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PREFACE

The purpose of this volume, which is the second of the current series, is to broaden the technical scope of clinical chemists.

Through the use of the talents and knowledge of a distinguished Board of International Editors and consequently an international authorship, the cutting edge of the science and philosophy of clinical chemistry will hopefully be presented with a more panoramic view.

There are five chapters contained in this book. Each one is written by a recognized expert in the field. The areas of focus are Ion-Selective Electrodes, the Somatomedins, Synthetic Peptide Substrate Assays for Hemostasis Testing, Bile Acids, and Folic Acid.

It continues to be the aspiration of the Editorial Board that the readership will be served by its efforts. We welcome your suggestions for further topics of interest.

HERBERT E. SPIEGEL

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ION-SELECTIVE ELECTRODES

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1. Overview

Ion-selective electrodes (ISEs) are relatively simple membrane-based potentiometric devices which are capable of accurately measuring the activity of ions in solution. Selectivity of these transducers for one ion over another is determined by the nature and composition of the membrane materials used to fabricate the electrode. While many scientists are quite familiar with the glass membrane pH electrode first described by Cremer (C10), most are far less aware of the other types of ISEs which may be prepared with crystalline, liquid, and polymer membranes and which allow for the selective measurement of a wide variety of cations and anions (e.g., Na^+, K^+, Ca^{2+}, Ag^+, Cl^-, Br^-, F^-, and organic ions). Moreover, in recent years, the range of measurable species has been further extended to include dissolved gases and

even biochemicals (e.g., amino acids and metabolites) by adding discriminating membrane and biocatalytic layers to the surfaces of various ISE devices to form gas sensors, enzyme electrodes, etc.

As analytical tools, ISEs offer attractive advantages for direct measurement in complex samples. Indeed, in many instances potentiometric measurements can be made *in situ* without interference from the chemical or physical properties of the sample (i.e., color, turbidity). Further, membrane electrodes are inexpensive devices and when used in conjunction with commercially available high-input impedance voltmeters, the entire method provides a low-cost yet effective means of performing a wide range of analyte determinations. It is these practical advantages along with the fact that ion activities are detected, not concentrations, that has prompted biomedical researchers to use ISEs in fundamental research efforts relating to the study of physiological processes. Since ion activity levels within cells play a vital role in cell function, the ability to measure these activities directly with microelectrodes has provided researchers with the information necessary to better understand the mechanism of cellular actions (e.g., nerves, muscles). Similarly, physicians have found that in some cases, the free activity of electrolytes in blood samples is a better diagnostic indicator of disease states than the total concentration values provided by traditional spectroscopic techniques, and this has resulted in the incorporation of ISEs into most modern instrumentation used in clinical chemistry laboratories (i.e., "ionized calcium").

In this article, we will attempt to provide a general overview of the basic principles and practical aspects of ISE measurements with particular emphasis on the use of ISEs in biological studies and in clinical chemistry. Information concerning the classification of ISE devices, the concept of electrode selectivity, instrumentation required for ISE measurements, and some of the limitations and concerns involved in such measurements will be covered in the introductory section. In keeping with the overall theme of this article, further emphasis will be placed on presenting the current state of ISE technology with regard to the incorporation of these devices in modern clinical chemistry analyzers. In addition, the fabrication of miniature and microsized ISE devices useful for *in vivo* and intracellular studies or for *in vitro* measurements in small volumes of biological samples will also be covered. This will include discussions on liquid membrane-based micropipets, ISE catheters, gas sensors, and coated wire ISEs. Some key examples of successful applications of these miniature and microtransducers will be cited along with pertinent details concerning the actual construction of the various electrodes. Finally, a discussion on the design and function of newly devised enzyme and bacterial bioelectrodes suitable for the direct measurement of amino acids, metabolites, etc. will be presented along with some speculation

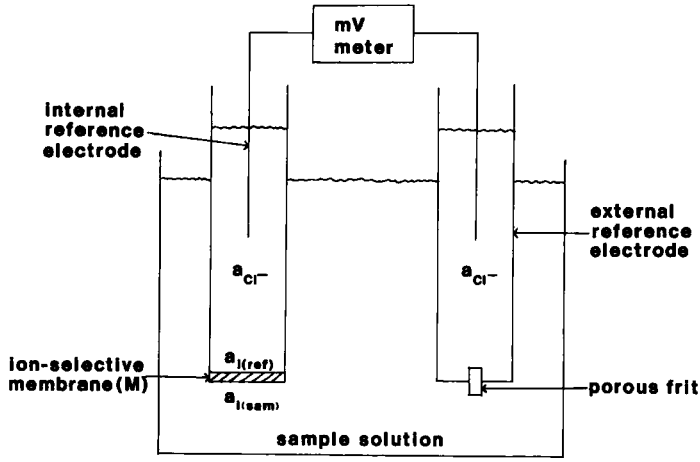

FIG. 1. Schematic of classical ISE arrangement.

as to the future prospects of preparing similar sensors for the detection of drugs and hormones.

2. An Introduction to Ion-Selective Electrodes (ISEs)

As potentiometric devices, ISEs obey thermodynamic principles. In practice, one wishes to relate the electrochemical potential of a working ISE to the activity or concentration of an analyte ion. To do this, let us consider the classical ISE experimental setup shown in Fig. 1. As already mentioned, the key component of this electrochemical cell is the ion-selective membrane (M) used to prepare the ISE probe which may consist of glass, crystalline, liquid, or polymer materials. The membrane is chosen so that it is permselective to the migration of only certain ions, or better yet, to only one ion. Regardless of the membrane type, the same fundamental equations apply. Since the potential of a single electrode can never be evaluated independently, all ISE measurements require an external reference electrode system of some type in addition to the ISE device. From traditional electrochemical convention, the galvanic cell shown in Fig. 1 can be represented by the following notation:

$$\underset{\text{electrode}}{\overset{\text{external}}{\overset{\text{reference}}{}}} \;\|\; \underset{a_{i(\text{sam})}}{\text{sample solution}} \;|\; \underbrace{\underset{\text{membrane}}{\text{ion-selective}} \;|a_{i(\text{ref})},\ a_{Cl^-},\ \text{AgCl(s)} \;|\; \text{Ag}}_{\text{complete ISE}}$$

The potential of this galvanic cell containing a membrane is always measured with a high-input impedance meter as:

$$E_{cell} = E_{ise} - E_{ref} \tag{1}$$

However, E_{ise} is composed of two potential generating systems: (1) the potential of the internal reference electrode, typically Ag/AgCl (as shown), and (2) the membrane potential, E_m, of the ion-selective membrane. $E_{Ag/AgCl}$ is solely dependent on the activity of chloride ions within the internal reference compartment of the ISE in accordance with the Nernst equation

$$E_{Ag/AgCl} = E^0_{Ag/AgCl} + \frac{2.3\ RT}{zF} \log a_{Cl^-} \tag{2}$$

where E^0 is the standard electrochemical potential of the electrode when the activity of chloride is unity, R is the gas constant (8.316 J mol^{-1} deg^{-1}), T is the temperature of the system in degrees Kelvin, F is the Faraday constant (96,491 coulombs) and z is the charge of the chloride ion (-1). The membrane potential E_m, is governed by the difference in the activity of analyte ions (i) on each side of the membrane. In simplest terms, if the membrane (M) is permselective only toward one ion i, then:

$$E_m = \frac{2.3\ RT}{zF} \log \frac{a_{i(sam)}}{a_{i(ref)}} \tag{3}$$

where z is now the charge on ion i. While for practical purposes, the membrane potential may be written as shown above, it should be noted that in reality, this potential is the result of two phase boundary potentials which exist at the two solution-membrane interphases. Moreover, for certain membrane systems, diffusion potentials arising from the difference in mobilities of ions within the membrane phase contribute to the absolute value of the membrane potential. More detailed descriptions of the actual physical interfacial processes that give rise to the membrane potential may be found in several excellent review articles by Buck (B9, B10) and a comprehensive book by Morf (M10). Regardless of the discrete physical processes that cause charge separation at the membrane surfaces, Eq. (3) is always valid for conventional ISE devices, e.g., devices with internal reference solutions. Thus, when $a_{i(sam)} = a_{i(ref)}$, the membrane potential is zero since thermodynamically the membrane system is at equilibrium. Since $a_{i(ref)}$ is fixed by the composition of the internal reference solution, the membrane potential can only change as the activity of the analyte ion in the sample changes.

Similarly, the external reference electrode portion of the galvanic cell is also composed of two potential-generating processes: (1) the half-cell reaction potential of the reference electrode (e.g., Ag/AgCl, Hg/Hg_2Cl_2), and (2) the

liquid junction potential (E_j), which develops at the interface between the sample solution and the filling solution within the reference electrode. The half-cell potential remains constant, being fixed by the activity of chloride ions in the filling solution of the external electrode. Liquid junction potentials originate from the differences in the rates of cation and anion migration across solution–solution interfaces (e.g., within the porous frit of the reference electrode). Since chloride and potassium ions have very similar ionic mobilities in solution, E_j values are minimized somewhat by using high concentrations of KCl as the electrolyte within the external reference electrode. While E_j values can be minimized, they can never be eliminated completely, and in conjunction with measurements in biological and clinical samples, they become the major source of error in direct ISE determinations. A further reduction in this error can be achieved by preparing calibrant solutions with a similar ionic composition as that of the unknown sample. This procedure ensures that E_j values, even if large in magnitude, remain similar for the calibrant and unknown solutions.

Normally, by taking the above precautions when working with ISEs, one, in fact, assumes that E_j values do not change appreciably in going from the calibrants to the sample solution and thus Eqs. (1)–(3) can be rearranged into the typical operational Nernst equation for ISEs

$$E_{\text{cell}} = K + \frac{2.3\ RT}{zF} \log a_{i(\text{sam})} \tag{4}$$

where K is a constant composed of all the fixed potentials within the electrochemical cell, a_i is the activity of ion i in the sample or calibrating solution, and z is the charge on the analyte ion. Thus, as written, E_{cell} will increase for cation responsive ISEs and decrease for anion responsive ISEs as the activity of the ion increases.

In theory, Eq. (4) is valid for ISEs in which the membrane phase can only transport the charge of a single ion. In reality, however, no membrane phase is completely specific for a single ion. Other ions can migrate into the membrane phase to various degrees and the sensitivity of an ISE to other ions can be quantitated via an expanded version of Eq. (4) (Nicolsky Eq.):

$$E_{\text{cell}} = K + \frac{2.3\ RT}{zF} \log (a_i + k_{ij} a_j^{z/x}) \tag{5}$$

where a_j is the activity of an interferent ion j with charge x, and k_{ij} is the selectivity constant of the electrode toward that ion relative to the principal analyte ion i. Thus, small k_{ij} values are desirable in order to avoid error from potential interferent ions. For example, potassium ISEs based on liquid membrane phases containing the antibiotic valinomycin exhibit k_{KNa} values in the order of 10^{-4}–10^{-5}. Consequently, even though Na^+ is present in

blood at levels about 50 times that of K^+, accurate blood K^+ values can still be obtained with such an electrode. Although not detailed here, Moody and Thomas (M9) provide an excellent overview of the various experimental procedures which may be used to evaluate selectivity constants for ISEs.

The selectivity and detection limits of any ISE is governed by the membrane phase involved. ISEs may be classified into three major categories: (1) glass, (2) solid state, and (3) liquid membrane. Naturally, glass electrodes include the common pH electrode which possesses extraordinary selectivity for H^+ over Na^+ or K^+. Indeed, values for k_{HNa} in the order 10^{-10}–10^{-13} are common, depending on the exact elemental composition of the glass material. Altering the composition of the glass membrane can lead to membrane electrodes responsive to Na^+, Ag^+, or NH_4^+. These glass membrane electrodes function as fixed site ion-exchanger systems with selectivity dependent on the equilibrium constant for the cation binding to the silicon oxide matrix and on the mobility of the ion within the outer hydrated layer of the glass surface (E3). The high electrical impedances of glass membranes and their fragile nature make them more difficult to work with on a miniature or microscale. Nonetheless, a large number of studies involving miniature and micro glass membrane systems have been described in the literature. Indeed, an entire monograph has appeared on the subject of glass microelectrodes (L2).

Solid-state electrodes are the second major class of ISEs. In this category, the active membrane phases consist of single crystal or pressed pellets of highly insoluble salt materials. For example, if the membrane phase shown in Fig. 1 were a single crystal of lanthanum fluoride doped with europium for improved conductivity, the resulting ISE would function as an extremely selective fluoride responsive device and may be used to determine fluoride ion activity in biological and clinical samples (M6, K11). Similarly, a pressed pellet of silver sulfide mounted into an appropriate outer body functions as pure membrane phase conductor of silver ions. An electrode of this type responds selectively towards the activity of Ag^+ down to extremely low levels. The same membrane electrode system may also be used to determine sulfide ion activities since increasing sulfide levels will reduce the residual silver ion activity at the surface of the membrane (determined by the solubility product constant, K_{sp} of Ag_2S). Further, by using mixtures of precipitates, e.g., Ag_2S/CuS or $Ag_2S/AgCl$, electrodes responsive to a wide range of cation and anions are possible (i.e., Cu^{2+}, Cl^-, Cd^{2+}, I^-, etc.). In all cases, however, the electrode remains most responsive to Ag^+ or S^{2-} as well as to ions which form more insoluble complexes than the analyte ion with Ag^+ or S^{2-}. For example, a bromide electrode has high selectivity over chloride ($k_{BrCl} = 2.5 \times 10^{-3}$, however, I^- is a major positive interferent since AgI is more insoluble than AgBr ($k_{BrI} = 5 \times 10^3$).

While solid-state electrodes based on Ag_2S membrane phases have been

used in biological and clinical studies, in most instances, *in situ* type measurements are difficult. This is because the sulfhydryl groups of proteins present in the sample bind silver ions at the membrane surface and foul the electrodes. In fact, Ag_2S electrodes respond directly to proteins in an amount proportional to the accessible sulfhydryl content of the proteins (D3). Such response can be reduced by working in acidic solutions where the cysteine sulfhydryl groups are protonated.

Liquid membrane-type electrodes are the third major class of ISEs and the ones most widely used in biomedical studies. Indeed, the very concept of liquid ISEs has its origin in the membrane transport processes that occur in all living cells. The ability to form electrodes in which either neutral carrier or ion-exchanger molecules are dissolved in thick (relative to the lipid bilayer membranes in cells) organic liquid membrane phases first appeared in the mid-1960s with the work of Bonner and Lunney (B6) on cation-responsive liquid ion-exchanger membranes, Ross's (R3) calcium electrode-based organic phosphate-type ion exchangers, and Pioda, Stankova, and Simon's (P3) initial work with valinomycin in diphenyl ether membranes as potassium-selective electrodes. In these systems, the ion exchangers or neutral carrier molecules (ionophores) provide a means of selectivity extracting particular ions into the otherwise ion-impermeable liquid membrane. Consequently, since the organic membranes can now transport the charge of a given ion, membrane potentials develop as a function of the activity of that ion on either side of the membrane. The selectivity of the resulting devices is a function of the respective binding equilibrium constants of the membrane carriers or exchangers toward the analyte and interferent ions. Structures of some of the newer organic neutral carrier molecules used to prepare cation-selective liquid membrane electrodes are shown in Fig. 2.

As originally developed, liquid membrane electrodes consisted of wet organic phases containing the ion exchanger or neutral carrier molecules supported by some hydrophobic porous membrane. For example, the original calcium-selective electrode (R3) was prepared by dissolving calcium didecyl phosphate in di-*n*-octylphenyl phosphonate and dispensing this solution as a membrane phase in a specially designed electrode body. For improved selectivity over Mg^{2+}, ligand ETH 1001 (Fig. 2) may now be used to prepare the membrane phase. In recent years, the use of wet liquid membranes has essentially been eliminated since it has been found that polymeric membranes, prepared with polyvinyl chloride (PVC), plasticizers, and the carrier or exchanger, exhibit potentiometric responses quite similar to those of the wet membranes. Indeed, diffusion coefficients for ionophores in the plastic membranes are not significantly lower than they would be in viscous liquid phases, e.g., 10^{-7}–10^{-8} cm^2 sec^{-1}, and therefore these membranes are still often classified as liquid type (S5).

