., Thermodynamics and molecular properties of some metal protein complexes. *In* "A Symposium on the Mechanism of Enzyme Action" (W. D. McElroy and B. Glass, eds.), p. 257. Johns Hopkins Press, Baltimore, Maryland, 1954.

K19. Kochman, M., Mastalerz, P., and Wolna, E., Phosphonic acids—a new group of

competitive inhibitors of intestinal alkaline phosphatase. *Arch. Immunol. Terapii Doswiadczalnej.* **12,** 106–112 (1964).

K20. Korner, N. H., Distribution of alkaline phosphatase in serum protein fractions. *J. Clin. Pathol.* **15,** 195–199 (1962).

K21. Korner, N. H., Distribution of alkaline phosphatase in the serum proteins in hypophosphatasia. *J. Clin. Pathol.* **15,** 200–205 (1962).

K22. Koshland, D. E., Jr., Mechanisms of transfer enzymes. *In* "The Enzymes" (P. D. Boyer, H. Lardy, and K. Myrbäck, eds.), 2nd ed., Vol. 1, p. 334, Academic Press, New York, 1959.

K23. Kowlessar, O. D., Haeffner, L. J., and Riley, E. M., Localization of serum leucine aminopeptidase, 5-nucleotidase and nonspecific alkaline phosphatase by starch-gel electrophoresis. Clinical and biochemical significance in disease states. *Ann. N.Y. Acad. Sci.* **94,** 836–843 (1961).

K24. Kowlessar, O. D., Pert, J. H., Haeffner, L. J., and Sleisinger, M. H., Localization of 5-nucleotidase and non-specific alkaline phosphatase by starch-gel electrophoresis. *Proc. Soc. Exptl. Biol. Med.* **100,** 191–193 (1959).

K25. Kreisher, J. H., Close, V. A., and Fishman, W. H., Identification by means of L-phenylalanine inhibition of intestinal alkaline phosphatase components separated by starch-gel electrophoresis of serum. *Clin. Chim. Acta* **11,** 122–127 (1965).

K26. Kress, L. F., Bono, V. H., Jr., and Noda, L., The sulfhydryl groups of rabbit muscle adenosine triphosphate-adenosine monophosphate phosphotransferase. Activity of enzyme treated with mercurials. *J. Biol. Chem.* **241,** 2293–2300 (1966).

K27. Kreutz, F. H., Determination of serum alkaline phosphatase activity. *Enzymol. Biol. Clin.* **6,** 185 (1966).

K28. Kuan, S. S., Martin, W. G., and Patrick, H., Alkaline phosphatases of the chick. Partial characterization of the tissue isozymes. *Proc. Soc. Exptl. Biol. Med.* **122,** 172–177 (1966).

L1. Landau, W., and Schlamowitz, M., Studies of factors related to the differentiation of alkaline phosphatases derived from several tissues. *Arch. Biochem. Biophys.* **95,** 474–482 (1961).

L2. Langman, M. J. S., Leuthold, E., Robson, E. B., Harris, J., Luffman, J. E., and Harris, H., Influence of diet on the "intestinal" component of serum alkaline phosphatase in people of different ABO blood groups and secretor status. *Nature* **212,** 41–43 (1966).

L3. Lapan, B., and Friedman, M. M., A comparative study of fetal and maternal serum enzyme levels. *J. Lab. Clin. Med.* **54,** 417–426 (1959).

L4. Latner, A. L., Phosphatase Isoenzymes. *In* "Enzymes in Clinical Chemistry" (R. Ruyssen and L. Vandendriessche, pp. 110–119. Elsevier, Amsterdam, 1965.

L5. Laurent, B., and Norberg, B., Phosphatase assay in serum with *p*-nitrophenyl phosphate as substrate. *Scand. J. Clin. Lab. Invest.* **12,** 154–157 (1960).

L6. Law, R. R. J., and Munro, S. S., Inheritance of two alkaline phosphatase variants in fowl plasma (starch-gel). *Science* **149,** 1518 (1965).

L7. Lawrence, S. H., and Melnick, P. J., Enzymatic activity related to human serum beta-lipoprotein: Histochemical, immuno-electrophoretic and quantitative studies. *Proc. Soc. Exptl. Biol. Med.* **107,** 998–1001 (1961).

L8. Lawrie, N. R., The excretion of phosphatase in the faeces. *Biochem. J.* **37,** 311–312 (1943).

L9. Lazdunski, M., and Ouellet, L., Intestinal alkaline phosphatase: Kinetics of *p*-nitrophenylphosphate hydrolysis. *Can. J. Chem.* **37,** 1298–1309 (1961).

L10. Lazdunski, M., and Ouellet, L., Inhibition de la phosphatase alcaline intestinale. *Can. J. Biochem. Physiol.* **40,** 1619–1639 (1962).

L11. Lazdunski, M., Brouillard, J., and Ouellet, L., Etude des effets electrostatiques sur le mechanism d'action catalytique de la phosphatase alcaline intestinale de veau. *Can. J. Chem.* **43,** 2222–2235 (1965).

L12. Lee, A. B. H., and Lewis, P. L., Increase of alkaline phosphatase in pregnancy. *Am. J. Obstet. Gynecol.* **87,** 1071 (1963).

L13. Levine, B., Mandell, G., Lipschutz, D., and Bernstein, J., Leukocyte alkaline phosphatase in gynecologic cancer. *Am. J. Obstet. Gynecol.* **95,** 692–695 (1966).

L14. Linscheer, W. G., Blum, A. L., Inglis, N. I., and Fishman, W. H., Intestinal alkaline phosphatase during glucose absorption in man. *Clinical Res.* **25,** 238 (1967).

L15. Linderstrom-Lang, K. U., and Schellman, J. A., Protein structure and enzyme activity *In* "The Enzymes" (P. D. Boyer, H. Lardy, and K. Myrbäck, eds.), 2nd. ed., Vol. 1, p. 443. Academic Press, New York, 1959.

L16. Lineweaver, H., and Burk, D., The determination of enzyme dissociation constants. *J. Am. Chem. Soc.* **56,** 658–666 (1934).