Such polymer membranes can be readily fabricated by dissolving the

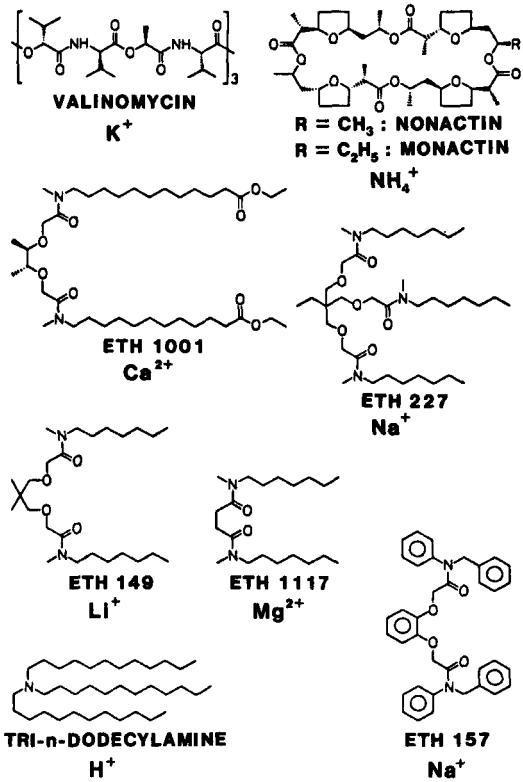

FIG. 2. Structures of some neutral carrier ionophores used in the preparation of liquid and polymer membrane ISEs [From ref. (S5) with permission. Copyright (1982), Pergamon Press.]

appropriate carrier or exchanger, PVC, plasticizer in tetrahydrofuran (THF) and casting this solution out into a glass ring placed on a glass slide. Typically, the casting solution contains the following composition: 1–3% ionophore or exchanger, 66–69% plasticizer, 30% PVC, and 0–1% additives. As the THF evaporates, the polymeric ion-selective membrane forms on the slide. Small disks of this membrane may be cut and glued to various tubes to form the body of the electrode. Such a procedure is shown schematically in Fig. 3. Alternatively, various types of plastic tubes can be dipped into the casting solution and, if suspended in a vertical position to dry, a polymeric membrane will form at the tip of the tube. Naturally, once the membrane is in place, the tube must be filled with an appropriate internal reference solution and a Ag/AgCl reference wire. Macro (5–15 mm o.d.) and miniature (0.5–3 mm o.d.) polymer membrane electrodes may be fabricated as described above or may be purchased from any number of commercial manu-

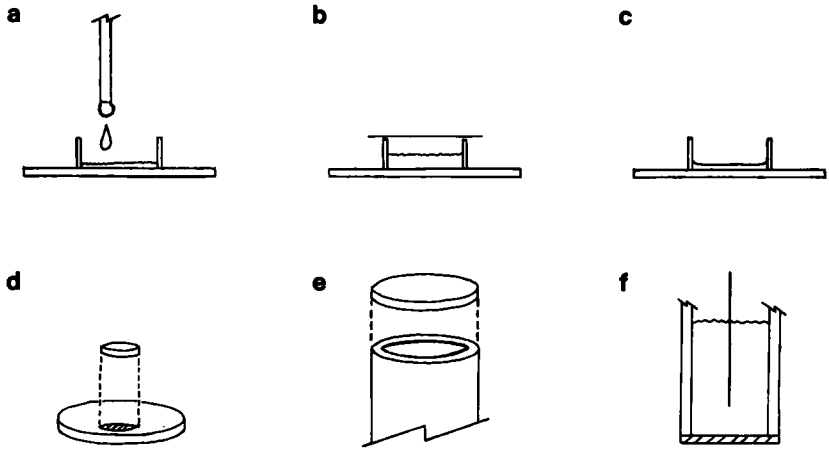

FIG. 3. Fabrication of polymer membrane ISEs: (a) dropwise addition of casting solution to glass ring resting on glass slide; (b) ring loosely covered, THF evaporates; (c) ion-selective polymeric membrane forms on the slide; (d) membrane removed from slide and small disk cut out; and (e) pasted onto a piece of PVC tubing; (f) a Ag/AgCl wire and internal reference solution complete electrode.

facturers. Pure liquid membrane systems are still widely utilized to fabricate capillary micropipet ISEs (0.1–10 μm) suitable for intracellular studies as will be described in Section 4 of this article.

An additional subclass of ISEs is the gas-sensing devices which, while not responsive directly to ionic species, do utilize common ISEs as inner sensing elements. The concept of modern potentiometric gas sensors was introduced by Severinghaus and Bradley (S3), who developed the pCO_2 sensor which is now routinely used in biomedical studies and within blood gas clinical instrumentation. In this device, a conventional glass membrane pH electrode and an external reference electrode (usually a Ag/AgCl wire) are incorporated behind a gas-permeable membrane. A thin film of internal electrolyte ($NaHCO_3$–NaCl) is sandwiched between the surface of the pH glass membrane and an outer gas-permeable membrane (e.g., silicone rubber). As CO_2 in the sample diffuses through the outer membrane, a pH change occurs in the thin electrolyte film which is sensed by the glass membrane electrode. Such devices are termed "sensors" since no other physical external reference electrode is required. Indeed, the complete electrochemical cell (both electrodes) is housed within the body confined by the gas-permeable membrane. In a similar fashion, sensors for SO_2, NO_2, and NH_3 also have been developed (R4).

In recent years, ISEs other than glass pH electrodes have also been utilized within gas-sensing configurations. For example, a highly selective

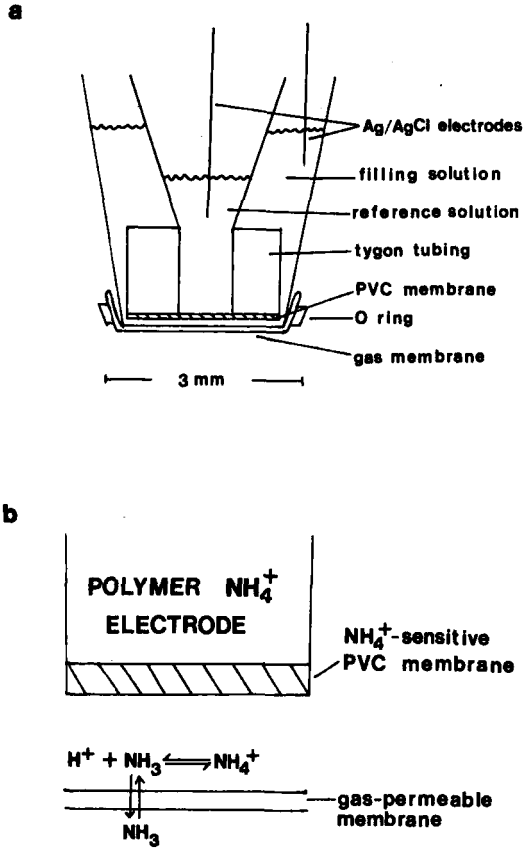

FIG. 4. (a) Schematic diagram of new polymer membrane-based NH_3 gas sensor. (b) Enlargement of sensing region and the processes occurring within [From Ref. (M4). Reprinted from *Analytical Chemistry* **52**. Copyright (1980) American Chemical Society.]

and sensitive NH_3 sensor has been fabricated by using a nonactin-based polymer membrane electrode to detect ammonium ions formed from diffusing NH_3 gas in a thin film of buffer within the sensor's tip (M4). Such a sensor is depicted in Fig. 4. Because the sensor is composed of all plastic components, essentially disposable, miniaturized versions may be readily prepared and used to measure ammonia directly in whole blood or plasma samples (M7).

At this point, in an effort to summarize the most important ISE systems available for biomedical studies, Table 1 is presented. The table lists the ISEs according to their classification and provides information on the membrane compositions used to construct the electrodes as well as some of their

TABLE I

COMMON ISE SYSTEMS USED IN BIOMEDICAL INVESTIGATIONS

	Analyte	Membrane composition	Linear response range	Selectivity constants
Glass	H^+ (Corning 015)	72.17% SiO_2, 6.44% CaO 21.39% Na_2O (mol %)	10^{-12}–10^{-2} *M*	$K_{HNa} \approx 10^{-11}$
	Na^+	11% Na_2O, 18% Al_2O_3, 71% SiO_2 (NAS 11–18)	10^{-6}–10^{-1} *M*	$K_{NaK} \approx 10^{-2}$ $K_{NaAg} = 4 \times 10^{2}$ $K_{NaNH_4} = 6 \times 10^{-5}$
Solid state	F^-	LaF_3 crystal	10^{-6}–saturated solution	$K_{FOH} = 10^{-1}$ $K_{FBr} = 10^{-4}$ $K_{FCl} = 10^{-4}$ $K_{FHCO_3} < 10^{-3}$
Liquid or polymer membrane	Na^+	Na^+ ionophore (ETH 227), 2-nitrophenyloctyl ether, sodium tetraphenylborate	10^{-3}–10^{-1} *M*	$K_{NaLi} = 2.5$ $K_{NaK} = 2 \times 10^{-2}$ $K_{NaCa} = 0.36$ $K_{NaMg} = 4 \times 10^{-3}$
	Cl^- (Corning 477315)	Tri-*n*-octylpropylammonium chloride, decanol	10^{-3}–10^{-1} *M*	$K_{ClOH} = 2.5$ $K_{ClBr} = 0.40$ $K_{ClF} = 0.67$ $K_{ClAc} = 4.55$
Liquid or polymer membrane	K^+	valimonycin, dioctyladipate, PVC	3×10^{-5}–1 *M*	$K_{KNa} = 6 \times 10^{-5}$ $K_{KNH_4} = 1.3 \times 10^{-2}$ $K_{KCa} = 5 \times 10^{-4}$ $K_{KMg} = 4 \times 10^{-5}$
	Ca^{2+}	Ca^{2+} ionophore (ETH 1001), 2-nitrophenyloctyl ether, sodium tetraphenylborate	10^{-7}–10^{-2} *M*	$K_{CaNa} = 3.2 \times 10^{-6}$ $K_{CaK} = 4 \times 10^{-6}$ $K_{CaMg} = 1.3 \times 10^{-5}$

(*continued*)

TABLE I (*Continued*)

	Analyte	Membrane composition	Linear response range	Selectivity constants
	Ca^{2+}	Calcium di-(*n*-decyl)phosphate, di-(*n*-octylphenyl)phosphonate, PVC	3×10^{-5}–1 *M*	$K_{CaNa} = 5.8 \times 10^{-5}$ $K_{CaK} = 3 \times 10^{-5}$ $K_{CaMg} = 0.14$–0.025
	acetylcholine	Tetra(*p*-chlorophenyl)borate, 3-nitro-o-xylene	10^{-5}–10^{-1} *M*	$K_{AcNa} = 10^{-4}$ $K_{AcNH_4} = 10^{-3}$ $K_{Ack} = 10^{-3}$ $K_{Ac/choline} = 6.6 \times 10^{-2}$
Gas sensors	CO_2	Combination glass pH electrode, 0.01–0.1 *M* $NaHCO_3$-NaCl filling solution; behind silicone rubber membrane	10^{-4}–10^{-1} *M*	Interferents are organic acids with similar solubilities to CO_2 in gas-permeable membrane
	NH_3	Combination glass pH electrode 0.1*M* NH_4Cl filling solution; behind porous Teflon gas-permeable membrane	10^{-5}–5×10^{-2} *M*	Interferents are low-molecular-weight volatile amines

response characteristics. It should be noted that the information given is primarily for commercially available macrosized ISEs, although in most instances, the micro-versions described in Section 4 of this article use the same membrane formulations.

We have already pointed out that membrane electrode systems involving glass membranes possess rather high electrical impedances. The conventional liquid and polymer membrane systems also exhibit resistances in the 10–250 MΩ range while solid-state electrodes typically have somewhat lower resistances (1–30 MΩ). In simplest terms, circuits used for the measurements of ISE cell potentials may be treated as a simple potential divider circuit with

$$E_{\text{meas}} = E_{\text{cell}} \left(\frac{R_{\text{m}}}{R_{\text{m}} + R_{\text{e}}} \right) \tag{6}$$

where E_{meas} is the measured potential of the ISE system, E_{cell} is the actual thermodynamic cell potential, R_{m} is the resistance of the measuring instrument, and R_{e} is the resistance of the ISE itself. Thus, to ensure that $E_{\text{meas}} = E_{\text{cell}}$, R_{m} must be very large relative to R_{e}. Consequently, when using most ISEs it is common practice to use potential measuring instruments with input impedances $>10^{12}$ Ω to ensure that there is no error in the potential measurement. Most modern pH/mV meters constructed with field-effect transistor-type input amplifiers fulfill this requirement. However, as the electrode surface area becomes smaller, the resistance of the ISE increases dramatically. Thus, for microsized electrodes, specially designed amplifier circuits with even higher input impedances are required to obtain accurate intracellular ion values and to help eliminate noise. In many instance, the micro-type measurements must also be made within the confines of a Faraday cage to reduce noise further by shielding the electrodes from environmental noise. In automated clinical chemistry analyzers, confinement of the electrodes within the outer metal cabinet of the instrument serves a similar purpose.

For the practical application of ISEs for measurement of ion activities in real samples there exist several concerns. We have already addressed the importance of liquid junction potentials and the need to either minimize these values or keep them constant between the calibrating solutions and unknowns. Also, in using ISEs one must fully realize the difference in measuring ion activities versus ion concentrations. The activity of an ion in solution is given by the expression

$$a_i = f_i c_i \tag{7}$$

where c_i is the total molar concentration of the ion and f_i is the activity coefficient. Since activity coefficients change as a function of ionic strength in

accordance with the Debye–Hückel equation (P2), differences in the ionic strength between standards and actual samples cause a major problem. For applications where the sample may be diluted prior to the electrode determination, this problem is eliminated by using a constant ionic strength adjustor as the diluent. Thus, if standards and samples are treated identically, calibration and actual measurement end up being made under the same ionic strength conditions, and plots of ion concentration versus E_{cell} may be used for quantitative purposes. If *in situ* type measurements are desired, then the standards must be made so that their ionic strength is identical to the unknown sample if concentration values are to be reported, or, if not, direct activities can be evaluated by using known activity standards and a direct comparison of the cell potential to these standards.

A further concern involves binding of the analyte ion to other components of the sample. Since ISEs sense ionic activity, bound ions go undetected. Comparison of ISE values to values obtained by atomic spectroscopy techniques may be useful in evaluating the fraction of analyte ions that are bound. Alternatively, a second ISE measurement, after the conditions have been changed to dissociate the ion complex, may be useful for such determinations. For example, free ionized calcium in blood samples can be measured directly with a polymer membrane Ca^{2+}-selective electrode at physiological pH. If a total calcium concentration value is required, such a measurement could be made by acidifying the blood sample to a pH <4 and then measuring the total Ca^{2+} using a new type of Ca^{2+} electrode which has improved selectivity over hydrogen ions (A5).

3. ISEs in Modern Clinical Chemistry Analyzers

Undoubtedly, advances in ISE technology over the past 15 years have had their greatest impact in the clinical chemistry laboratory. Indeed, flame photometers and atomic absorption instruments, for years the workhorses of most laboratories, have become obsolete as newer ISE-based instruments and methods now perform the task of determining electrolyte levels in blood and urine samples. Clearly, electrochemical measurement of fluid components is desirable, since in many cases no dilution or pretreatment of the sample is required before the actual measurement. Thus final test results are obtained more rapidly and economically.

In this section we review the current state of ISEs in connection with electrolyte and blood gas measurements including discussions on the specific membrane electrode systems used in electrolyte analyzers, the various approaches taken to incorporate the electrodes within biochemical instruments, and future prospects for new ISEs in the area of clinical analysis.

3.1. ISE-Based Electrolyte and Blood Gas Analyzers

3.1.1. *Membrane Systems Utilized for Clinical Analysis*

Because of the complex nature of the samples, direct measurements of ions and gases in physiological fluids requires highly selective membrane electrode systems. Electrodes utilized for such purposes must display Nernstian response to the analyte even in the presence of abnormally high levels of interferent ions in order to have accurate diagnostic test results. Fortunately, ISEs with appropriate selectivity now exist for the measurement of a wide range of species including sodium, potassium, ionized calcium, total calcium, pH, pCO_2, total CO_2, and chloride in blood and urine samples. The membrane systems utilized are, for the most part, those listed previously in Table 1. A large number of instruments are now commercially available, and some of these are listed in Table 2 along with the manufacturer.

Without question, sodium and potassium have been the analytes receiving the most attention in conjunction with the development of new analyzers. Almost all instruments on the market utilize the potassium-selective membrane system based on the antibiotic valinomycin in a PVC membrane matrix. For blood measurements, such a membrane is quite adequate. However, in undiluted urine samples, a negative error in the measurement of potassium has been reported (K10). Apparently, this interference comes from a negatively charged lipophilic component of the urine which can partition into the PVC membrane, reducing the membrane potential (i.e., the membrane is not permselective). Fortunately, this problem can be overcome by incorporating the valinomycin in a silicone rubber-based membrane matrix (A4) into which the unknown anionic component apparently has a less favorable partition coefficient.