L17. Lobel, B. D., Deane, H. W., and Romney, S. L., Enzymic histochemistry of the villous portion of the human placenta from six weeks of gestation to term. *Am. J. Obstet. Gynecol.* **83,** 295–299 (1962).

L18. Long, C. H., Ullrey, D. E., and Miller, E. R., Serum alkaline phosphatase in the post-natal pig and effect of serum storage on enzyme activity. *Proc. Soc. Exptl. Biol. Med.* **119,** 412–414 (1965).

L19. Lowry, O. H., and Lopez, J. A., The determination of inorganic phosphate in the presence of labile phosphate esters. *J. Biol. Chem.* **162,** 421–428 (1946).

L20. Lowry, O. H., and Passonneau, J. V., Kinetic evidence for multiple binding sites on phosphofructokinase. *J. Biol. Chem.* **241,** 2268–2279 (1966).

M1. Madsen, N. B., and Tuba, J., On the source of the alkaline phosphatase in rat serum. *J. Biol. Chem.* **195,** 741–750 (1952).

M2. Madsen, N. B., and Tuba, J., The role of alkaline phosphatase in intestinal absorption. I. The kinetics of phosphatase action on various substrates. *Can. J. Med. Sci.* **31,** 1–7 (1953).

M3. Malamy, M. H., and Horecker, B. L., Release of alkaline phosphatase from cells of Escherichia coli upon lysozyme spheroplast formation. *Biochemistry* **3,** 1889–1893 (1964).

M4. Malamy, M. H., and Horecker, B. L., Purification and crystallization of the alkaline phosphatase of Escherichia coli. *Biochemistry* **3,** 1893–1897 (1964).

M5. Manning, J. P., Meli, A., and Steinetz, B. G., Alkaline phosphatase and β-glucuronidase activity in the rat uterus during early pregnancy. *J. Endocrinol.* **35,** 385–391 (1966).

M6. Manson, L. A., Pelmont, J., Yapo, A., Roche, C., and Nisman, B., The biosynthesis of alkaline phosphatase with a particulate fraction of Escherichia coli. The mechanism of inhibition by inorganic phosphate. *Biochem. J.* **95,** 215–225 (1965).

M7. Markert, C. L., and Appella, E., Physiochemical nature of isozymes. *Ann. N.Y. Acad. Sci.* **94,** 678–690 (1961).

M8. Markert, C. L., and Moller, F., Multiple forms of enzymes: Tissue, ontogenetic and species specific patterns. *Proc. Natl. Acad. Sci. U.S.* **45,** 753–763 (1959).

M9. Marsh, W. H., Fingerhut, B., and Kirsch, E., Adaptation of alkaline phosphatase method for automatic colorimetric analysis. *Clin. Chem.* **5,** 119–125 (1959).

M10. Marshall, W. C., A chronic progressive osteopathy with hyperphosphatasia. *Proc. Roy. Soc. Med.* **55,** 238–239 (1962).

M11. Martin, A. J. P., and Porter, R. R., The chromatographic fractionation of ribonuclease. *Biochem. J.* **49,** 215–218 (1951).

M12. Martland, M., and Robison, R., Possible significance of hexosephosphoric esters in ossification. Part VI. Phosphoric esters in blood plasma. *Biochem. J.* **20,** 847–855 (1926).

M13. Mathies, J. C., Preparation and properties of highly purified alkaline phosphatase from swine kidneys. *J. Biol. Chem.* **233,** 1121–1127 (1958).

M14. McCance, R. A., Fairweather, D. V. I., Barrett, A. M., and Morrison, A. B., Genetic, clinical, biochemical and pathological features of hypophosphatasia. *Quart. J. Med.* [*N.S.*] **25,** 523–538 (1956).

M15. McMaster, Y., Tennant, R., Clubb, J. S., Neale, F. C., and Posen, S., The mechanism of the elevation of serum alkaline phosphatase in pregnancy. *J. Obstet. Gynecol. Brit. Commonwealth* **71,** 735–739 (1964).

M16. Meade, B. W., and Rosalki, S. B., The origin of increased maternal serum enzyme activity in pregnancy and labour. *J. Obstet. Gynecol. Brit. Commonwealth* **70,** 862–868 (1963).

M17. Meranze, T., Meranze, D. R., and Rothman, M. M., Blood phosphatase in pregnancy. *Am. J. Obstet. Gynecol.* **33,** 444–450 (1937).

M18. Michaelis, L., and Menten, M. L., Kinetics of invertase action. *Biochem. Z.* **49,** 333–369 (1913).

M19. Milicic, M., Der Einfluss von vitamin-A-mangel und uberdosierung auf niere, dunndarm und leber unter besonderer, berucksichtigung der alkalischen phosphatase. *Acta Anat.* **50,** 312–325 (1962).

M20. Milstein, C., The amino acid sequence around the reactive serine residue in alkaline phosphatase from Escherichia coli. *Biochem. J.* **92,** 410–421 (1964).

M21. Mithoefer, J., and Rapoport, S., Serum alkaline phosphatase in dogs following ligation of the hepatic veins. *J. Lab. Clin. Med.* **37,** 520–525 (1951).

M22. Mizutani, A., and Barrnett, R. J., Fine structural demonstration of phosphatase activity at pH 9. *Nature* **206,** 1001–1003 (1965).

M23. Moog, F., The physiological significance of the phosphomonoesterases. *Biol. Rev.* **21,** 41–59 (1946).

M24. Moog, F., Developmental adaptations of alkaline phosphatases in the small intestine. *Federation Proc.* **21,** 51–56 (1962).

M25. Moog, F., The regulation of alkaline phosphatase activity in the duodenum of the mouse from birth to maturity. *J. Exptl. Zool.* **161,** 253–368 (1966).

M26. Moog, F., Vire, H. R., and Grey, R. D., The multiple forms of alkaline phosphatase in the intestine of the young mouse. *Biochim. Biophys. Acta* **113,** 336–349 (1966).

M27. Morton, R. K., The lipoprotein particles in cow's milk. *Biochem. J.* **57,** 231–237 (1954).

M28. Morton, R. K., The purification of alkaline phosphatase of animal tissues. *Biochem. J.* **57,** 595–603 (1954).

M29. Morton, R. K., The substrate specificity and inhibition of alkaline phosphatase of cow's milk and calf intestinal mucosa. *Biochem. J.* **61,** 232–240 (1955).

M30. Morton, R. K., The kinetics of hydrolysis of phenylphosphate by alkaline phosphatases. *Biochem. J.* **65,** 674–682 (1957).

M31. Moskowitz, R. W., Klein, L., and Katz, D., Urinary hydroxyproline levels in an aged population. A study of nonosteoporotic and osteoporotic patients. *Arthritis Rheumat.* **8,** 61–68 (1965).

M32. Moss, D. W., Kinetics of phosphatase action on naphthyl phosphates determined by a highly sensitive spectrofluorimetric technique. *Biochem. J.* **76,** 32P (1960).

M33. Moss, D. W., Iso-enzymes of alkaline phosphatase in autolysed and butanol-extracted liver preparations. *Nature* **193,** 981–982 (1962).

M34. Moss, D. W., Heterogeneity of human intestinal alkaline phosphatase. *Nature* **200,** 1206–1207 (1963).

M35. Moss, D. W., Properties of alkaline phosphatase fractions in extracts of human small intestine. *Biochem. J.* **94,** 458–462 (1965).

M36. Moss, D. W., and King, E. J., Properties of alkaline phosphatase fractions separated by starch-gel electrophoresis. *Biochem. J.* **84,** 192–195 (1962).

M37. Moss, D. W., Campbell, D. M., Anagnostou-Kakaras, E., and King, E. J., Characterization of tissue alkaline phosphatases and their partial purification by starch-gel electrophoresis. *Biochem. J.* **81,** 441-447 (1961).

M38. Moss, D. W., Eaton, R. H., Smith, J. K., and Whitby, L. G., Alteration in the electrophoretic mobility of alkaline phosphatases after treatment with neuraminidase. *Biochem. J.* **98,** 32C (1966).

M39. Motzok, I., and Branion, H. D., Studies on alkaline phosphates. III. Influence of age of fowl and mammals on pH optima. *Biochem. J.* **80,** 5–9 (1961).

N1. Nagant de Deuxchaisnes, C., and Krane, S. M., Paget's disease of bone: Clinical and metabolic considerations. *Medicine* **43,** 233–266 (1964).

N2. Najjar, V. A., Phosphoglucomutase. *In* "The Enzymes" (P. D. Boyer, H. Lardy, and K. Myrbäck, eds.), 2nd ed., Vol. 6, p. 161. Academic Press, New York, 1962.

N3. Nath, R. L., and Das, I., Determination of serum alkaline phosphatase with substrates containing branched chain and straight chain alkyl groups. *Enzymologia* **31,** 155–162 (1966).

N4. Nath, R. L., and Ghosh, N. K., A preliminary report on the determination of the normal values of serum alkaline phosphatase activity by velocity constant method. *Bull. Calcutta School Trop. Med.* **10,** 71–72 (1962).

N5. Nath, R. L., and Ghosh, N. K., Effect of precipitation of serum alkaline phosphatase on the electronic influence of substituents in the enzymatic hydrolysis of substituted phenyl phosphates; a preliminary report. *Bull. Calcutta School Trop. Med.* **11,** 54–55 (1963).

N6. Nath, R. L., and Ghosh, N. K., Serum phenylphosphatase and fructose 1:6-diphosphatase in thalassaemia. *Bull. Calcutta School Trop. Med.* **11,** 140–141 (1963).

N7. Nath, R. L., and Ghosh, N. K., Studies on serum phosphatases: Effect of dilution on acid and alkaline phosphatases of human serum. *Enzymologia* **25,** 182–195 (1963).

N8. Nath, R. L., and Ghosh, N. K., Studies on serum phosphatases. II. Increase in active site of serum alkaline phosphatase on dilution. *Enzymologia* **25,** 350–358 (1963).

N9. Nath, R. L., and Ghosh, N. K., Studies on serum phosphatases. III. Influence of structure on the hydrolysis of substituted phenyl phosphates by serum alkaline phosphatase. *Enzymologia* **26,** 141–154 (1963).

N10. Nath, R. L., and Ghosh, N. K., Studies on serum phosphatases. IV. Energy changes during complex formation between substituted phenylphosphates and serum alkaline phosphatase. *Enzymologia* **26,** 297–309 (1963).

N11. Nath, R. L., and Ghosh, N. K., Paper electrophoresis of alkaline phosphatase of rachitic serum. *Bull. Calcutta School Trop. Med.* **12,** 59–60 (1964).

N12. Nath, R. L., and Ghosh, N. K., Use of 16-beta-methyl-9-alpha-fluoro-prednisolone (beta-methasone)-phosphate as a substrate for the estimation of human serum alkaline phosphatase. *Bull. Calcutta School Trop. Med.* **12,** 111–112 (1964).

N13. Nath, R. L., and Ghosh, N. K., Studies on serum phosphatases. V. Non-specific human alkaline phosphatase and FDPase in normal and pathological conditions. *Enzymologia* **32,** 278–290 (1967).