For sodium measurements, either a glass membrane or a PVC-type liquid membrane electrode system may be employed. The PVC-neutral carrier systems are generally based on ionophore ETH 227 (Fig. 2 and Table 1) or methylmonensin, although recent work with bis-crown components (S4) has yielded electrodes with similar selectivities and clinical results. All three ionophores possess selectivity coefficients for sodium over potassium on the order of $k_{NaK} = 0.01$, which is quite acceptable considering that, as previously mentioned, sodium is present in blood at levels about 50 times that of potassium. However, significant response to lithium by these ionophore systems poses a problem if the patient is on any form of lithium therapy. The glass membrane sodium electrode has about the same selectivity over potassium but better selectivity over lithium. Obviously, this type of membrane is still most responsive to hydrogen ions ($k_{NaH} = 250$), although this is not a significant problem since the hydrogen concentration of blood is about 6 orders of magnitude lower than sodium levels.

TABLE 2
SOME COMMERCIALLY AVAILABLE ISE-BASED CLINICAL ANALYZERS

Model	Species measured by ISEs	Manufacturer
Spectrum	Na, K, Cl	Abbott
IL 501	Na, K	Allied Instrumentation Laboratory
IL 502	Na, K	
IL 508	Na, K	
IL 446	Total CO_2	
IL-Genesis	Na, K, Cl	
AVL-980	Na, K, ionized Ca	AVL
E-4-A	Na, K, Cl, total CO_2	Beckman
Astra-4	Na, K, total CO_2	
Astra-8	Na, K, total CO_2	
Corning 178	pH, pCO_2	Corning Medical
Corning 614	Na, K	
Corning 634	pH, ionized Ca	
Ektachem 400	Na, K, Cl, total CO_2	Eastman Kodak
Ektachem 700	Na, K, Cl, total CO_2	
DT60 (Doctor's office)	Na, K	
Hitachi 702	Na, K, Cl	Hitachi
Hitachi 772	Na, K, Cl	
Individual manual electrode units	Na, K, ionized Ca	Ionetics
Chem-Lite	Na, K, Cl	Kone
Nova-1	Na, K	Nova Biomedical
Nova-2	Ionized Ca	
Nova-3	Cl, total CO_2	
Nova-4	Na, K, Cl, total CO_2	
Nova-5	Na, K, Cl	
Nova-6	Na, K, ionized Ca	
Nova-7	Ionized Ca, total Ca, pH	
Nova-8	Ionized Ca, pH	
Orion 1020	Na, K	Orion Research
SS-20	ionized Ca	
Biological alkali micro-analyzer	Na, K	Radelkis
KNA-1	Na, K	Radiometer
ICA-1	pH, ionized Ca	
SMAC	Na, K	Technicon
RA-1000	Na, K, total CO_2	

As expected, all instruments designed to measure pH in physiological samples utilize the classical pH-sensitive glass membrane which has high selectivity over sodium. This same glass membrane is also used as the internal sensing element for pCO_2 and total CO_2 sensors. In this so called Severinghaus design, a combination glass electrode is housed within a sensor body

which contains a $NaHCO_3$–NaCl solution. The tip of the sensor is covered by a CO_2-permeable silicone rubber membrane as described earlier in Section 2. For pCO_2 measurements, undiluted blood is utilized as the sample and calibration is normally performed with 5 and 10% mixtures of CO_2 in an inert gas (pCO_2 = 38 and 76 mm Hg). When total CO_2 is measured with a gas-sensing electrode system, the sample is first diluted with acid (40 mM H_2SO_4) to convert all forms of CO_2, including bicarbonate, into the free gas. Calibration in this case is performed with $NaHCO_3$ standards. Equilibrium response times for these gas-sensing arrangements are typically much longer (e.g., 25–50 sec) than for isolated membrane electrodes, and this can limit the sample throughput of instruments based on these devices.

As an alternative to glass membrane pH electrodes, Simon and co-workers (A3) have recently investigated the use of a tri-*n*-dodecylamine based PVC membrane electrode for the measurement of blood pH. This membrane system has selectivity coefficients over sodium and potassium which are comparable to the glass electrode, yet the electrode is much easier to fabricate. They found that whole blood pH measurements made with this new electrode correlated quite well with values obtained with the conventional glass devices. For total CO_2 measurements, one alternative to the use of a gas sensor is to use a PVC membrane electrode which detects one of the ionic forms of dissolved CO_2. Indeed, instruments are now available which employ a carbonate-ion-selective membrane for this purpose. The membrane is composed of Aliquat 336 (a general anion exchanger), trifluoroacetyl-*p*-butylbenzene, a plasticizer, and PVC (H1, R2, G1). The trifluoro compound is the key ingredient which imparts selective response toward carbonate over chloride. Thus, for detection of total CO_2 in blood, the sample is first diluted with a pH 8.6 buffer to generate a constant fraction of the total CO_2 as CO_3^{2-}. Interference from lipophilic anions in the sample can be a problem, especially salicylate; however, special absorbant layers have been formulated to remove such interferents from the sample (K6).

Ionized calcium and total calcium measurements are both performed with PVC-type electrodes. Membranes based on lipophilic alkyl phosphates with phosphonate plasticizers have only marginal selectivity over magnesium but have been used in the past to determine ionized calcium (i.e., free calcium) in undiluted blood samples (M8). Interference from protons at low pHs prevents such membranes from being employed for total calcium determinations on samples diluted with acid. Use of ionophore ETH 1001 (see Fig. 2) overcomes any concerns about selectivity, whether from magnesium or pH, and is now the neutral carrier system most often utilized within analyzers to detect ionized or total calcium.

The reliable determination of chloride in physiological fluids by ISEs represents a significant problem. A simple Ag/AgCl wire type electrode or a

pressed pellet of metallic silver with AgCl (not Ag_2S/AgCl) may be employed; however, such electrodes are subject to fouling by protein components of the sample and thus require frequent recalibration. In addition, the presence of bromide or iodide in samples causes significant positive errors. In some instrument designs fouling and interference are minimized by covering the sensing region of the electrode with a semipermeable membrane (S1). A better approach is to utilize a PVC-type liquid membrane electrode system which has adequate selectivity for chloride over bicarbonate and salicylate. Efforts to develop such a membrane have been underway for many years, and just recently Nova Biomedical, Inc., has introduced a polymer membrane chloride electrode within their new electrolyte analyzer which appears suitable for detecting chloride in blood and urine samples (W6).

3.1.2. *ISE Configurations and Analyzer Designs*

Ion-selective electrode-based electrolyte and gas analyzers are quite varied in their design. Most fully automated instruments incorporate the appropriate ISEs in flow-through arrangements. Calibrants, samples, and flush solutions can be rapidly delivered to the electrodes via pumping manifolds. The flow-through electrodes assemblies may be of conventional or tubular design as shown in Fig. 5. For multiple electrolyte sensing systems, the individual electrodes are normally placed in series within the flow path of the sample solution. A single reference electrode in contact with the sample stream can be used to evaluate the potentials of each ISE in the system (except for CO_2 sensors, which must have their own reference element behind the gas-permeable membrane). The reference electrode configuration is critical to avoid significant junction potential problems. Free-flowing junctions, in which a stream of solution is pumped through a reference

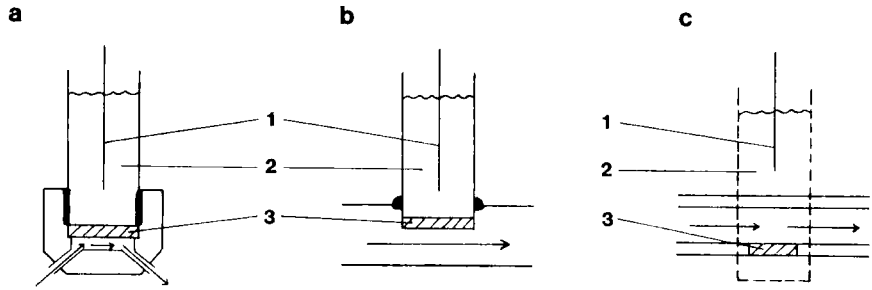

FIG. 5. Typical flow-through configurations for ISEs in clinical analyzers: (1) internal Ag/AgCl wire; (2) internal reference solution; (3) ion-selective membrane.

electrode and then merged with the sample stream after the latter has passed all the ISE indicator electrodes, appear to be the best approach in avoiding erythrocyte junction potential problems in whole blood measurements. Static-type junctions, in which reference filling solution contacts the sample stream by means of a porous plug or membrane is another possible arrangement, although clogging of such junctions can be a problem, and proper choice of reference fill solution is mandatory to avoid serious differences between junction potentials present when measuring undiluted samples (whole blood, urine, serum) and those present when using aqueous calibrants. Indeed, to mimic the undiluted samples and thereby minimize junction potential differences, calibrants are typically made up with constant total ionic strengths which approximately match those of the unknown physiological samples (i.e., 0.15 *M* for undiluted blood). For instruments which dilute the sample before measurement, concerns about junction potentials are lessened since the diluted samples have essentially the same ionic compositions as the standards. For fully automated systems, electrode drift problems are solved by frequent calibration, sometimes a one-point calibration after each sample. This enables the instrument to keep track of changes in baseline potential values [i.e., K in Eq. (5)]. However, two-point calibration to correct for slope changes is still periodically required (several per hour).

Manual and some automated analyzers utilize electrodes which operate under static conditions. For example, the Kodak Ektachem system utilizes disposable ISE slides (B3). These slides are prepared by coating appropriate polymeric ISE membranes over internal reference gel/electrode coatings on conductive substrates. The slides contain two ISE membranes, one used for a reference solution of the analyte and the second for the sample. When a small volume of reference solution with known analyte concentration and a similar volume of sample are placed on the slide, a differential measurement can be used to determine the unknown electrolyte level. Electrolyte contact between the two solution wells is made through a paper strip. A new slide is used for each measurement. The entire process of placing the samples and reference solutions on the slides is fully automated. Smaller and less expensive analyzers are also available which make use of manual-probe-type ISEs, which can be placed directly into unmeasured volumes of undiluted serum samples (in a test tube). These are essentially combination electrodes because the reference electrode is housed within the same body. Using proper calibrating solutions, accurate values of the electrolytes (particularly Na^+ and K^+) can be read directly from a digital ion meter which directly converts the cell potential to concentration units. These inexpensive units are ideally suited for performing clinical measurements in a small clinical laboratory or doctors' offices.

The question of sample dilution for a given electrolyte measurement is an

important one. It has been shown that ISE-based measurements of potassium and sodium in undiluted serum samples give values that are more valid but higher than those obtained by diluted ISE systems or flame photometry (L5). In principle, the values should be about 7% higher owing to the effective volume occupied by proteins and lipids in the undiluted sample. It is argued that direct (undiluted) measurement of sodium and potassium is better because in instances of hyperlipidemia and hyperproteinemia, electrolyte values obtained will not decrease as seen with the diluted techniques. In practice, the difference in values obtained on undiluted samples in the case of sodium measurements is somewhat less than the 7% expected (3–5%), and this difference has been attributed to either liquid junction potential problems (C8, C12) and/or sodium ion binding to bicarbonate (C6).

The future of ISEs in the clinical chemistry instrumentation is quite exciting. As described in subsequent sections of this article, the coupling of enzyme and immunological reagents to ISE detectors to form bioelectrode systems appears to offer manufacturers a new approach toward the detection of metabolites such as creatinine and urea directly in blood and urine samples. Ultimately, such biosensors will be placed into complete electrode-based automated clinical analyzers. In addition, continued research on new membrane formulations, particularly liquid membrane ionophore systems, will result in the development of addition electrodes which can be incorporated into current analyzer systems to expand the electrolyte menu. Indeed, recent efforts have indicated that membranes selective for bicarbonate (F5) and lithium (Z2) are likely additions in the near future.

4. Miniature and Micro-ISEs

Ever since the inception of modern ISEs, researchers have realized the potential value of using smaller versions of the original macrodesigns for *in situ* type measurements. Thus, efforts to determine ion activities in very small volumes of samples or even within single intact cells, and the desire to utilize ISEs for continuous *in vivo* monitoring of blood electrolytes during surgical procedures, have prompted considerable research into the development of appropriate miniature and microsized devices. In this section, we will summarize the various approaches that have been taken in fabricating these devices and further discuss the fabrication techniques involved, the operating principles of the electrodes, and the analytical performance of the resulting probes. Some examples of biomedical applications with these devices will also be given.

4.1. Coated-Wire Electrodes

The concept of coated-wire electrodes (CWEs) was first introduced by Freiser and co-workers (C4, J1) in an attempt to simplify the preparation of polymer membrane ISEs. The response mechanism of such electrodes is based on the same ion extraction principles found in conventional liquid membrane systems (involving neutral carriers or ion exchangers). These types of electrodes have been the focus of several recent reviews (F2, F3, C3) and, over the past 10 years, numerous devices of this type have been prepared for species ranging from simple inorganic ions (i.e., K^+, Na^+, Ca^{2+}) to organic ion species including quaternary ammonium compounds, various drugs, etc. In principle, CWEs can be made for any ion for which there already exists a known liquid or polymer membrane electrode. The unique feature of these devices is the absence of an internal reference solution common to the conventional ISE design (see Fig. 1). By eliminating this solution, smaller (typically 0.5–2 mm o.d.) and more rugged electrodes may be fabricated. This has certainly been of interest to the manufacturers of the clinical chemistry analyzers discussed in the previous section.

Preparation of CWEs is simple enough. The method normally involves soldering a piece of copper, platinum, or gold wire to the central conductor of a piece of coaxial cable (whose other end has the proper pin to connect to a pH/mV meter). Alternatively, the inner conducting wire of the cable itself may be used directly after stripping away the outer shielding. The exposed wire is then dipped repeatedly into a PVC membrane casting solution con-

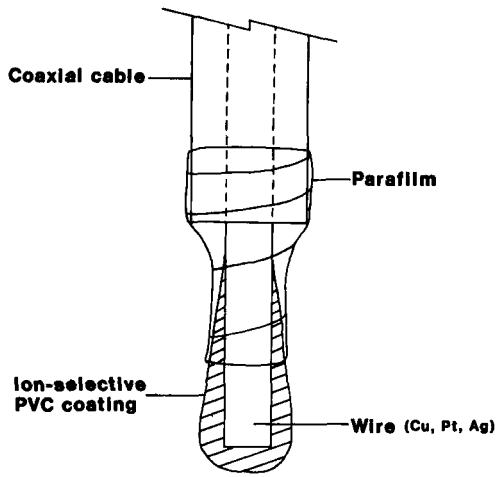

Fig. 6. Sensing tip of a coated wire ion-selective electrode.

taining the appropriate ionophore or exchanger (the same solution used to cast traditional polymer ISE membranes). After repeated dipping and drying steps, a polymer membrane bead forms at the base of the wire, as shown in Fig. 6. Any exposed metal still remaining may be wrapped in Parafilm to insulate the electrode completely.

As far as detection limits, selectivity, and Nernstian response ranges, the performance of CWEs is essentially analogous to that of conventional polymer membrane electrodes. However, one major drawback to their use appears to be the lack of reproducible potentials. Even within a single day, cell potentials can vary substantially for the same standard solution. As a result, CWEs must be recalibrated often, or better yet, standard addition or titration techniques must be utilized to ensure accurate results. Obviously, such analytical techniques could not be employed if *in situ* determinations are desired.

The problem of drifting cell potentials seems to arise from the fact that no defined reference potential exists at the interface between the internal metal wire and the polymeric membrane. Some workers postulate an oxygen-dependent half-cell potential at this interface with the required oxygen being able to permeate the PVC film (S2). Clearly, the question as to what is happening at the interface of the metal and the membrane needs to be resolved before CWEs of the above type gain widespread acceptance as useful analytical devices.

More stable CWE-type devices can be prepared by fabricating the electrodes with appropriate thermodynamically reversible redox reference systems. For example, workers at Miles Laboratories (S6) have developed a very stable potassium-selective CWE by coating a valinomycin-PVC-plasticized membrane over a Ag/AgCl wire which had first been repeatedly dipped in a polyvinyl alcohol (PVA) solution containing potassium chloride. In this design, the chloride ion in the hydrophilic polymer coating (PVA) adjacent to the Ag/AgCl wire, provides a classical and stable internal reference potential for the resulting CWE device. Thus, this type of CWE is really a conventional polymer membrane electrode in which the internal reference solution is immobilized as a thin gel of electrolyte coated onto the internal reference wire. Electrodes with tip diameters ranging from 0.5 to 2.0 mm can readily be fabricated with the use of this approach. In this design, however, care must be taken to exactly match the osmolarity of the PVA layer with that of the sample solution so that water vapor, which can permeate the outer PVC membrane, will not swell the inner PVA layer. When the osmolarities are properly matched, this type of electrode may be used for the direct measurement of blood electrolytes. Not surprisingly, Ionetics, Inc. (Santa Ana, California), now uses a very similar design to

manufacture miniature Na^+, K^+, and Ca^{2+} electrodes for use in physicians' offices and small clinical laboratories (see Table 2).