N14. Nath, R. L., and Pradhan, T. K., On the mechanism of the enzymatic hydrolysis of substituted phenyl phosphates by phosphatase from French bean leaves. Phaseolus Vulgaris L. *Enzymologia* **25,** 116–124 (1962).
N15. Nath, R. L., and Rydon, H. N., The influence of structure on the hydrolysis of substituted phenyl-β-D-glucosides by emulsin. *Biochem. J.* **57,** 1–10 (1954).
N16. Nayudu, P. R. V., and Moog, F., Intestinal alkaline phosphatase: Regulation by a strain-specific factor in mouse milk. *Science* **152,** 656–657 (1966).
N17. Neale, F. C., Clubb, J. S., and Posen, S., Artificial elevation of the serum alkaline phosphatase concentration. *Med. J. Australia* **2,** 684 (1963).
N18. Neale, F. C., Clubb, J. S., Hotchkis, D., and Posen, S., Heat stability of human placental alkaline phosphatase. *J. Clin. Pathol.* **18,** 359–363 (1965).
N19. Neims, A. H., DeLuca, D. C., and Hellerman, L., Studies on crystalline D-amino acid oxidase. III. Substrate specificity and σ-ρ relationship. *Biochemistry* **5,** 203–213 (1966).
N20. Newton, M. A., A distinctive fraction of alkaline phosphatase in health and disease. *J. Clin. Pathol.* **19,** 491–495 (1966).
N21. Nikkila, E. A., Serum alkaline phosphatase activity in pulmonary infarction. *Scand. J. Clin. Lab. Invest.* **11,** 405–406 (1959).
N22. Nisselbaum, J. S., Schlamowitz, M., and Bodansky, O., Immunochemical studies of functionally similar enzymes. *Ann. N.Y. Acad. Sci.* **94,** 970–987 (1961).
N23. Nitowsky, H. M., and Herz, F., Alkaline phosphatase activity of human cell cultures. *Proc. Soc. Exptl. Biol. Med.* **107,** 532–534 (1961).
N24. Nitowsky, H. M., and Herz, F., Alkaline phosphatase in cell cultures of human origin. *Nature* **189,** 756–757 (1961).
N25. Nitowsky, H. M., Herz, F., and Geller, S., Induction of alkaline phosphatase in dispersed cell cultures by changes in osmolarity. *Biochem. Biophys. Res. Commun.* **12,** 293–299 (1963).
N26. Nyc, J. F., Kadner, R. J., and Crocken, B. J., A repressible alkaline phosphatase in Neurospora Crassa. *J. Biol. Chem.* **241,** 1468–1472 (1966).
O1. Ormerod, W. E., Hydrolysis of benzoylcholine derivatives by cholinesterase in serum. *Biochem. J.*, **54,** 701–704 (1953).
O2. Ornstein, L., Disc electrophoresis. I. Background and theory. *Ann. N.Y. Acad. Sci.* **121,** 321–349 (1964).
O3. O'Sullivan, M. A., and Pryles, C. V., A comparison of leukocyte alkaline phosphatase determinations in 200 patients with mongolism and in 200 "familial" controls. *New Engl. J. Med.* **268,** 1168–1170 (1963).
P1. Peacock, A. C., Brecher, G., and Highsmith, E. M., A simplified procedure for quantitative measurement of alkaline phosphatase in white blood cells. *Am. J. Clin. Pathol.* **29,** 180–185 (1958).
P2. Peacock, A. C., Reed, R. A., and Highsmith, E. M., Ethanol fractionation of human serum alkaline phosphatase. *Clin. Chim. Acta* **8,** 914–917 (1963).
P3. Pelichová, H. Kraml, J., and Veĉerek, B., Precipitating and binding antibodies against human intestinal alkaline phosphatase. *Folia Biol. (Prague)* **11,** 208–214 (1965).
P4. Pigretti, M. M., and Milstein, C., Acid inactivation of and incorporation of phosphate into alkaline phosphatase from Escherichia coli. *Biochem. J.* **94,** 106–113 (1965).
P5. Pimstone, B., Eisenberg, E., and Silverman, S., Hypophosphatasia: Genetic and dental studies. *Ann. Internal Med.* [*N.S.*] **65,** 722–729 (1966).
P6. Plocke, D. J., and Vallee, B. L., Interaction of alkaline phosphatase of E. coli with metal ions and chelating agents. *Biochemistry* **1,** 1039–1043 (1962).

P7. Plocke, D. J., Levinthal, C., and Vallee, B. L., Alkaline phosphatase of Escherichia coli: A zinc metalloenzyme. *Biochemistry* **1,** 373–378 (1962).

P8. Plosscowe, R. P., Berg, G. C., and Segal, H. L., Enzyme histochemical studies of human gastric and jejunal biopsy specimens in normal and disease states. *Am. J. Digest. Diseases* **8,** 311–318 (1963).

P9. Plotch, S., and Lukton, A., Active site studies of alkaline phosphatase. *Biochim. Biophys. Acta* **99,** 181–182 (1965).

P10. Polin, S. G., Spellberg, M. A., Teitelman, L., and Okumura, M., The origin of elevation of serum alkaline phosphatase in hepatic disease. *Gastroenterology* **42,** 431–438 (1962).

P11. Pontremoli, S., Luppis, B., Wood, W. A., Traniello, S., and Horecker, B. L., Fructose diphosphatase from rabbit liver. II. Changes in catalytic properties induced by dinitrofluorobenzene. *J. Biol. Chem.* **240,** 3464–3468 (1965).

P12. Pontremoli, S., Luppis, B., Traniello, S., Wood, W. A., and Horecker, B. L., Fructose diphosphatase from rabbit liver. III. Nature of the groups reactive with dinitrofluorobenzene. *J. Biol. Chem.* **240,** 3469–3472 (1965).

P13. Pope, C. E., and Cooperband, S. R., Protein characteristics of serum and bile alkaline phosphatase. *Gastroenterology* **50,** 631–636 (1966).

P14. Port, C., and Van Venrooy, M. J. W., Detection of enzymes in agar electropherograms. *Nature* **204,** 684 (1964).

P15. Porter, G. R., Rydon, H. N., and Schofield, J. A., Mechanism of esterase action: Nature of the reactive serine residue in enzymes inhibited by organophosphorus compounds. *Nature* **182,** 927 (1958).

P16. Portmann, P., Zur Kenntis der alkalischen darmphosphatase. *Z. Physiol. Chem.* **309,** 87–128 (1957).

P17. Portmann, P., Rossier, R., and Chardenners, H., Zur Kenntnis der alkalischen darmphosphatase. II. Untersuchungen uber die Homogenität und den chemieschen Aufbau der reinen alkalischen Phosphomonoesterase. *Helv. Physiol. Pharmacol. Acta* **18,** 414-427 (1960).