4.2. Catheter-Type ISEs for *in Vivo* Measurements

Clearly, the ability to monitor ion activities and blood gases continuously during surgical procedures or at the bedside of intensive care patients would provide a means of alerting clinicians as to the instantaneous condition of their patients. Further, an ability to monitor *in vivo* would also aid in many basic biomedical studies concerning the short-term effects of drugs, exercise, etc. on the electrolyte levels in blood. While research in this area has been in progress for more than 10 years, the use of ISE-type devices for *in vivo* measurements is still at the experimental stage.

Two main approaches have been taken with regard to the fabrication of *in vivo* ISEs. One route of investigation has resulted in the development of ion-selective field-effect transistor devices (ISFETs) in which catheters with simultaneous multi-ion sensing capabilities are possible. The concept is a spinoff of coated wire electrodes (J2) except that the ion-selective membrane materials are coated directly on the gates of field-effect transistors, the devices that detect electrode potentials in modern pH/mV meters, instead of at the end of a wire leading from such gates. This approach has received considerable attention in recent years, although drift and encapsultation problems still plague these devices for practical applications.

Since size limitations for catheter devices are not stringent, e.g., < 2 mm o.d., miniaturized conventional designs for ISEs offer the second approach to preparing such devices. In the earliest work, miniature glass membrane pH and Na^+ electrodes were mounted at the end of catheter tips for *in vivo* sensing (S10). Unfortunately, the high electrical resistances of the miniature glass electrodes coupled with the noisy environment of an *in vivo* measurement situation, including streaming potentials due to the flow of blood, yielded erroneous results. In view of the increasing number of ionic species which can be sensed with appropriate liquid and polymer membrane electrode systems (including H^+ with tridodeclyamine as the neutral carrier; see Fig. 2), miniaturized versions of the polymer membrane electrodes have become the most widely researched avenue for the development of useful ISE catheters.

Unfortunately, it has been suggested that pulsating pressures which occur *in vivo* can affect traditional PVC-membrane electrode systems by causing large potential oscillations. Through the efforts of Band and co-workers in England (B2), it has been found that casting the PVC membrane on a porous ceramic plug at the end of the catheter tube solves this problem, since the

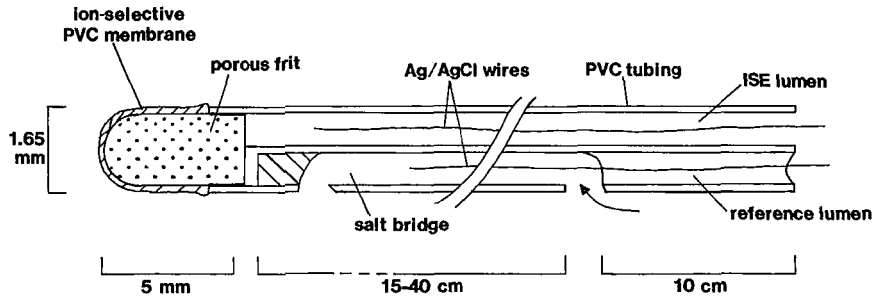

FIG. 7. Potassium-sensing catheter for *in vivo* use. [From ref. (T1) with permission.]

plug acts as a solid support for the membrane. A typical K^+ catheter of this type is shown schematically in Fig. 7. The catheter is a double lumen design with one lumen serving as the internal reference compartment behind the ISE membrane (Ag/AgCl electrode and KCl solution) and the second lumen as the external reference electrode containing a salt solution or a gelled salt solution to serve as a salt bridge with the sample. It is important that both electrodes be as close together as possible to reduce the resistance of the electrochemical cell. The same design can readily be used to prepare catheters for pH, Na^+, K^+, Cl^-, and Ca^{2+} by utilizing an appropriate carrier or ion-exchanger impregnated PVC membranes at the tip of the device. Such a design yields stable and reproducible cell potentials since a defined internal reference couple exists.

Coated-wire electrode (CWE)-type devices for *in vivo* monitoring have also been described. For example, workers at General Electric Inc. (N3), have patented a catheter for *in vivo* pH measurements which was based on the approach used by the Miles workers to prepare the previously mentioned K^+ CWE. The ISE portion of the pH catheter consisted of a Ag/AgCl wire coated first with a hydrophilic polymer containing buffer components and chloride ions and then with a polymer membrane containing a H^+ carrier molecule. A second tube with appropriate Ag/AgCl wire and electrolyte served as the external reference element. Once again, stable potentials can only be obtained if the osmolarity of the hydrophilic polymer layer matches that of whole blood.

With regard to *in vivo* gas-sensing devices, the majority of the work reported to date has involved oxygen-sensitive devices which operate as an electrolytic, not galvanic, type of electrochemical cell (i.e., current measured, not potential). Since such oxygen-sensing catheters are not based on ISEs, they will not be considered in this review. There has been, however, some limited work concerning the development of potentiometric sensors, particularly for *in vivo* CO_2 measurements. One approach has been to devise

transcutaneous-type devices based on the Severinghaus design for CO_2 sensing (e.g., a glass pH electrode behind a gas-permeable membrane). In principle, these devices need not be very small since they are mounted outside the skin, and thus the resulting procedure is considered a noninvasive blood gas measurement method. It would seem that problems arising from variations in skin thickness from individual to individual as well as temperature gradients resulting from the fact that the sensors are external to the CO_2 source may limit the practical utility of these kinds of devices (including problems regarding proper calibration procedures, etc.). Nonetheless, several successful reports of their use in neonatal subjects have appeared recently (e.g., K12).

Actual catheter-type CO_2 sensors have also been fabricated. Workers at General Electric (M1) have patented a dual pH/pCO_2-sensing catheter based on a polymer pH membrane through which CO_2 is permeable and an internal palladium/palladium oxide pH-responsive electrode. The catheter was prepared by the dip coating processes previously used to fabricate CWE-type devices. The use of nonglass pH electrodes for the construction of these devices reduces the technological difficulties normally encountered when trying to miniaturize the conventional glass membrane system. Indeed, the resulting metal pH electrode-based sensors are inexpensive and rugged. Unfortunately, such metal–metal oxide pH electrodes may be subject to interferences from the redox environment of the sample and more detailed studies regarding the effect of oxygen and other interferents on these sensors need to be undertaken. A more promising approach may be to use the polymer membrane type pH electrodes prepared by the multiple coated wire process as internal sensing elements for the fabrication of CO_2, and even NH_3, gas-sensing catheters. Investigations in this direction are currently underway (O1).

In an effort to reduce the noise problems often encountered during *in vivo* type measurements (which limit the reliability of the final determination), telemetry-type systems have been proposed (B1). In this approach the implanted catheter is connected to a miniature radio transmitter which transmits the *in vivo* ISE cell potentials to a receiving unit placed far from the patient or animal. By replacing the wires normally required to transmit the potential information to the external instrument, far less interference from environmental and electrical noise normally found in rooms used for surgical procedures occurs. This approach appears to hold great promise for the future of *in vivo* ion and gas monitoring with ISEs.

While there have been several reports on the use of the above type catheters (see Section 4.4.1.), *in vivo* measurements with ISE-type devices are not yet routine. Problems regarding electrode drift when in prolonged contact with whole blood, the effect of blood clots, the use of appropriate

external reference electrodes to reduce junction potential errors, and the development of adequate standards for calibration purposes need to be further addressed by researchers. These problems, however, do not appear to be insurmountable. Certainly, recent progress in the development of reference electrode systems and calibrants used within clinical analyzer instruments based on modern ISE technology (used for whole blood measurements as described in Section 3) could be adapted to solve some of the problems encountered with the *in vivo* sensing systems.

In view of the existing concerns with direct *in vivo* type devices, one alternative approach appears to be the development of continuous on-line monitoring instrumentation. In these systems, small volumes of blood (i.e., 2 ml/hour) are continuously removed from the patient and pumped through appropriate ISE devices situated at the patient's bedside. The main advantage of this approach is that the blood can be diluted within the instrument as required to eliminate junction potential, ionic strength, and clotting problems. At the same time, the desired continuous readout of the *in vivo* electrolyte levels are obtained. Workers at Miles Laboratories have developed a so-called "Biostator" instrument capable of continuously monitoring Ca^{2+} and K^+ by ISE technology (as well as glucose) (F1) during surgical procedures. Experiments with dogs have demonstrated the reliability of this interesting approach.

4.3. Micro-ISEs for Intracellular Measurements

The size requirements for ISEs utilized for intracellular measurements varies greatly depending on the size of the cells under investigation. Historically, the term micro-ISE has been used to describe electrodes with tip diameters ranging from 200 μm down to 0.1 μm. Fabrication of such devices, particularly those <10 μm in tip diameter is a real art, and highly specialized equipment is required. Over the years, micro-ISEs of the glass, solid-state, and liquid membrane classes have been proposed, although the liquid membrane type are the ones most extensively employed at this time.

Before discussing specific micro-ISE designs and the fabrication techniques involved, let us briefly discuss the fundamental principles of intracellular measurements. For physiologists and biologists, the goal is to detect ion activities accurately within intact living cells and relate these levels to cell function. If the cell is relatively large, a neuron for example, it may be possible to place both the ISE and the external reference electrode into the same cell simultaneously and determine the ion activity from a separate calibration curve made with the same electrodes under appropriate conditions (i.e., ionic strength, interferent ion activities, etc.). In most instances, however, it is difficult to impale the same cell with two separate electrodes.

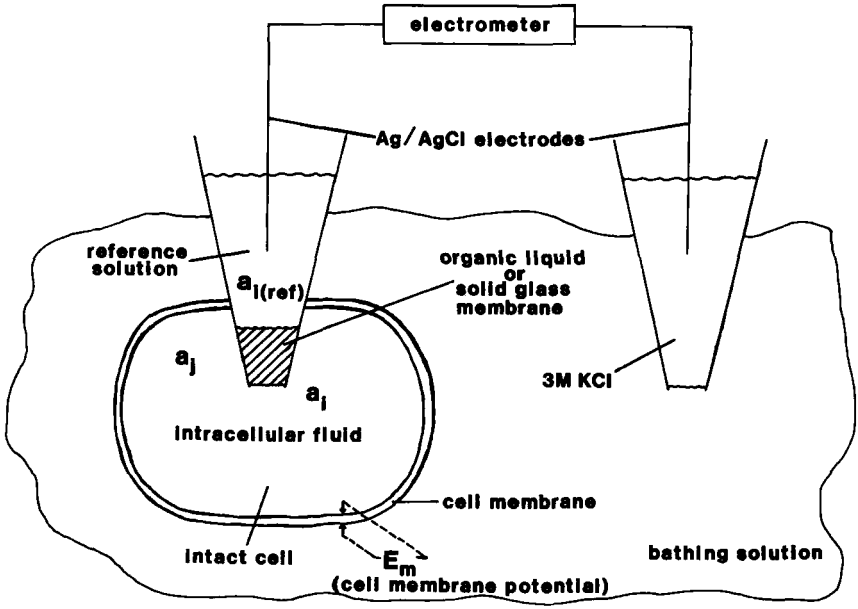

FIG. 8. Typical arrangement for intracellular ion activity measurements using micro-ISEs.

In these cases, the reference electrode is placed in the bathing solution outside the cell and two separate electrode measurements must be made. The situation is depicted in Fig. 8. When the ISE is within the cell and the reference electrode outside (as shown), the potential measured is given by the expression

$$E_{\text{cell}} = K + \frac{2.3\ RT}{zF} \log (a_i + k_{ij}a_j^{z/x}) + E_m \tag{8}$$

where E_m is the membrane potential of the living cell and the other terms are as defined previously for Eq. (5). Therefore, to accurately measure the intercellular activity of ion i, not only does one need to have some idea of ion activities of the potentially interferent ions (e.g., a_j) found within the cell, but also the membrane potential of the intact cell must be known. The latter can be evaluated by using two micropipet-type reference electrodes filled with 3.0 M KCl, the kind of electrodes normally used to measure bioelectrical potentials in tissues, cells, etc. (external electrode in Fig. 7). If one uses two identical reference electrodes, one inside the cell and one in the bathing solution, the potential of the galvanic cell would be

$$E_{\text{cell}} = E_{\text{ref(1)}} - E_{\text{ref(2)}} + E_m \tag{9}$$

and since

$$E_{\text{ref(1)}} = E_{\text{ref(2)}} \tag{10}$$

then

$$E_{\text{cell}} = E_{\text{m}} \tag{11}$$

Once E_{m} is known, Eq. (8) can be used to evaluate the intracellular ion activity. Usually, E_{m} is measured several times in many cells, and an average value is used to calculate the unknown ion activity. If the microelectrode is highly selective for the analyte ion, little attention need be given to the selectivity coefficient term of Eq. (8). However, when less selective electrodes are employed, selectivity coefficient values and estimates as to the interferent ion activities within the cells must be made. Sometimes, actual measurement of these interferent ion activities can be made first with other micro-ISEs, and the resulting values used in the calculations.

In discussing the size of micro-ISEs, we referred to tip diameter as the property used to compare the devices. However, tip diameter alone is not the only characteristic which determines the size of the cell which can be impaled with the electrode. If one is working with microglass or solid-state type electrodes, the ion-sensing area of the electrode usually extends up the length of the device. For example, a microglass pH electrode may have a tip diameter of 1 μm, yet the pH-sensing area of the electrode may extend 10 μm or more up from the tip. Thus, while the tip may fit into a given cell, the entire pH sensing area may not, and this will result in a mixed and erroneous potentiometric measurement of the intracellular pH.

Microelectrodes based on closed-end pH glass membranes were the first to be described in the literature (C1). Hinke (H2) further developed glass membrane microelectrodes with tip diameters of 10 μm for the measurement of sodium and potassium. Various designs and approaches to the fabrication of these all-glass microelectrodes have been taken over the years, and specific fabrication procedures may be found in the literature (H3, K3). Similarly, solid-state type electrodes based on pressed pellets of Ag_2S with tip diameters on the order of 100 μm have been reported for the determination of Ag^+, S^{2-}, I^-, Cl^-, Cu^{2+}, Br^-, etc. (C13). We have already discussed that such solid-state electrodes can foul when applied for direct measurements in biological systems, so the fabrication of these will not be discussed here.

At present, the most widely used type of micro-ISEs are the liquid membrane type. As has been the case with the previously mentioned clinical chemistry systems and catheter designs, the need for many of the glass membrane electrode systems has been obviated thanks to the increasing number of highly selective neutral carrier molecules that have become avail-

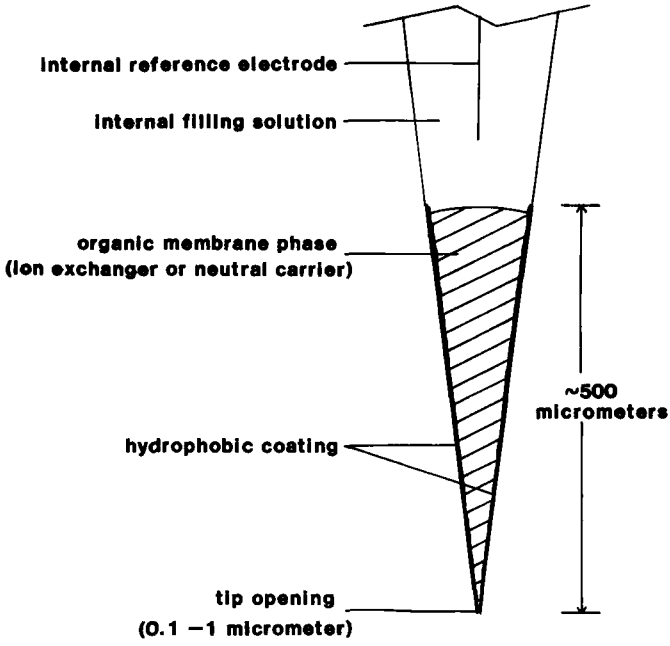

FIG. 9. Typical liquid membrane micropipet ISE.

able over the past 10 years (see Fig. 2). However, unlike in the earlier flow-through and catheter designs, liquid membrane micro-ISEs still predominantly use pure liquid membrane phases rather than polymeric phases. This is because the fabrication techniques developed involve the use of micropipets which can most easily be filled with the wet organic solutions. The basic design for liquid membrane micro-ISEs was originally described by Walker (W1). While initially termed "liquid ion-exchanger microelectrodes," most current electrodes utilize the neutral carrier type molecules to obtain enhanced selectivity. A typical liquid membrane micropipet ISE is shown in Fig. 9. The electrode is prepared from a glass capillary tube (1–2 mm o.d., 0.3 mm walls, borosilicate). After thoroughly cleaning and drying the glass, the tube is placed in pipet puller and pulled to obtain the desired tip diameter. The key to the construction of the final electrode is then to render the inner lower walls of the micropipet hydrophobic. Normally, the glass surface is hydrophilic, and, if an organic membrane phase were placed in the tip, the organic layer would be displaced by water from the sample or the internal reference solution. Therefore, the inner surface of the pipet must be made hydrophobic via a silanizing procedure, normally accomplished by dipping the micropipet tip into an organic silanizing reagent (e.g., 5% trimethylchlorosilane in carbon tetrachloride, 2–4% tributylchlorosilane

in 1-chloronapthalene, etc.) which can react with the hydroxyl groups present on the glass surface. According to Walker (W2), the actual silanizing reagent used can have an effect on the selectivity characteristics of the final electrode. After such treatment, the residual silanizing solution is removed by drying the micropipet in an oven at 250°C for 1 hour. The tips of the pipets are then filled with the liquid membrane phase, usually so that less than a 500 μm length of organic phase exists in the tip. This process is done under a microscope and with the aid of a pulled glass needle (<1 μm) to help remove any air bubbles in the organic membrane. Finally, the top portion of the micropipet is filled with internal reference solution and a Ag/AgCl electrode is introduced.