P18. Posen, S., Clubb, J. S., Neale, F. C., and Hotchkis, D., The measurement of plasma volume of enzyme dilution. *J. Lab. Clin. Med.* **65,** 530–538 (1965).

P19. Posen, S., Neale, F. C., and Clubb, J. S., Heat inactivation in the study of human alkaline phosphatases. *Ann. Internal Med.* [*N.S.*] **62,** 1234–1243 (1965).

P20. Posen, S., Neale, F. C., Brudenell-Woods, J., and Birkett, D. J., Continuous determination of enzyme activity during heat inactivation. *Lancet* **I,** 264–265 (1966).

P21. Poulik, M. D., and Smithies, O., Comparison and combination of the starch-gel and filter-paper electrophoretic methods applied to human sera: Two dimensional electrophoresis. *Biochem. J.* **68,** 636–643 (1958).

P22. Powell, M. E. A., and Smith, M. J. H., The determination of serum acid and alkaline phosphatase activity with 4-aminoantipyrine (A. A. P.) *J. Clin. Pathol.* **7,** 245–248 (1954).

P23. Price-Evans, D. A., Confirmation of association between ABO blood groups and salivary ABH Secretor phenotypes and electrophoretic patterns of serum alkaline phosphatase. *J. Med. Genet.* **2,** 126–127 (1965).

R1. Rasmussen, B. A., Inheritance of R-e-i Blood groups and alkaline phosphatase polymorphism in sheep. *Genetics* **51,** 767–770 (1965).

R2. Reale, E., Electron microscopic localization of alkaline phosphatase from material prepared with the cryostat-microtome. *Exptl. Cell Res.* **26,** 210–211 (1962).

R3. Reis, J., Uber die Aktivitat der 5-Nukleotidase in den tierischen und menschlichen Geweben. *Enzymologia* **2,** 183–190 (1937).

R4. Rendel, J., and Stormont, C., Variants of ovine alkaline serum phosphatases and their association with the R-O blood groups. *Proc. Soc. Exptl. Biol. Med.* **115,** 853–856 (1964).

R5. Roberts, W. M., Variations in the phosphatase activity of the blood in disease. *Brit. J. Exptl. Pathol.* **11,** 90–95 (1930).

R6. Roberts, W. M., Blood phosphatase and the van den Bergh reaction in the differentiation of the several types of jaundice. *Brit. Med. J.* **I,** 734–738 (1933).

R7. Robinson, J. C., and Pierce, J. E., Differential action of neuraminidase on human serum alkaline phosphatases. *Nature* **204,** 472–473 (1964).

R8. Robinson, J. C., and Pierce, J. E., Prevention of background staining of starch gels. Preliminary treatment of the starch with streptomyces griseus protease. *Am. J. Clin. Pathol.* **40,** 588–590 (1963).

R9. Robinson, J. C., Pierce, J. E., and Blumberg, B. S., The serum alkaline phosphatase of pregnancy. *Am. J. Obstet. Gynecol.* **94,** 559–565 (1966).

R10. Robinson, J. C., Pierce, J. E., and Goldstein, D. P., Leucocyte alkaline phosphatase: Electrophoretic variants associated with chronic myelogenous leukemia. *Science* **150,** 58–60 (1965).

R11. Robison, R., The possible significance of hexosephosphoric esters in ossification. *Biochem. J.* **17,** 286–293 (1923).

R12. Robison, R., Bone phosphatase. *Ergeb. Enzymforsch.* **1,** 280–294 (1933).

R13. Robison, R., and Soames, K. M., The possible significance of hexose phosphoric esters in ossification. Part II. The phosphoric esterase of ossifying cartilage. *Biochem. J.* **18,** 740–754 (1924).

R14. Robison, R., and Soames, K. M., Possible significance of hexosephosphoric esters in ossification. III. Action of the bone enzyme on the organic phosphorus compounds in blood. *Biochem. J.* **18,** 755–764 (1924).

R15. Robson, E. B., and Harris, H., Genetics of the alkaline phosphatase polymorphism of the human placenta. *Nature* **207,** 1257–1259 (1965).

R16. Robson, E. B., and Harris, H., Further studies on the genetics of placental alkaline phosphatase. *Ann. Human Genet. (London)* **30,** 219–232 (1966).

R17. Roche, J., Blood phosphatases. *Biochem. J.* **25,** 1724–1733 (1931).

R18. Roche, J., Phosphatases. *In* "The Enzymes" (J. B. Summer and K. Myrbäck, eds.), Vol. 1, Part 1, pp. 473 and 507. Academic Press, New York, 1950.

R19. Roche, J., and Sarles, H., Organ specificity of alkaline phosphatases and (the phosphatase of) hyperphosphatasemia. *Bull. Soc. Chim. Biol.* **33,** 667–672 (1951).

R20. Roche, J., and Sarles, H., Nature and origin of the alkaline phosphomonoesterase of the serum. *Compt. Rend. Soc. Biol.* **147,** 1588–1561 (1953).

R21. Roche, J., and Thoai, N. V., Phosphatase alcaline. *Advan. Enzymol.* **10,** 83–122 (1950).

R22. Resalki, S. B., and Wilkinson, J. H., Urinary lactic dehydrogenase in renal disease. *Lancet* **II,** 327–328 (1959).

R23. Rose, R. G., and Wilson, A. C., Peafowl lactate dehydrogenase: Problem of isoenzyme identification. *Science* **153,** 1411–1413 (1966).

R24. Rosenberg, I N., Zone electrophoretic studies of serum alkaline phosphatase. *J. Clin. Invest.* **38,** 630–644 (1959).

R25. Rosenkranz, H. S., Unusual alkaline phosphatase level in streptomycin-dependent strains of E. Coli. *Biochemistry* **2,** 122–125 (1963).

R26. Ryan, W. L., and Carver, M. J., Inhibition of antibody synthesis by L-phenylalanine. *Science* **143,** 479–480 (1964).

R27. Rydon, H. N., Mechanism of esterase action: A possible mechanism of action

of esterases inhibitable by organophosphorus compounds. *Nature* **182,** 928–929 (1958).