A wide variety of electrodes of this type have been fabricated, including ones selective for K^+ (based on valinomycin), Cl^- (based on quaternary ammonium ion-exchanger molecules) and more recently, acetylcholine [based on ion exchangers (J3)], Na^+ (S11), Mg^{2+} (L1), and H^+ (A2) microelectrodes have been introduced with the latter three based on the neutral carriers illustrated in Fig. 2 of this article. In general, these liquid membrane micro-ISEs possess response characteristics including selectivities comparable to the macroelectrode designs prepared with the same organic liquid membrane phases.

When making actual intracellular measurements, the microelectrodes are mounted in micromanipulators for cell penetration. It has been found that beveling the tip of the micropipet ISE aids in the ability to enter the cell and also enables the fabrication of electrodes with smaller tip diameters of 0.1 μm. Once inside the cell, single-barrel liquid membrane micro-ISEs (as described above) allow for the measurement of only steady-state ion activities. For excitable cells, where ion levels change rapidly, one cannot differentiate the potential changes resulting from variations in the intracellular activity of a specific ion and the living cell membrane potential. For such situations, double-barrel-type liquid membrane micro-ISEs have been developed (K2).

The main advantage of the double-barrel configuration is that it enables one to measure transient changes in intracellular ion activities. A schematic of this design is shown in Fig. 10. The microelectrode is fabricated from two glass capillary tubes that are initially glued together side by side, heated and twisted around one another, and then pulled in a conventional pipet puller. Once pulled, one of the barrels needs to be filled with the organic membrane phase. A special positive pressure technique is used to avoid silanizing the second barrel. After silanization of the ISE barrel, the organic phase may be added, followed by the aqueous internal reference solution. The second barrel is used as a reference electrode by filling it with 3 *M* KCl and a Ag/AgCl wire. A more detailed description of the fabrication techniques involved may be found in an excellent review by Zeuthen (Z1).

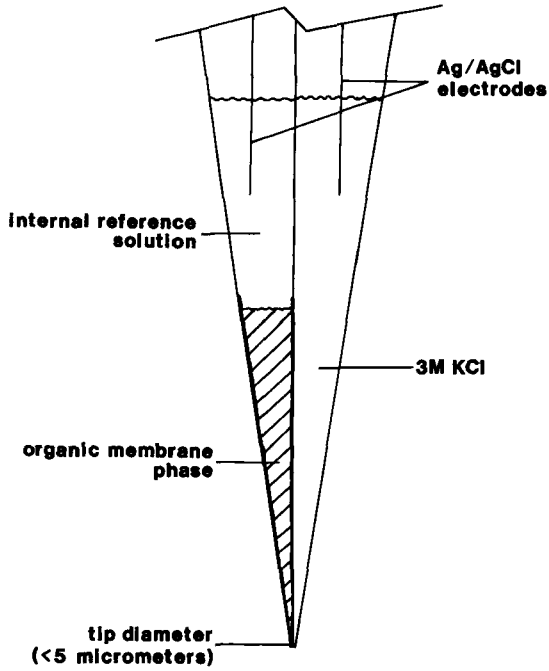

FIG. 10. Double-barrel micropipet ISE for measuring transient intracellular ion activities.

The purpose of the reference barrel is to allow for the simultaneous measurement of the cell membrane potential while measuring the intracellular ion activities with the ISE portion of the device. Thus, in practice, a second single-barrel reference micropipet electrode is placed in the bathing solution outside the cell so that the potential between the two KCl-filled electrodes can always be monitored to obtain the instantaneous cell membrane potential (E_m). The potential of the liquid membrane ISE barrel can also be monitored versus the external reference electrode. In this manner, potential changes due to variations in the cell membrane potential can be taken into account when calculating the intracellular ion activities. Alternatively, only the potential difference between both barrels of the electrode could be monitored. This potential should only be dependent on the intracellular activity of the analyte ion (not affected by the cell membrane potential). For certain ion measurements, e.g., K^+ using a valinomycin based liquid micropipet electrode, leakage of K^+ from the reference barrel could present a problem. In such cases, the reference barrel and the outer reference pipet should be filled with a solution other than 3 *M* KCl.

Even further improvements in intracellular ISE measurements are on the horizon. For example, a four-barrel configuration for microelectrodes was proposed (K1) in which three barrels were used to prepare three different

liquid membrane ISEs (Na^+, Ca^{2+}, and K^+) and the fourth barrel was used as the reference electrode. The entire assembly had a tip diameter of 1 μm. While not yet reported for intracellular studies, this arrangement may ultimately enable researchers to monitor the activities of three different ions simultaneously in the same cell with a single device.

Aside from intracellular work, the liquid membrane micro-ISEs described above may also be used for extracellular studies involving a wide range of tissues. For example, accumulation and depletion of ions that flow across extracellular space between nerve groups in the brain can be easily monitored by these types of microelectrodes (N2). The size of the electrodes used in such studies need only be 2–4 μm. In these experiments, the ISEs are placed in the extracellular fluid and ion activities may be directly monitored without concern for the cells' membrane potentials.

4.4. Some Applications of Miniature and Micro-ISE Devices

4.4.1. *In Vivo*

As indicated earlier, there have been only a limited number of reports concerning the use of ISE devices for continuous intravascular monitoring. Band and co-workers (T1) in London have found that the polymer membrane type devices, in which the PVC-ISE membrane is cast over a porous frit at the end of the catheter (Fig. 7), appear to function quite well for *in vivo* studies. Figure 11 shows a typical tracing of an actual *in vivo* experiment in which two potassium-selective catheters were placed in the venous circulation of an anesthetized dog to monitor the time course of blood potassium levels following injections of KCl. In other experiments, simultaneous determinations of K^+ by flame photometry have correlated well with the values obtained *in vivo* with the ISE catheter. A very similar K^+ catheter, used in conjunction with a telemetry monitoring system, has also been employed to monitor epinephrine-induced changes in plasma K^+ in anesthetized greyhounds (L6). Band and Treasure (B2) further used Ca^{2+}-selective catheters of the PVC membrane type to determine pH-dependent changes of Ca^{2+} levels in the plasma of cats.

The multiple coated wire type catheters developed at General Electric have also been evaluated *in vivo*. For example, the pH-sensing device was implanted in the femoral artery of beagle dogs for up to 6 hours (L3). Over that time span, the pH of the blood as measured by the *in vivo* coated wire electrode was nearly identical to that measured *in vitro* with conventional glass electrode instrumentation. In addition, the same catheter also has been used percutaneously to measure the muscle pH of dogs in shock (K14). The

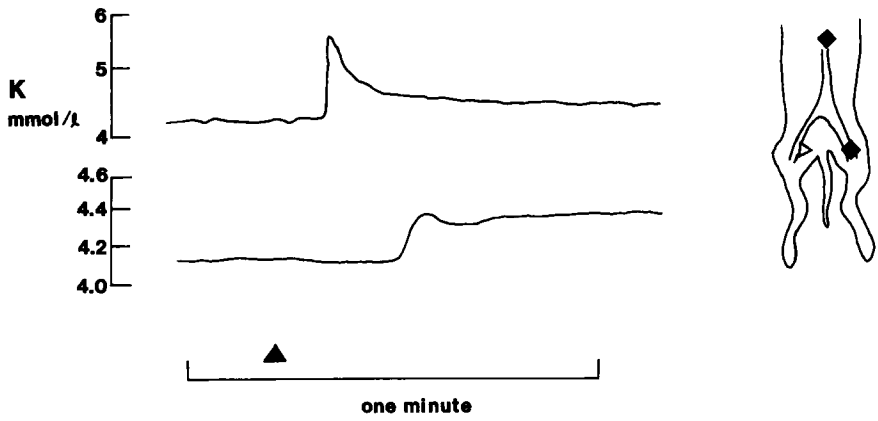

FIG. 11. *In vivo* application of K^+ catheters in anesthetized dogs; response to an injection of 2 ml of 0.1 *M* KCl (at △) recorded by potassium catheters (at ♦). (From ref. (T1) with permission.]

pCO_2 sensing catheters developed by General Electric also have been tested in animals (C7) and in human subjects (N1). Again, the correlation of *in vivo* and *in vitro* assays appeared to be satisfactory, indicating that these devices could ultimately be used routinely for *in vivo* monitoring situations, although additional reports in this direction have not been made in recent years.

4.4.2. *Intracellular Measurements*

There have been an extensive number of intracellular ISE measurements made over the past 25 years, initially using the all-glass type microelectrodes (e.g., for pH, Na^+, and K^+), and more recently, the liquid membrane micropipet-type devices. Several excellent reviews including one on the measurement of ions in excitable cells (H4) and one emphasizing measurements in nerve and muscle cells (W3) have appeared. Brown and Owen (B8) have surveyed the intracellular applications of the various types of micro-ISEs, including ones responsive to pH, Na^+, Ca^{2+}, Cl^-, and K^+. For convenience, some representative intracellular applications of these micro-ISEs are presented in Table 3.

5. ISE Devices for Biochemical Species

Up until this point, we have reviewed the state of ISE technology with regard to the measurement of inorganic ions and certain gases. In view of the advantages offered by an ISE-based analytical determination, biomedical

TABLE 3
SOME REPRESENTATIVE INTRACELLULAR APPLICATIONS OF MICRO-ISEs

Cell type	Electrode type	Analyte ion activity	Reference
Frog heart ventricle	Cl^- liquid membrane micropipet (Corning exchanger #477915)	Cl^-; 17.6 ± 0.57 m*M*	W5
	K^+ liquid membrane micropipet (valinomycin)	K^+; 86.2 ± 0.65 m*M*	W4
Rabbit heart muscle sarcoplasm	Ca^{2+} liquid membrane micropipet (neutral ligand—ETH 1001)	Ca^{2+} (resting muscle); 38 ± 17 n*M*	L4
Neurons of the mollusc, *Aplysia californica*	Ca^{2+} liquid membrane micropipet (ion-exchanger type with PVC added)	Ca^{2+}; 540 n*M*	O2
Vacuoles of plant cells (*Acer pseudoplatanus*)	H^+ liquid membrane micropipet (tridodecylamine as neutral ligand)	pH; 6–6.5	K15
Renal tubules of rats	K^+ liquid membrane micropipet—double-barrel design (valinomycin)	K^+ (proximal tubule); 54.4 ± 2.5 m*M*	K4
Renal tubules of bull frogs	Cl^- liquid membrane micropipet (ion-exchanger type)	Cl^-; 9.2 m*M*	F4
Giant snail neurons	Ca^{2+} liquid membrane micropipet (alkyl phosphate ion-exchanger type)	Ca^{2+}; 450 n*M*	C5
Squid axons (*Lollgo pealii*)	H^+ sealed-end all-glass microelectrode	pH; 7.3	B7
Muscle fiber of frog satorius	K^+ liquid membrane micropipet—double barrel design (valinomycin as carrier)	K^+; 105 m*M*	K5
Giant neuron of *Aplysia californica*	Na^+ closed-end all-glass microelectrode	Na^+; 40.3 m*M*	E1
Sheep heart Purkinje fibers	Na^+ closed end, recessed tip—all-glass microelectrode	Na^+; 7.3 m*M*	E4
	Cl^- liquid membrane micropipet (ion-exchanger based)	Cl^-; 13.8 m*M* (quiescent state)	V1
Chironomus parvae salivary gland cells	Na^+ liquid membrane micropipet [ion-exchanger based on tetra(-*p*-chlorophenyl)borate]	Na^+; 16 m*M*	P1

researchers have recognized that it would be desirable to have similar electrochemical sensors which are directly responsive to biologically important molecules including amino acids, metabolites, drugs, hormones, nucleotides, etc. Obviously, such sensors could be utilized as *in vitro* devices in clinical chemistry laboratories and automated instruments to replace classical spectrophotometric methods. Moreover, if miniaturized and microsized biochemical sensors were available, they could be used for continuous *in vivo* monitoring of biochemicals, (e.g., drug metabolism studies) or for intracellular studies.

There have been two main approaches taken toward developing such biosensing devices, and the current state of each will be described below.

5.1. ISE Devices Responsive to Organic Ions

If the biochemical species one wishes to detect is, at certain pHs, a charged ion, then it is possible to devise liquid or polymer membrane type ISEs for that ion. As is the case when measuring inorganic ions with these electrodes, selectivity for one organic ion over another is imparted by the nature of the carrier or ion-exchanger molecule used within the organic membrane phase. A large number of reports concerning the development of conventional polymer membranes or CWE type devices responsive to biochemical species has appeared in recent years. We have already alluded to an acetylcholine electrode prepared with a membrane phase of acetylcholine–tetrachlorophenylborate ion exchanger in 3-nitro-*o*-xylene (B4). In addition, electrodes responsive to methadone and cocaine (C11), codeine (H5), atropin and novatropine (D1), ethidium (B5), phencyclidine (M2), oxalate and acetate (C2), histamine (K13), creatinium (D2), and nicotine (E2) have been suggested. Cosofret (C9) has cataloged a large number of additional ISEs that have been prepared to detect drug-type substances.

Most of these liquid or polymer membrane electrodes were prepared by forming a complex between the analyte ion and a large hydrophobic ion exchanger molecule which is soluble in the organic membrane (e.g., tetraphenylborate, alkyl-ammonium species). Selectivity for one organic ion over another similar in structure is often poor since partition coefficients for the analyte ion into the membrane are not distinctly different from those of potential interferent ions. Consequently, the analytical use of these electrodes has been limited to relatively simple samples with fairly well-defined compositions (i.e., pharmaceutical preparations) or in specialized fundamental studies involving the pure analyte of interest. For example, the ethidium-responsive electrode was used to study the binding of ethidium ions to DNA. Similarly, the acetylcholine electrode was employed to measure the binding between acetylcholine and adenosine triphosphate in hopes of solv-

ing the controversy as to how acetylcholine is stored in the synaptic vesicle (K9). Also, an electrode responsive to butyltriphenylphosphonium cation was utilized to determine living cell membrane potentials of *Paracoccus denitrotrums* since aerobic respiration of these cells drives the uptake of this ion (M3). Such studies can be performed under physiologcial conditions because the selectivity of the organic ion electrodes over common inorganic electrolyte ions is often quite good. Unfortunately, in view of the non-specificity toward the full range of organic ions that may be present in an actual biological sample, these organic ion electrodes do not offer great promise as accurate detectors for *in situ* type biomedical investigations.

5.2. Bioselective Electrodes

Bioselective electrodes are a class of hybrid devices which combine the selective properties of biocatalytic reactions with ISE detection of liberated ions or gases. In contrast to the organic ion liquid membrane electrodes described above, the bioelectrodes can often possess extraordinary selectivity over molecules which are very similar in structure to the analyte. In addition, these devices can respond to nonionic species. Consequently, such electrodes can be used directly in biological samples to determine a wide variety of biochemicals accurately.