S1. Sadovsky, E., and Zuckerman, H., An alkaline phosphatase specific to normal pregnancy. *Obstet. Gynecol.* **26,** 211–214 (1965).

S2. Sandler, M., and Bourne, G. H., Histochemical studies of phosphatases separated by starch-gel electrophoresis. *Exptl. Cell Res.* **24,** 174–177 (1961).

S3. Sandler, M., and Bourne, G. H., Starch-gel electrophoresis of alkaline phosphatases using a histochemical method. *Nature* **194,** 389–390 (1962).

S4 Saraswathi, S., and Bachhawat, B. K., Phosphatases from human brain. I. Purification and properties of pyridoxal phosphate phosphatase. *J. Neurochem.* **10,** 127–133 (1963).

S5. Saraswathi, S., and Bachhawat, B. K., Heterogeneity of alkaline phosphatase in sheep brain. *J. Neurochem.* **13,** 237–246 (1966).

S6. Sarles, H., Sur la phosphomonoesterase alcaline du serum et sa caracterisation au moyen de sa constant d'affinite. *Compt. Rend. Soc. Biol.* **147,** 1688–1690 (1953).

S7. Sarles, H., Role of the liver in the regulation of the alkaline phosphatase activity of the blood. *Compt. Rend. Soc. Biol.* **147,** 1747–1750 (1953).

S8. Saunders, S. J., and Isselbacher, K. J., Intestinal absorption of amino acids. *Gastroenterology* **50,** 586–595 (1966).

S9. Schain, R. J., Copenhaver, J. H., and Carver, M. J., Inhibition by phenylalanine of the entry of 5-hydroxy-tryptophan-1-C^{14} into cerebrospinal fluid. *Proc. Soc. Biol. Med.* **118,** 184–186 (1965).

S10. Schlamowitz, M., Production of antibodies against dog intestinal phosphatase. *J. Biol. Chem.* **206,** 361–367 (1954).

S11. Schlamowitz, M., Specificity of dog intestinal phosphatase antiserum. *J. Biol. Chem.* **206,** 369–374 (1954).

S12. Schlamowitz, M., Immunochemical studies on alkaline phosphatase. *Ann. N.Y. Acad. Sci.* **75,** 373–379 (1958).

S13. Schlamowitz, M., The reaction of dog intestinal phosphatase with its antibodies in the region of excess antibody. *J. Immunol.* **80,** 176–181 (1958).

S14. Schlamowitz, M., and Bodansky, O., Tissue sources of human serum alkaline phosphatase as determined by immunochemical procedures. *J. Biol. Chem.* **234,** 1433–1437 (1959).

S15. Schlang, H. A., and McHenry, L. E., Jr., High serum alkaline phosphatase. *N.Y. State J. Med.* **62,** 3811–3813 (1962).

S16. Schlesinger, M. J., The reversible dissociation of the alkaline phosphatase of Escherichia coli. II. Properties of the subunit. *J. Biol. Chem.* **240,** 4293–4298 (1965).

S17. Schlesinger, M. J., and Barrett, K., The reversible dissociation of the alkaline phosphatase of Escherichia coli. *J. Biol. Chem.* **240,** 4284–4292 (1965).

S18. Schlesinger, M. J., and Levinthal, C., Hybrid protein formation of E. Coli alkaline phosphatase leading to in vitro complementation. *J. Mol. Biol.* **7,** 1–12 (1963).

S19. Schmidt, G., and Thanhauser, S. J., Intestinal phosphatase. *J. Biol. Chem.* **149,** 369–385 (1943).

S19a. Schmidt, G., and Laskowski, M., Sr., Phosphate ester cleavage. *In* "The Enzymes" (P. D. Boyer, H. Lardy, and K. Myrbäck, eds.), Vol. 5, p. 3. Academic Press, New York, 1961.

S20. Schneiderman, H., Young, W. J., and Childs, B., Patterns of alkaline phosphatase in developing drosophila. *Science* **151** 461–463 (1966).

S21. Schultze, H. E., Influence of bound sialic acid on electrophoretic mobility of human serum proteins. *Arch. Biochem. Biophys.* Suppl. 1, 290–294 (1962).