In most instances, bioselective electrodes are prepared by immobilizing biocatalysts, e.g., enzymes, intact cells, or organelles, at the surface of conventional ISE devices. Selectivity of the final bioprobe is dependent on the selectivity of the biological catalyst and on the innate selectivity of the ISE transducer used to construct the electrode. Thus, in order to avoid ionic interferences, gas-sensing devices are often used to fabricate such electrodes.

5.2.1. *Enzyme Electrodes*

Most work to date has involved the immobilization of purified enzymes at the surface of various ISEs and gas sensors. Guilbault (G2) and Kobos (K7) have provided excellent reviews on what has been accomplished in this area along with the methods used to prepare the devices. Over the last 10 years, electrodes responsive to urea, creatinine, glutamine, histidine, phenylalanine, amygdalin, adenosine, adenosine monophosphate, tyrosine, acetylcholine, as well as to a host of other biological molecules have been prepared. The required enzyme is either chemically immobilized or physically entrapped at the surface of the ISE transducer. For example, an electrode capable of measuring urea concentrations in blood may be prepared by immobilizing the enzyme urease in a thin gelled layer at the surface of a conventional ammonia gas sensor (see Fig. 12). When the electrode is placed

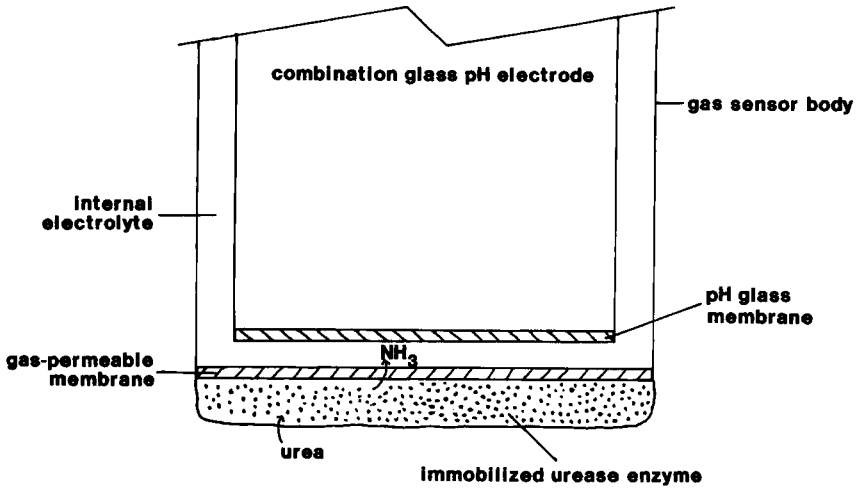

FIG. 12. Schematic of urea enzyme electrode utilizing conventional NH_3 gas sensor.

into a sample containing urea, the urea diffuses into the immobilized enzyme layer and is converted to ammonia gas via the reaction: urea + $H_2O \rightleftharpoons 2NH_3 + CO_2$. Liberated ammonia then passes through the gas-permeable membrane of the gas sensor and changes the pH of the internal filling solution. This pH change is sensed by the combination pH glass electrode (Severinghaus-type gas sensor). A steady-state pH in the film is achieved when the rate of ammonia production is equal to the rate of gas diffusion away from the immobilized enzyme layer. The same principle of operation applies to the other potentiometric electrodes reported, although the internal sensing electrode may be different [e.g., glass pH electrode, CO_2 gas sensor, cyanide electrode (for amygdalin)]. Most of the enzyme electrodes prepared have steady-state response times ranging from 2 to 5 minutes with detection limits on the order of 10^{-5} *M*.

While the majority of enzyme electrodes fabricated have been rather large devices, there have been some recent reports concerning the development of miniaturized and even microsensors. For example, Meyerhoff (M5) prepared an essentially disposable urea sensor (tip diameter 3 mm) by immobilizing urease at the surface of a new type of polymer-membrane electrode-based ammonia sensor (see Fig. 4). Alexander and Joseph (A1) have also prepared a new miniature urea sensor by immobilizing urease at the surface of pH-sensitive antimony wire. Similarly, Iannello and Ycynych (I1) immobilized urease on a pH-sensitive iridium dioxide electrode. In these latter investigations, ammonia liberated from the enzyme-catalyzed reaction alters the pH in the thin film of enzyme adjacent to the pH-sensitive wire.

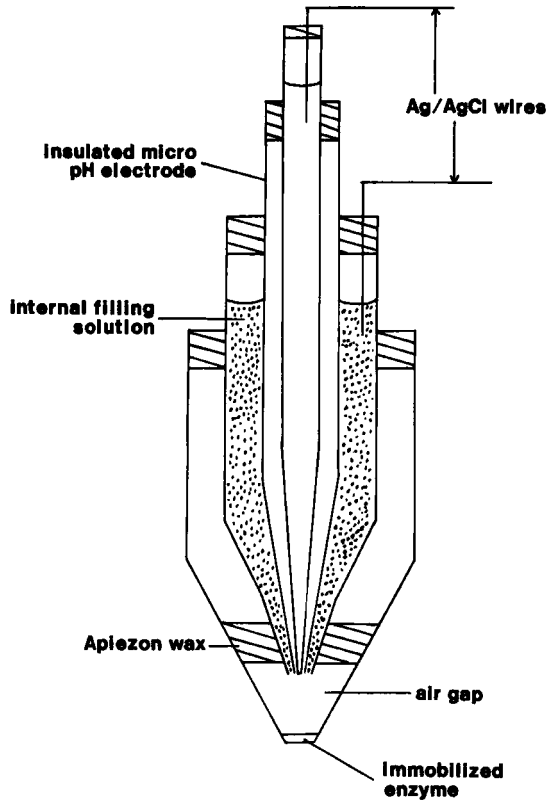

FIG. 13. Schematic diagram of microenzyme electrode [From ref. (P4). Reprinted from Analytical Chemistry **50**. Copyright (1978) American Chemical Society.]

Perhaps, realizing the need to make intracellular measurements of biochemical species, Pui, Rechnitz, and Miller (P4) fabricated the first truly microenzyme electrode. Their design was based on inserting a microglass pH electrode into an outer open tipped micropipet (tip diameter 10 μm). Figure 13 schematically illustrates this device. The bottom of the outer pipet is silanized so that an air gap is formed between the inner pH electrode and the sample. When internal electrolyte solution (NH_4Cl) is placed in the outer pipet (above the air gap), the resulting device functioned as a micro-ammonia sensor with the air gap replacing the gas-permeable membrane normally found in the larger sensors. To prepare the enzyme electrode for urea, urease enzyme was immobilized as a gel by cross-linking it with bovine serum albumin within the open tip of the sensor (using a glutaraldehyde procedure). The resulting micro-urea sensor exhibited rapid and selective response toward urea in solution. While not applied for intracellular type

measurements, it is clear that this design, although quite fragile, may provide the means of preparing a wide range of enzyme electrodes for intracellular measurements simply by immobilizing different enzymes at the tip.

5.2.2. *Bacterial and Tissue Electrodes*

The development of analytically useful enzyme electrodes is limited by the availability of purified and stable enzyme preparations. In an effort to extend the range of measurable species using ISE devices further, Rechnitz and co-workers (R1) recently introduced bacterial- and tissue-based bioselective electrode systems. These sensors are prepared in much the same manner as the enzyme probes except that whole intact cells are utilized as the immobilized reagents. There are several potential advantages to this novel approach, including (1) no need to extract and purify the enzymes involved, i.e., low cost; (2) enzymes which are unstable when extracted from the cell may be used *in situ* to maximize and preserve their activity; (3) if desired enzyme reactions require cofactors, these cofactors need not be added to the assay mixture because they are already present in the intact cell; and (4) analytical reactions involving multistep enzyme sequences already present in the cells may be used to detect given analytes.

Thus far, several bacterial and tissue electrodes have been reported, including ones responsive to glutamine, arginine, tyrosine, adenosine, glutamate, nitrilotriacetic acid, nitrate, guanine, and serine. In general, response characteristics for such probes are similar to enzyme electrodes except that response times are typically longer (e.g., 3–10 minutes) owing to the additional diffusion and/or transport steps involved. An advantage, however, is that bacterial and tissue electrodes usually have much longer analytically useful lifetimes due to the enhanced stability of the enzyme systems involved. This added reagent stability could be important if such sensors were to be adapted to clinical analysis instrumentation.

Kobos (K8) has recently reviewed the current state of the art with regard to the use of bacterial cells with ISEs while Arnold (A6) and Solsky (S7) have provided good introductory reviews of bioselective electrodes in general (including the tissue-based probes). These sensors have proven to be practical for clinical analysis. For example, Arnold and Rechnitz (A7) demonstrated that a highly selective electrode for glutamine, based on the use of immobilized porcine kidney cells at the surface of an ammonia gas sensor, could be used directly to assay glutamine in cerebral spinal fluid.

While selectivity in certain instances has been excellent (i.e., the above glutamine sensor), most tissue and bacterial electrodes are plagued by simultaneous response to several biochemical species. This is because there are often several enzymes present in a given cell which liberate the electrode-detectable product. Consequently, *in situ* type measurements with such

electrodes must be undertaken with caution in respect to the accuracy of the results obtained. On the other hand, errors for *in vitro* type assays could be minimized by addition of inhibitors to the sample diluent buffers (A8) which reduce the response from interferent metabolic pathways within the cells.

6. Future Prospects

The future of ISEs in medicine, biology, and clinical chemistry is bright. We are now at a stage where ISEs have become routinely used within clinical chemistry instrumentation to assay blood electrolytes. While satisfactory electrodes now exist for many cations, additional work needs to be undertaken with regard to the development of more selective anion-responsive membranes (e.g., for HCO_3^-, HPO_4^{2-}). Perhaps the synthesis of novel organic anion exchangers with size exclusion cavities (similar to crown compounds used for cation detection) will prove useful for this purpose. If so, not only would such electrodes be welcomed in clinical chemistry laboratories for detection of anions in blood, but also, once fabricated in micro form, these electrode systems could provide the tools necessary for more accurate measurements of anion levels within intact living cells.

Within the next 5–10 years the use of ISE type devices for continuous monitoring during surgical procedures and at the bedside of critically ill patients should become commonplace. While there are still problems to overcome, recent experiments with animals have already demonstrated that such measurements are feasible. It is probable that biomedical instrument manufacturers will produce both extracorporeal (e.g., the Miles "Biostator" already mentioned) and catheter-type systems for continuous detection of blood electrolyte levels. The use of telemetry for monitoring ISE potentials will undoubtedly play a major role in the development of the catheter devices. Ideally, one can envision, in the near future, operating rooms equipped with *in vivo* or extracorporeal devices and video screens which continuously display the patient's electrolyte levels just as blood pressure and heart rates are currently monitored.

We are also at a point in time where recently developed bioselective electrode systems will gain widespread use in clinical chemistry instrumentation as well as for basic biomedical research efforts in which *in situ* assays of biochemical species are required. While enzyme electrodes offer the most hope with regard to highly selective assay techniques, continued research into the development of new tissue and bacterial membrane electrode systems may lead to devices with improved operational lifetimes and sensitivity to compounds not amenable to enzymatic analysis (e.g., antibiotics). Indeed, considering the recent strides made in recombinant DNA technology, it may

be possible to tailor bacterial strains for use in conjunction with ion- and gas-sensing electrodes which could offer exceptional analytical properties including selectivity. At the same time, there will also be substantial efforts made aimed at further miniaturizing the present design for bioelectrodes so that such electrodes could be used routinely for *in vivo* and intracellular measurement of biochemicals.

Aside from the efforts to miniaturize existing bioelectrode designs and to devise new membrane systems for ion and gas sensing, the development of more universal bioselective immunosensors is on the immediate horizon. Already, liquid membrane electrode systems directly responsive to selective antibodies have been reported (S8, S9). In the near future, through the use of monoclonal antibodies in conjunction with enzyme-immunoassay arrangements, it may be possible to devise miniature electrochemical sensors capable of detecting extremely low concentrations of drugs, hormones, cyclic nucleotides and other biologically important substances with the selectivity required for *in situ* type measurements. At present, the biocatalytic electrodes, e.g., enzyme, bacterial, do not possess the detection limits necessary for such determinations. However, by combining the chemical amplification innate to the enzyme immunoassay system and the high specificity of monoclonal antibodies, it seems likely that electrochemical sensor measurements of biological species in the nanomolar range will be possible. Obviously, the prospect of using such devices *in vivo* for continuous monitoring of hormones and drugs at these levels is an exciting one to biomedical researchers. Consequently, fabrication of suitable ISE-based immunosensors will be one area vigorously pursued in the upcoming years as ISE techniques in general continue to become more widely accepted in medicine and biology.

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THE SOMATOMEDINS: INSULIN-LIKE GROWTH FACTORS

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1. Introduction

The term *somatomedin* was coined just over a decade ago to describe partly characterized plasma factors which stimulated sulfate incorporation into chondroitin sulfate of cartilage, had insulin-like activity (not neutralized by anti-insulin serum) in adipose tissue and diaphragm, and caused increased thymidine incorporation into DNA in various tissues (D10). It is the aim of this article to present an account of recent advances in the understanding of the origins, functions, and clinical significance of these factors. Although several excellent general reviews have appeared in the past two years (D1, H2, H14), adding to a number of others published previously, there has recently been a proliferation of interest in this area, leading to an ever-expanding output of published work. A variety of reasons may explain the increased interest in the somatomedins. First, cell and tissue culture techniques, once the domain of a restricted number of laboratories, are now carried out very widely, allowing access to a wealth of new information on the production and actions of these growth factors and many others, such as epidermal growth factor, fibroblast growth factor, nerve growth factor, and platelet-derived growth factor. Second, the time-consuming analytical methods of the past have been supplemented by relatively simple radioligand assays, permitting the analysis of many more samples than previously possible. Third, highly purified peptides are now being prepared and distributed by a number of centers, giving many laboratories an opportunity to study their actions. The sections of this article fall into two groups: those describing where and how somatomedins are produced, how they are transported in

the circulation, and how they interact with their target tissues, and those concerned with the measurement of somatomedins, and the physiological and clinical significance of their levels in the circulation. While it is impossible to make such a review totally comprehensive, it is hoped that it will nevertheless be a useful source of information on some recent advances in the area.

1.1. Background and Nomenclature

In the past decade, many preparations possessing a range of anabolic activities have been designated somatomedins. Somatomedin-A was defined by its activity in stimulating sulfate incorporation into glucosaminoglycans ("sulfation factor activity") in chick cartilage (S25); somatomedin-B was a stimulator of DNA synthesis in glial cells (U2); and somatomedin-C had sulfation factor activity in rat cartilage (V4). A related peptide, basic somatomedin, appeared to resemble somatomedin-C (B1). Other peptide preparations also fitted the somatomedin definition: multiplication-stimulating activity or MSA (M26), which had mitogenic activity in a number of different cell types, and acid–ethanol soluble nonsuppressible insulinlike activity (NSILAs), which subsequently yielded two active peptides, NSILA I and II (R8), later renamed insulinlike growth factors (IGF-I and -II). Another insulinlike peptide, ILAs, appeared similar to IGF-II (P13). All of these preparations were isolated from human plasma except for MSA, which was derived from culture medium conditioned by the rat liver cell line BRL 3A. They are now known to share all three of the originally described biological activities: stimulation of sulfate incorporation into cartilage, insulinlike activity, and multiplication-stimulating activity.

The interrelationships within this rather confusing array of peptides have become clearer with the purification, sequencing, and immunological characterization of several of the species. IGF-I is a basic, single-chain polypeptide of 70 amino acids, molecular mass 7649, containing three disulfide bridges (R10). IGF-II, a slightly acidic peptide with a molecular mass of 7471, contains 67 amino acids, over 60% of them corresponding to those in IGF-I (R9). Both peptides also have a high structural homology with human proinsulin. In brief, the A and B chains of insulin, with their interchain disulfide bridges, are closely paralleled in the IGF peptides, while the proinsulin C-peptide region is represented by much shorter sequences in IGF-I and IGF-II. In addition, the growth factors have short carboxy-terminal extensions (termed the D region) not present in proinsulin. Three-dimensional models of both peptides have been constructed using interactive molecular computer graphics, highlighting the similarities with the insulin molecule and accounting for some of the antibody- and receptor-binding properties of the IGF peptides (B30).

Following its purification (S34), sequence analysis of somatomedin-C has indicated that it is identical to IGF-I (K10), confirming radioreceptor assay (RRA) and radioimmunoassay (RIA) studies which suggested identity or near-identity (V3). The term somatomedin-C/IGF-I (SM-C/IGF-I) is now used to designate this peptide. A basic somatomedin isolated from rat serum is strikingly similar in its amino-terminal sequence, and may be regarded as rat SM-C/IGF-I (R16). Purification and analysis of one of the MSA fractions has revealed 93% homology with human IGF-II (M5); this peptide is now regarded as rat IGF-II. Somatomedin-B is no longer included as a member of the somatomedin group, as its mitogenic activity is due to contamination by epidermal growth factor (H11). Characterization of the most recently isolated preparation of somatomedin-A has revealed apparent identity with SM-C/IGF-I, with the possible exception of a glutamine deamidation at position 40 (E3). Thus the known members of the somatomedin group are now confined to SM-C/IGF-I and IGF-II.

There may, however, be other related peptides which are as yet uncharacterized. For example, forms of rat IGF-II have been described with molecular masses of 8700 and 16300 (M25); these are immunologically related to the characterized peptide of molecular mass 7500 (M5). A "big IGF-II" has also been reported in human spinal fluid and serum (H9). It is conceivable that these species are incompletely processed precursor forms of IGF-II. In addition to such variants of known IGFs, variants of different charge have also been described. One, an acidic peptide (pI 4.8) first detected by its insulin-like activity, cross-reacts in two radioimmunoassays for SM-C/IGF-I, but not in an assay for IGF-II (H13). Finally, the existence of a fetal somatomedin has been postulated (H2), but whether this differs from IGF-II remains to be established.

1.2. Regulation by Growth Hormone

One of the definitive characteristics of the somatomedins is their regulation by growth hormone (GH). As discussed in detail in Section 7, circulating levels of SM-C/IGF-I in humans are dependent upon the GH secretory status, being elevated in states of GH hypersecretion, and often almost undetectable in total GH deficiency. Human fibroblasts in culture, which release SM-C/IGF-I (see Section 2.1), also show responsiveness to GH (C11). Regulation by GH can equally be demonstrated in animals. For example, in chronically cannulated, conscious rats there is a highly significant association between secretory pulses of GH and integrated SM-C/IGF-I levels (B19), and in hypophysectomized rats, or rats bearing GH-secreting tumors, the GH status is reflected not only by the circulating SM-C/IGF-I level, but also by the hepatic production rate of the peptide (S23). A variety

of *in vitro* systems have been used to demonstrate GH regulation in the rat: isolated perfused liver preparations (P9, S9), cultured liver explants (B25), and primary cell cultures (S23, S28).

Dependence of IGF-II on the circulating concentration of GH is less easily demonstrated. GH hypersecretion in man does not cause elevated IGF-II levels; however, significantly decreased IGF-II levels are seen in hyposomatotropism, suggesting at least partial GH dependence (see Section 7). Thus, with this slight qualification, both SM-C/IGF-I and IGF-II possess properties which allow them to be classified as somatomedins: they show sulfation factor, insulinlike, and mitogenic activities, and they are regulated by GH.

2. Production, Properties, and Actions

2.1. Sites of Production

A wide variety of data from many laboratories indicates that the liver is a major source of somatomedin peptides. This has been demonstrated directly in studies of isolated perfused livers (F3, K13, M10, P9, S9, S21, W6), fetal (D14, R5) and adult (B25, S13) liver in organ culture, rat liver cell lines (M5, M26, S27), and primary hepatocyte cultures (K14, S22, S28). These studies are supported by the observations that partial hepatectomy (U3) or liver disease (S14, T5) results in low circulating somatomedin activity. It has not always been clear, however, which members of the somatomedin family were being assayed in some of these studies, due to the broad specificity of the assays used (see Section 5). For example, whereas it has been well established that the BRL (buffalo rat liver) cell line, and fetal rat liver in organ culture (R5), produce peptides of the MSA or IGF-II family (M5, M26), it is not known whether normal adult liver produces IGF-II. Indeed, production of SM-C/IGF-I by adult liver has been demonstrated specifically in only two studies. In these, Schwander *et al.* (S21) found that a ^{35}S-labeled product from perfused liver could be immunoprecipitated with an IGF-I antiserum, and Scott *et al.* (S22) used a specific SM-C/IGF-I RIA to demonstrate production of the peptide by adult hepatocytes in primary culture. In both studies, the measured hepatic production rate was calculated to be sufficient to account fully for circulating SM-C/IGF-I levels.

Second to the liver, fibroblasts are perhaps the most widely studied source of somatomedins. The idea that these cells might produce their own growth factors was suggested by the observation that hypopituitary serum was as effective as normal serum in stimulating thymidine incorporation into skin fibroblasts, a possible indication that they did not require exogenous somato-

medins (M21). Atkinson *et al.* (A8) first demonstrated that the human embryonic lung fibroblast line WI-38 produces a substance detectable in a SM-C/IGF-I RIA. Human skin fibroblasts also release immunoreactive SM-C/IGF-I (C11), the production rate being enhanced by the addition of GH and a range of other hormones and growth factors (C9). In an interesting study by Adams *et al.* (A3), an age-related shift from MSA/IGF-II to SM-C/IGF-I production by rat lung and skin fibroblasts was seen in cell cultures derived from embryonic, neonatal, and adult animals. The production rate of SM-C/IGF-I by adult fibroblasts, about 50 ng per million cells per 48 hours, is of a similar magnitude to that reported for adult hepatocytes (S22), suggesting a significant role for fibroblasts in somatomedin production.

A wide range of other body fluids and tissues are also known to produce or contain somatomedins. For example, SM-C/IGF-I has been reported in human milk (B18) and semen (B17), somatomedin A in Sertoli cell cultures (H4), and MSA/IGF-II in ovarian follicular fluid (H7) and amniotic fluid (M14). Many fetal tissues, in particular, seem capable of synthesizing SM-C/IGF-I when grown in organ culture (D14). Direct extraction of adult tissues also indicates significant concentrations of these peptides throughout the body. In addition to the liver (D16, K8, V5), kidney, lung, pancreas, submaxillary gland, testes, neural tissues, heart, and other organs all contain acid-extractable somatomedins (D16, K8, S8). D'Ercole *et al.* (D16) found that in hypophysectomized rats treated with GH, the concentrations of SM-C/IGF-I in various tissues peaked and declined before the maximal increase was seen in the serum concentration, suggesting that all of these tissues might contribute to the circulating levels of the peptide. Conversely, in diabetic or malnourished rats, the fall in hepatic content and production of bioactive somatomedin preceded the fall in serum levels (V7). In the former study (D16), the liver was calculated to account for about 55% of the total amount in the circulation, with no other single tissue making a major contribution.

It may be concluded from a number of the studies described above that the liver is the source of the majority of the SM-C/IGF-I in the circulation (the main source of circulating IGF-II in adults is entirely unknown). Since most other tissues appear to produce their own somatomedins, this raises the question of the function of the circulating peptide. It has been postulated that, particularly in the fetus, these growth factors exert their biological effects on the cells which produce them, or on nearby cells; that is, that they act by autocrine or paracrine, rather than endocrine, mechanisms (D14, D16). If this is so in adult tissues, the reason for the high hepatic production rate is unexplained, since liver cell membranes contain very few SM-C/IGF-I receptors (see Section 4.2) and, with the exception of one study in which an incompletely characterized somatomedin preparation was used (B8), no ac-

tions of this peptide have been demonstrated in normal liver. Indeed, the observation that SM-C/IGF-I production by hepatocytes is greatest at highest cell densities is evidence against a role for the peptide in liver cell replication (S23). An alternative view is that the liver and possibly a few other tissues act as endocrine organs in producing SM-C/IGF-I mainly for export to cells at distant sites. The activity extractable from the nonproducing tissues could represent peptide which has accumulated on cell receptors, and the early rise in tissue levels following GH treatment after hypophysectomy (D16) might simply indicate that these receptors, depleted of ligand by hypophysectomy, are extremely efficient at extracting newly synthesized peptide from the serum. Whether growth factor secretion by cultured fibroblasts and other continuously dividing cell lines represents the true function of these cells *in vivo*, or merely an adaptation to continuous culture, also remains to be established. The use of hybridization histochemistry (H25) to identify sites of SM-C/IGF-I gene transcription in various tissues may help to resolve these questions.

Recent reports from several laboratories describing a variety of cell-free somatomedin-producing systems should also be mentioned. Acquaviva *et al.* (A2) have described the use of a cell-free system to translate mRNA extracted from BRL 3A cells. The main product immunoprecipitable by a specific rat IGF-II antiserum had a molecular mass of 21,600, and was postulated to be a pre-pro-IGF-II. Using an entirely different approach, Li *et al.* (L5) produced the complete SM-C/IGF-I molecule by solid-state synthesis. It was reported that, by chemical analysis, biological activity, and immunoreactivity, the product was indistinguishable from the peptide extracted from human plasma. Finally, Jansen *et al.* (J3) have reported the complete nucleotide sequence of a human liver cDNA coding for SM-C/IGF-I, and, independently, the gene has been synthesized and cloned (J5). Several laboratories appear to be making advances in IGF-I production by recombinant DNA techniques, and it seems only a matter of time before the peptides are available in plentiful quantities.

2.2. Actions on Cartilage

It is almost three decades since Salmon and Daughaday (S3) described a GH-dependent serum factor which could stimulate sulfate incorporation by cartilage *in vitro*, an activity apparently not inherent in GH itself. This "sulfation factor" was postulated to mediate the known effects of GH *in vivo* on skeletal growth. Subsequent studies revealed a range of GH-dependent activities in rat cartilage: stimulation of sulfate and leucine uptake into glucosaminoglycans (S4), proline conversion to collagen hydroxyproline (D3), and incorporation of uridine into RNA and thymidine into DNA (G1,

S4). Inhibition of adenylate cyclase activity has also been reported (T9), raising the possibility that changes in cyclic AMP levels might mediate some of the other observed effects. In chick cartilage, both amino acid uptake (A5) and sulfate incorporation (H1) were stimulated. These biological actions have been used in many laboratories to monitor the purification of somatomedins, and led to the isolation of somatomedin-C and somatomedin-A, both now known to be identical to IGF-I. However, IGF-II–related peptides are also capable of eliciting increases in proteoglycan and nucleic acid synthesis in cartilage, as demonstrated both in cultured chondrocytes (A6, T14) and cartilage segments (Z1). In the latter study IGF-I and IGF-II were found to have equal potency in stimulating sulfate incorporation.

Despite the inability in early experiments to demonstrate direct effects of GH on cartilage, leading to the discovery of the somatomedins, it has recently been demonstrated that GH, when administered directly to the tibial cartilage growth plate in hypophysectomized rats, stimulates bone elongation (I1). Although this observation does not exclude mediation of the effect of GH by somatomedins, it is clear that if they have a role in this system they must act locally rather than after transport in the circulation. Consistent with the idea of a local action in cartilage, Kato *et al.* (K5) have isolated a somatomedinlike growth factor from bovine cartilage.

2.3. Effects on Adipose Tissue

The recognition that much of the insulinlike biological activity in serum cannot be accounted for by insulin immunoreactivity led to the discovery of NSILA, later purified to yield both IGF-I and IGF-II. In these studies, insulinlike effects were generally monitored in adipose tissue. The range of activities of somatomedins in this tissue is wide, including stimulation of glucose metabolism to CO_2, lipids, and glycogen, and inhibition of epinephrine-stimulated lipolysis and of glycogen breakdown (O1, R5, U1, V4, Z1), all known actions of insulin. IGF-II has 2–3 times the potency of SM-C/IGF-I in these actions (Z1). It is interesting to note, however, that the stimulation of glucose transport in fat cells by GH cannot be mediated by the somatomedins, since in hypophysectomized rats, in which 3-*O*-methylglucose efflux is maximal, GH infusion returns the transport rate to the basal level of intact animals, while neither SM-C/IGF-I nor IGF-II infusion has this effect (S19).

2.4. Mitogenic Activity

Apart from anabolic actions in cartilage and insulinlike effects in fat cells, the third historically recognized action of somatomedins is their ability to

stimulate DNA synthesis and cell proliferation in fibroblasts and other cell types, termed multiplication-stimulating activity (M26, P10). In chick embryo fibroblasts, IGF-I and -II have equal potency in stimulating thymidine incorporation (Z1). Mitogenic activity (or at least stimulation of thymidine incorporation into DNA) has been described in a diverse range of cells other than fibroblasts, among which are included Sertoli cells (B31), fetal brain cells (L4), myoblasts (F2), lens epithelium (R6), pancreatic β-cells (R1), and lectin-activated lymphocytes (S15).

Observations that the mitogenic effect of pure somatomedins on cultured cells is less than that seen when serum is added to the culture medium led to the concept that other factors acting synergistically with somatomedins must be required for a maximal mitogenic response. Stiles *et al.* (S31) confirmed this postulate by demonstrating that a variety of somatomedin preparations, of both IGF-I and IGF-II types, could stimulate density-arrested BALB/c 3T3 cells to synthesize DNA only after the cells had been rendered "competent" by exposure to platelet-derived growth factor or fibroblast growth factor. Several actions of these so-called competence factors have been described which may be associated with their ability to facilitate the expression of the mitogenic activity of somatomedins. In BALB/c 3T3 cells they induce several cytosol proteins whose appearance seems intimately associated with the induction of competence (P11), whereas in hamster lung fibroblasts they stimulate the phosphorylation of a characteristic peptide of molecular mass 27,000 (C4). Perhaps the most clear-cut mechanism by which they facilitate the action of somatomedins is by causing the induction of receptors for SM-C/IGF-I, thus allowing optimal expression of its mitogenic effects (C12).

2.5. Other Actions

In addition to the sulfation factor, insulinlike, and mitogenic activities originally ascribed to the somatomedins, many other biological responses to various somatomedin preparations have been demonstrated in recent years. Most importantly, growth effects have been demonstrated in whole animals, adding to the significant body of less direct evidence that these factors mediate somatic growth. The first such demonstration (V1) was in Snell dwarf mice, in which it was shown that impure somatomedin preparations administered subcutaneously over 4 weeks were capable of increasing body weight and length as well as the weight of several organs. In more recent experiments involving the use of pure peptide preparations, infusion of SM-C/IGF-I caused hypophysectomized rats to gain weight, increased the width of their tibial epiphyses, and increased DNA synthetic activity in costal cartilage (S20). All effects were dose-related over the range 43–103 μg/day.

It is not possible in this review to present more than a brief cross-section

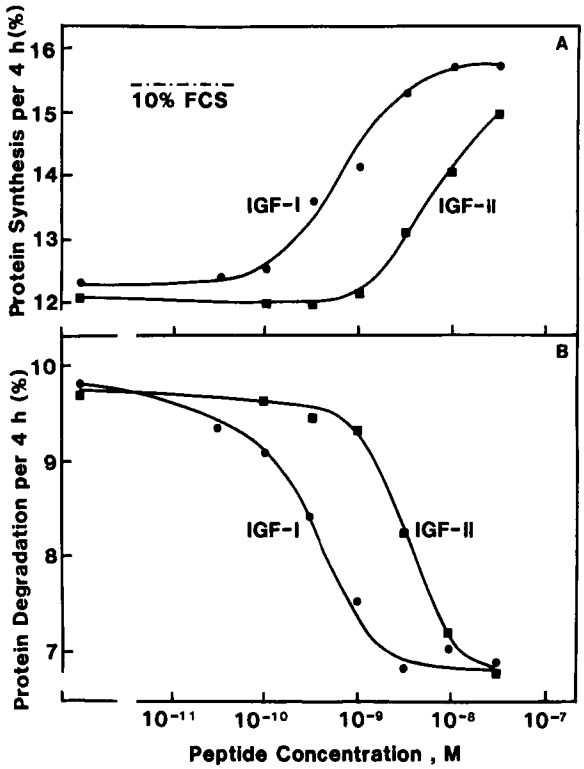

FIG. 1. Stimulation of protein synthesis (A) and inhibition of protein degradation (B) over a 4-hour period in L6 myoblasts by IGF-I and IGF-II prepared in the author's laboratory. The dashed line indicates stimulation of protein synthesis by 10% fetal calf serum. Assays performed by F. J. Ballard and L. C. Read, using published methods (B5).

of other recently described effects of somatomedins, which cover a broad range of growth-related functions. For example, both IGF-I and -II stimulate phosphatidylcholine biosynthesis in 3T3 fibroblasts (W1), MSA increases transferrin secretion by Sertoli cells (S26) and ovalbumin gene expression by chick oviduct (E5), and SM-C/IGF-I stimulates aminoisobutyric acid uptake in human fibroblasts (K1). In rat L6 myoblasts, IGF-I and -II increase protein synthesis and inhibit protein degradation (Fig. 1), with IGF-I showing about ten times the potency of IGF-II. Inhibition of protein degradation has also been demonstrated in a variety of fibroblasts, hepatomas, and other cell lines (B6). Bothwell (B32) has reported that MSA promotes nerve growth factor-independent neurite formation in chick dorsal root ganglion sensory neurons, while IGF-I and -II or MSA enhance the differentiation of myoblasts in various cell systems (S17, T15). This differ-

TABLE 1
STIMULATION OF CELL PROTEIN PHOSPHORYLATION BY SOMATOMEDINS

Cell or tissue type	Peptide tested	Size, location of phosphoprotein	Phosphorylated amino acid	Reference
CC139 lung fibroblasts	MSA	33K ribosomal protein S6		C4
Chick embryo fibroblasts	MSA	40K	Tyrosine	N1
3T3 L1 adipocytes	MSA	22K, cytosol	Serine, threonine	B27
IM-9 lymphocytes	SM-C	SM-C/IGF-I receptor, 95K subunit	Tyrosine	J2
BRL 3A2 liver cells	IGF-I	SM-C/IGF-I receptor, 98K subunit	Tyrosine	Z7
Human placenta	IGF-I	SM-C/IGF-I receptor, 90K subunit	Tyrosine	R15

entiation has been shown to occur even under conditions where over 95% of DNA synthesis was blocked (T15). Somatomedins also promote the phosphorylation of a number of cell proteins which may be involved in cell replication and other processes. As shown in Table 1, these phosphorylations occur in various cell compartments and on several different amino acids. In brief, a continually increasing number of studies indicate that the somatomedins play a vital role in the growth and differentiation of a variety of tissues.