S22. Schwartz, J. H., The phosphorylation of alkaline phosphatase. *Proc. Natl. Acad. Sci. U.S.* **49,** 871–878 (1963).
S23. Schwartz, J. H., Crestfield, A., and Lipmann, F., The amino acid sequence of a tetradecapeptide containing the reactive serine in E. Coli alkaline phosphatase. *Proc. Natl. Acad. Sci. U.S.* **49,** 722–729 (1963).
S24. Schwartz, M. K., Kessler, G., and Bodansky, O., Comparison of serum alkaline phosphatase activities determined with sodium beta glycerophosphate and sodium phenylphosphate as substrates. *Am. J. Clin. Pathol.* **33,** 275–280 (1960).
S25. Scott, W. W., and Huggins, C., The acid phosphatase activity of human urine, an index of prostatic secretion. *Endocrinology* **30,** 107–120 (1942).
S26. Sebesta, D. G., Bradshaw, F. J., and Prockop, D. J., Source of the elevated serum alkaline phosphatase activity in biliary obstruction: Studies using isolated liver perfusion. *Gastroenterology* **47,** 166–170 (1964).
S27. Seelich, F., and Ehrlich-Gomolka, H., Uber du "Saure" und "alkalische" phosphatase in placenta and fruchtwasser. *Enzymologia* **15,** 96–97 (1951).
S28. Sherlock, S., "Diseases of the Liver and Biliary System," 3rd. ed., p. 43. Blackwell, Oxford, 1963.
S29. Sherlock, S., and Walshe, V., Hepatic alkaline phosphatase: Histological and microchemical studies on liver tissue in normal subjects and in liver and bone disease. *J. Pathol. Bacteriol.* **59,** 615–630 (1947).
S30. Shinowara, G. Y., Jones, L. M., and Reinhart, H. L., The estimation of serum inorganic phosphate and "acid" and "alkaline" phosphatase activity. *J. Biol. Chem.* **142,** 921–933 (1942).
S31. Shishova, O. A., The role of phosphorylation in the process of enteric absorption of amino acids. *Biokhimiya* **21,** 111–118 (1956); *Chem. Abstr.* **50,** 10221 (1956).
S32. Shreffler, D. C., Genetic studies of blood group-associated variations in a human serum alkaline phosphatase. *Am. J. Human Genet.* **17,** 71–86 (1965).
S33. Shreffler, D. C., Relationship of alkaline phosphatase level in intestinal mucosa to ABO and secretor blood groups. *Proc. Soc. Exptl. Biol. Med.* **123,** 423–427 (1966).
S34. Siebert, G., Kesselring, K., and Fischer, F., Phosphoenolpynevat als substrat der alkalischen phosphatase: Konkurrenz mit Pyruvatkinase. *Z. Physiol. Chem.* **341,** 44–75 (1965).
S35. Sims, M., Methods for detection of enzymatic activity after electrophoresis on Polyacrylamide Gel in Drosophila species. *Nature* **207,** 757–758 (1965).
S36. Sizer, I. W., The action of certain oxidants and reductants upon the activity of bovine phosphatase. *J. Biol. Chem.* **145,** 405–414 (1942).
S37. Sizer, I. W., Chemical aspects of enzyme inhibitors. *Science* **125,** 54–59 (1957).
S38. Smithies, O., An improved procedure for starch-gel electrophoresis: Further variations in the serum proteins of normal individuals. *Biochem. J.* **71,** 585–587 (1959).
S39. Smithies, O., Zone electrophoresis in starch-gels: Group variations in the serum proteins of normal human adults. *Biochem. J.* **61,** 629–641 (1955).
S40. Smithies, O., Zone electrophoresis in starch-gels and its application to studies of serum proteins. *Adv. Protein Chem.* **14,** 65–113 (1959).
S41. Smithies, O., Molecular size and starch-gel electrophoresis. *Arch. Biochem. Biophys.* Suppl. 1, 125–131 (1962).
S42. Spink, W. W., and Landeryou, M. J., Diminution in renal alkaline phosphatase in endotoxin shock. *J. Clin. Invest.* **39,** 302–309 (1960).
S43. Stadtman, T. C., Alkaline phosphatases. *In* "The Enzymes" (P. D. Boyer, H.

Lardy, and K. Myrbäck, eds.), 2nd. ed., Vol. 5, p. 71. Academic Press, New York, 1961.

S44. Stein, S. S., and Koshland, D. E., Mechanism of action of alkaline phosphatase. *Arch. Biochem. Biophys.* **39,** 229–230 (1952).

S45. Stemmerman, G. N., An histologic and histochemical study of familial osteoectasia. *Am. J. Pathol.* **48,** 641–651 (1966).

S46. Sterling, R. E., Wilcox, A. A., Ware, A. G., and Umchara, M. K., An automated determination of alkaline phosphatase utilizing p-nitrophenol phosphate. *Clin. Chem.* **10,** 1112–1116 (1964).

S47. Stevenson, D. E., Demonstration of alkaline phosphatase activity following agar-gel electrophoresis. *Clin. Chim. Acta* **6,** 142–143 (1961).

S48. Stolbach, L. L., Nisselbaum, J. S., and Fishman, W. H., A simplified technic for measuring serum acid phosphatase of prostatic origin (Fishman-Lerner method). *Am. J. Clin. Pathol.* **29,** 379–384 (1958).

S49. Stolbach, L. L., Krant, M. J., Inglis, N. I., and Fishman, W. H., Correlation of serum L-phenylalanine sensitive alkaline phosphatase, derived from intestine, with the ABO blood group of cirrhotics. *Gastroenterology* **103,** 819–827 (1967).

T1. Taleisnik, S., Paglini, S., and Zeitung, V., Localisation de la phosphatase alcaline du serum dans les fractions protéiniques separées par electrophorese. *Compt. Rend. Soc. Biol.* **149,** 1790 (1955).

T2. Taswell, H. F., and Jeffers, D. M., Isoenzymes of serum alkaline phosphatase in hepatobiliary and skeletal disease. *Am. J. Clin. Pathol.* **40,** 349–356 (1963).

T3. Teaford, M. E., and White, A. A., Alkaline phosphatase and osteogenesis in vitro. *Proc. Soc. Exptl. Biol. Med.* **117,** 541–546 (1964).

T4. Tietz, N. W., and Green, A., An automated procedure for the determination of phosphorus and alkaline phosphatase (Bodansky) in serum. *Clin. Chim. Acta* **9,** 392–399 (1964).

T5. Triantaphyllopoulos, E., and Tuba, J., Changes in intestinal and serum alkaline phosphatase levels during absorption of certain amino acids. *Can. J. Biochem. Physiol.* **37,** 711–719 (1959).

T6. Trubowitz, S., Feldman, D., Morganstern, S. W., and Hunt, V. M., The isolation purification and some properties of the alkaline phosphatase of human leucocytes. *Biochem. J.* **80,** 369–374 (1961).

T7. Tsou, K. C., and Su, H. C. F., A new colorimetric method for the determination of alkaline phosphatase with indoxyl phosphate. *Anal. Biochem.* **11,** 54–64 (1965).

V1. Vaes, G., and Jacques, P., Studies on bone enzymes. The assay of acid hydrolases and other enzymes in bone tissue. *Biochem. J.* **97,** 380–388 (1965).

V2. Vaes, G., and Jacques, P., Studies on bone enzymes. Distribution of acid hydrolases, alkaline phenylphosphatases, cytochrome oxidase and catalase in subcellular fraction of bone tissue homogenate. *Biochem. J.* **97,** 389–392 (1965).

V3. Valentine, W. N., and Beck, W. S., Biochemical studies on Leucocytes. I. Phosphatase activity in health, leucocytosis and myelocytic leucemia. *J. Lab. Clin. Med.* **38,** 39–55 (1951).

V4. Valentine, W. N., Follette, J. H., Hardin, E. B., Beck, W. S., and Lawrence, J. S., Studies on leukocyte alkaline phosphatase activity: Relation to "stress" and pituitary adrenal activity. *J. Lab. Clin. Med.* **44,** 219–228 (1954).

V5. Vallee, B. L., Metal and enzyme interactions: Correlation of composition, function, and structure. *In* "The Enzymes" (P. D. Boyer, H. Lardy, and K. Myrbäck, eds.), 2nd ed., Vol. 3, Part B, pp. 225–276. Academic Press, New York, 1960.

V6. Večerek, B., Pelichová, H., Štěpán, J., Štípek, S., Chmelař, M., and Kraml, J.,

Changes in human intestinal and kidney phosphatases during isolation. *Proc. 3rd. Natl. Congr. Czech. Biochem. Soc.*, p. 40. Czech. Acad. Sci., Olaomoue, 1963.

V7. Večerek, B., Kraml, J., Pelichová, H., Štěpán, J., Chmelár, M., and Štípek, S., Phosphatases. II. Changes in the composition of human intestinal and kidney alkaline phosphatase during purification. *Collection Czech. Chem. Commun.* **30,** 3964–3968 (1965).

V8. Verzar, F., Sailer, E., and Richterich, R., Einfluss der Nebennierenrinde auf die alkalische phosphatase der Dunndarmschleimhaut. *Helv. Physiol. Pharmacol. Acta* **10,** 231–246 (1952).

V9. Vesell, E. S., Significance of the heterogeneity of lactic dehydrogenase activity in human tissues. *Ann. N.Y. Acad. Sci.* **94,** 655–1030 (1961).

V10. Vesell, E. S., pH dependence of lactate dehydrogenase isoenzyme inhibition by substrate. *Nature* **210,** 421–422 (1966).

V11. Vesell, E. S., and Brody, I. A., Biological applications of lactic dehydrogenase isoenzymes: Certain methodological considerations. *Ann. N.Y. Acad. Sci.* **121,** 544–559 (1964).

W1. Wachstein, M., and Bradshaw, M., Histochemical localization of enzyme activity in the kidneys of three mammalian species during their postnatal development. *J. Histochem. Cytochem.* **13,** 44–56 (1965).

W2. Wachstein, M., and Meisel, E., Histochemistry of hepatic phosphatases at a physiological pH with special reference to the demonstration of bile canaliculi. *Am. J. Clin. Pathol.* **27,** 13–23 (1957).

W3. Wachstein, M., Meagher, J. G., and Ortiz, J., Enzymatic histochemistry of the term human placenta. *Am. J. Obstet. Gynecol.* **87,** 13–29 (1963).

W4. Wacker, W. E. C., and Dorfman, L. E., Urinary lactic dehydrogenase activity I. Screening method for detection of cancer of kidneys and bladder. *J. Am. Med. Assoc.* **181,** 972–978 (1962).

W5. Walsh, E. O., and Walsh, G., Inhibition of hexosediphosphatase by sulfhydryl reagents and by ascorbic acid. *Nature* **161,** 976–977 (1948).

W6. Warnock, M. L., Characterization of tissue and serum alkaline phosphatases. *Clin. Chim. Acta* **14,** 156–165 (1966).

W7. Watanabe, K., and Fishman, W. H., Application of the stereospecific inhibitor L-phenylalanine to the enzymorphology of intestinal alkaline phosphatase. *J. Histochem. Cytochem.* **12,** 252–260 (1964).

W7a. Watson, W. C., Murray, E. S., and Gardner, M. D., Regulation of intestinal alkaline phosphatase levels in the rat. Role of the adrenal cortex. *J. Clin. Path.* **20,** 185–189 [1967].

W8. Wieland, T. H., and Pfleiderer, G., Chemical differences between multiple forms of lactic acid dehydrogenases. *Ann. N.Y. Acad. Sci.* **94,** 691–700 (1961).

W9. Wilson, I. B., and Dayan, J., The free energy of hydrolysis of phosphoryl phosphatase. *Biochemistry* **4,** 645–649 (1965).

W10. Wilson, I. B., Dayan, J., and Cyr, K., Some properties of alkaline phosphatase from Escherichia coli. Transphosphorylation. *J. Biol. Chem.* **239,** 4182–4185 (1964).

W11. Winters, R. S., Graham, J. B., Williams, T. F., McFalls, V. W., and Burnett, C. H., A genetic study of familial hypophosphatemia and Vitamin D resistant rickets with a review of the literature. *Medicine* **37,** 97–142 (1958).

W12. Wolfson, W. Q., Location of alpha-2 globulin by demonstration of alkaline phosphatase during paper electrophoresis. *Nature* **180,** 550–551 (1957).

W13. Woodward, H. Q., Acid and alkaline glycerophosphatase in tissue and serum. *Cancer Res.* **2,** 497–508 (1942).

W14. Woodward, H. Q., Quantitative studies of beta glycerophosphatase in normal and neoplastic tissues. *Cancer* **9,** 352–366 (1956).

W15. Woodward, H. Q., Escher, G. C., and Farrow, J. H., Changes in the blood chemistry of patients with disseminated carcinoma of the breast during therapy *Cancer* **7,** 744–757 (1954).

W16. Wray, S., The significance of the blood acid and alkaline phosphatase values in cancer of the prostate. *J. Clin. Pathol.* **9,** 341–345 (1956).

Y1. Yong, J. M., Cause of raised serum alkaline phosphatase after partial gastroectomy and other malabsorption states. *Lancet* **I,** 1132–1134 (1966).

Z1. Zinkham, W. H., Blance, A., and Clowry, L. J., Jr., An unusual isoenzyme of lactic dehydrogenase in mature testes: Localization, ontogeny and kinetic properties. *Ann. N.Y. Acad. Sci.* **121,** 571–588 (1964).

Z2. Zuckerman, H., and Sadovsky, E., Thermostable alkaline phosphatase in pregnancy. *Israel J. Med. Sci.* **1,** 230–234 (1965).

Z3. Zwaig, N., and Milstein, C., The amino acid sequence around the reactive serine residue in alkaline phosphatase of Serratia marcescens. *Biochem. J.* **92,** 421–422 (1964).

AUTHOR INDEX

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