2.6. INHIBITION OF PITUITARY GROWTH HORMONE SECRETION

The hypothesis that somatomedins are produced by GH target organs in response to GH stimulation, and that they mediate the growth-promoting actions of GH, has the corollary that pituitary GH secretion should be inhibited by somatomedins, in a classical endocrine negative feedback loop. Consistent with the existence of this regulatory loop, Laron dwarfs, with defective somatomedin generation apparently due to the lack of GH receptors, have elevated circulating GH levels (L2). In the past few years, it has been possible to demonstrate directly the regulation of GH secretion by somatomedins. Two studies in conscious intact rats, in which ILAs, an IGF-II-like peptide (T8), and SM-C/IGF-I (A1) were injected intracerebroventricularly, have indicated that the characteristic secretory pulses of GH seen in conscious rats are markedly suppressed by these peptides.

The mechanism of this suppression is uncertain. Berelowitz *et al.* (B23) have reported that SM-C/IGF-I causes the stimulation of somatostatin re-

lease from rat hypothalamus preparations, a possible mechanism for the inhibition of pituitary GH secretion *in vivo*. Alternatively, since centrally administered peptides are capable of entering the peripheral blood (P2), somatomedins injected intracerebroventricularly might reach the pituitary and exert their effect directly on GH secretion. Basal GH release by cultured anterior pituitary cells has been shown to be inhibited by SM-C/IGF-I added to the culture medium for 24 hours (B23). In a more recent report, Brazeau *et al.* (B33) described the inhibition by SM-C/IGF-I of GH release from pituitary cell monolayers stimulated by the addition of hypothalamic GH releasing factor. The effect was seen in both short-term (3 hour) and long-term (24 hour) incubations, and IGF-II was less potent as an inhibitor than SM-C/IGF-I, whereas epidermal growth factor and fibroblast growth factor were without effect. Whatever the precise mechanism for the inhibitory effect of somatomedins on GH secretion, its demonstration adds considerable weight to the original hypothesis that the somatogenic effects of GH are mediated by the somatomedins.

3. Somatomedin Binding Proteins

3.1. Circulating Forms and Biological Actions

The somatomedins are unusual among the peptide hormones in that they appear in the circulation bound to carrier proteins. The existence of these carrier proteins has been demonstrated in several ways. Addition of radioiodinated somatomedin peptides to human serum either *in vitro* or *in vivo*, and subsequent fractionation by gel chromatography, results in the appearance of radioactivity associated with fractions of 60,000 or higher molecular mass (H15, K7, Z2). In rat serum, tracer somatomedin was shown to be associated with two protein fractions, roughly corresponding in mass with immunoglobulins and albumin (M23). These two peaks of binding activity, generally assigned molecular masses of 150,000 and 40–60,000 respectively, have also been identified in many studies of human serum (C17, H20, W4). In healthy subjects, the smaller protein appears in all of these studies to bind about twice as much radioactivity as the larger.

Given the capacity of serum to bind added somatomedin tracers, it is not surprising that endogenous somatomedins in plasma are exclusively associated with high-molecular-mass complexes. This was first indicated by the demonstration that sulfation factor activity in plasma chromatographed at neutral pH is all of high molecular mass (H15). Following gel chromatography at pH 2.3, the sulfation activity is found in the 5–10,000 molecular mass range (H15), suggesting acid dissociation of the larger complex. More recent studies

on size-fractionated human serum, subjected to SM-C/IGF-I RIA after extraction of each fraction to remove binding activity, indicate that some 72% of the endogenous peptide is associated with the 150,000-molecular-mass fraction and 25% with the 50,000-molecular-mass fraction (D9). The majority of MSA bioactivity, measured in chick embryo fibroblasts, is also present in the 150,000-molecular-mass form (W4).

The exact role of somatomedin binding proteins in the circulation, and how they act to deliver somatomedins to their target tissues, are poorly understood. One well-established function is to increase the plasma half-life of somatomedins from 8 to 30 minutes when not protein-bound to 3–6 hours in the presence of binding proteins (C18). Another apparent role is to provide a reservoir for somatomedin peptides which, unlike insulin, are not stored in high concentrations in any tissue (D16). Since serum is the main storage site, and the total serum SM-C/IGF-I plus IGF-II concentration—about 800 ng/ml or 100 nmol/l (1000 times higher than the concentration of insulin)—is much greater than the apparent biologically active amount, it appears that the peptides when protein-bound exert little of their potential activity. Consistent with this idea, partially pure human somatomedin binding protein preparations have been shown to block sugar transport, lipogenesis, and other insulin-like activities (D20, Z6; see Section 3.5), and a binding protein purified from BRL 3A rat liver cell culture medium was also able to inhibit DNA synthesis in chick embryo fibroblasts (K11). However, this does not necessarily indicate that the naturally occurring high-molecular-mass complexes are devoid of activity; indeed, evidence exists which suggests that a portion of the total NSILA in serum exerts its biological action when protein-bound (C21).

An important key to the regulation of somatomedin activity *in vivo* may, then, be the mechanism by which the peptides are released from their macromolecular complexes to interact with cell surface receptors and initiate intracellular events. Daughaday *et al.* (D9) observed that the binding capacity of serum was markedly dependent upon its concentration, and postulated that dilution of serum in the interstitial fluid may facilitate dissociation of bound somatomedins. A more specific mechanism has been proposed by Chatelain (C5), Clemmons (C13), and co-workers. They demonstrated that, when serum is incubated at neutral pH, the exposure of SM-C/IGF-I antigenic sites is increased, although the distribution of the peptide between the different binding protein species is not greatly altered (C5). This apparent increase in the exposure of somatomedin is blocked by protease inhibitors. Clemmons (C13) extended this study to show that incubation of heparinized plasma in a buffer containing heparin resulted in the redistribution of a major fraction of the SM-C/IGF-I from the 140,000-molecular-mass form to its low-molecular-mass, unbound form, a change also caused by acidification

of plasma. The conclusion from these studies was that, at neutral pH, serum proteolytic enzymes reduce the affinity of somatomedin binding proteins for their ligands, and that heparin, or other polyanions, prevent the reassociation, allowing the free peptide to remain available to its target tissues.

3.2. Sites of Production

In several laboratories it has been shown that normal liver and liver-derived cell lines in tissue culture release somatomedin binding proteins into the surrounding medium. In the rat, primary hepatocyte cultures (S22, S28), liver explants (B25) and isolated perfused liver (S9, S21) produce a protein of molecular mass 40–50,000. A binding species of similar size is released by the BRL 3A2 cell line (M24). Whereas somatomedin peptides could also be detected in all of these studies, human hepatoma (M22) and mammary carcinoma (B20) cell lines have been described which release the 40–50,000 molecular mass binding protein without any detectable low-molecular-mass somatomedins. Human amniotic fluid also contains a binding species of similar size (C8, D20) but, contrary to an original report that there was no detectable somatomedin (C8), it appears that both IGF-I and -II are also present (M14).

It is particularly interesting that no cell or tissue preparation, and no biological fluid except for serum and milk (B18), has been reported to produce or contain somatomedin binding activity in the 150,000-molecular-mass form. This raises the very important question of the origin of this species. Although the possibility cannot be excluded that some as yet unknown tissue synthesizes all of the high-molecular-mass binding protein found in the circulation, it seems most likely that the liver, which can account for most or all of the SM-C/IGF-I in the circulation (D16, S21, S22) and which releases it in association with binding protein (S21, S22), also makes a major contribution to the total circulating binding protein. How the 50,000-molecular-mass form is converted to the larger species is unknown, although it may involve combination with an acid-labile subunit (see Section 3.3). Presumably the conversion takes place at some extrahepatic site, unless it is assumed that perfusion and tissue culture media lack some components which the liver requires to make the conversion itself. The exact nature of such a factor, and whether glycosylation or another posttranslational modification is involved, remain intriguing questions.

3.3. Molecular Structure

Treatment of normal serum with acid, in addition to releasing somatomedins from their macromolecular complexes, also has the effect of reducing

the 150,000-molecular-mass species to about 60,000 (F7, M24). This reduction in mass is apparently not dependent upon the loss of bound ligands, since it has been observed even if the acidification is performed within a dialysis membrane which retains low-molecular-mass peptides. Agents known to effect this size conversion include dilute acetic and formic acids, 8 mol/liter urea at pH 4.75, and 0.1 mol/liter lithium diiodide salicylate (M24). Furlanetto (F7) has described the isolation by ion exchange chromatography of an acid-labile serum fraction which, when combined with either a native binding protein species of Stokes' radius 36 Å (i.e., approximate molecular mass 60,000) or the acid-treated binding species of similar size, results in the re-formation of a complex of Stokes' radius 43 Å (i.e., 150,000 molecular mass). An additional, smaller binding protein, of Stokes' radius 28.5 Å (i.e., 40–50,000 molecular mass), was incapable of combining with the acid-labile protein to form the large complex. These data were interpreted as indicating that the high-molecular-mass binding complex contains at least two subunits, one of which is acid labile.

The 60,000-molecular-mass protein which results from acidification of human serum has been further analyzed by Morris and Schalch (M20). Exposure of this fraction, containing bound iodinated SM-C/IGF-I, to pH 8.0 buffer was found to yield binding complexes of apparent masses 46,000 and 30,000. Affinity labeling of native rat serum, followed by SDS gel electrophoresis and autoradiography, yields a somewhat more complex picture, with labeled bands corresponding to 95,000, 49,000, 36,000, 33,000, 26,000, and 23,000 molecular masses (D13). Similarly, two subunits of 30,000 to 35,000 molecular mass have been identified by SDS polyacrylamide gel electrophoresis of a purified 60,000-molecular-mass binding protein from culture medium conditioned by the BRL cell line (K12), and perfusates of normal rat liver contain a binding species of 35,000 molecular mass (S21). If the 30–35,000-molecular-mass binding protein subunits from human serum and rat liver are similar, these experiments establish a link between the 150,000-molecular-mass protein found in serum (or its 60,000-molecular-mass acidified form) and the smaller protein released by the liver and other tissues.

3.4. Growth Hormone Dependence

The circulating half-life of exogenous somatomedin in the hypophysectomized rat has been shown to be greatly prolonged in the presence of normal rat serum compared to serum from hypophysectomized rats (C18). This observation can be explained by a comparison of the somatomedin-binding profile of rat serum from normal and hypophysectomized animals, which indicates that the 150,000-molecular-mass species is GH dependent,

since it is absent in GH deficiency and returns with GH therapy (M23, M24). The smaller protein produced by rat liver shows a similar dependence, as indicated by a 93% decrease in the release of binding protein by cultured hepatocytes from hypophysectomized rats compared to cells from normal animals, and 2- to 3-fold higher production by cells from rats bearing GH-secreting tumors (S23). Cultured hepatocytes from normal rats are also responsive to GH *in vitro*, increasing their output of binding protein by 50% in the presence of 200 ng/ml GH (S23).

Human somatomedin binding proteins are also regulated by GH. Using a variety of analytical techniques, higher levels of total binding activity can be detected in the serum of healthy subjects than in samples from hypopituitary patients (B26, D19, Z5). In hypopituitarism, GH treatment results in an increase in somatomedin binding by serum proteins in the 150,000-molecular-mass range, as demonstrated either by binding of SM-C/IGF-I followed by gel chromatography (C19), or by gel chromatography of serum and measurement of MSA binding capacity of each fraction (W4). However, Binoux *et al.* (B26) were unable to demonstrate an increase in binding protein when GH treatment was of short duration (1–3 days). In acromegaly the total binding capacity of human serum is increased 2- to 3-fold (B26), the increase apparently being found predominantly in the 150,000-molecular-mass region (H3). In contrast to these attempts to measure the total binding capacity of serum, Hintz *et al.* (H20) measured unsaturated binding sites. Not surprisingly, binding to unoccupied sites, predominantly in the 40–50,000 molecular mass range, was increased in hypopituitary subjects, who had lower circulating somatomedin levels. Scatchard analysis suggested that the increased binding in these subjects was due to a 2- to 3-fold increase in affinity of the binding proteins. The conclusions to be drawn from these studies are that, as in the rat, the 150,000-molecular-mass binding species in human serum, which binds most of the endogenous somatomedin, is under GH regulation, while the species of 40,000 molecular mass, which has the majority of unoccupied sites, is not.

3.5. Somatomedin Inhibitors

In a variety of metabolic states where somatomedin action *in vivo* appears to be reduced (see Section 8), circulating factors have been identified which are capable of inhibiting the various biological activities of somatomedins *in vitro*. Inhibitors of sulfate incorporation into cartilage have been described in the plasma of children with severe malnutrition (V2), and in acutely starved rats (S2). The factor in starved rat serum was slightly antagonistic to the action of insulin as well as somatomedin, and was inactivated at 60°C, though surprisingly stable at 100°C if first diluted and acidified (S5). Phillips

and co-workers found a similar inhibitory factor in the serum of rats with experimental diabetes (P5, P7). In addition to its effect on cartilage, this factor inhibited the stimulatory effect of both insulin and an impure somatomedin preparation on glucose oxidation by adipose tissue, and blocked the stimulation by insulin of glucose incorporation into glycogen in rat diaphragm (P5). The inhibitors have been poorly characterized to date, being identified only as proteins due to their heat lability and trypsin sensitivity. Like SM-C/IGF-I and somatomedin binding proteins, they appear to be of hepatic origin, since neutral rat liver extracts were found to be inhibitory in the cartilage assay (V6). The liver factor was heat and acid labile, had a molecular mass above 45,000 and, surprisingly, could be extracted in equal amounts from livers of normal, diabetic, fasted and hypophysectomized rats (V6).

A somewhat different inhibitor has been isolated from Cohn fraction IV-I of human serum by Herington and Kuffer (H12, K16). Although devoid of bioactivity itself, this substance was first identified as an antagonist of the stimulatory effects of a partially pure somatomedin preparation in both cartilage and fat-cell assays (H12). In contrast to the factors from rat serum and liver described above, it is acid stable, and has no effect on the stimulatory action of insulin, as measured in the fat-cell bioassay (H12), but competitively inhibits the actions of both IGF-I and IGF-II (K16). An acidic protein ($pI \approx 4.4$), its molecular mass appears to be only 16–18,000. Although no somatomedin binding protein or subunit with this molecular mass has been described, the inhibitor appears to interact directly with somatomedins, and has been purified by chromatography on a somatomedin affinity column (K16), raising the possibility that it is a binding protein subunit not previously described. Its exact relationship to the serum carrier proteins, and to the inhibitors extracted from rat serum and liver, remain important areas for investigation.

4. Receptors for Somatomedins

4.1. Molecular Structure

Like insulin and other hormones, the actions of SM-C/IGF-I and IGF-II on their target cells are thought to be initiated by binding to specific cell surface receptors. In view of the high degree of structural homology between the two peptides, it might be expected that their receptors would also show structural similarities. It is somewhat surprising, therefore, that major differences have been found between the two receptor types. A powerful tool in these structural studies has been the use of affinity labeling of recep-

tors by chemically cross-linking bound radioligands, followed by gel electrophoresis under reducing conditions. Locating the positions of labeled binding sites by autoradiography, Kasuga *et al.* (K3) identified a receptor for MSA (rat IGF-II) on rat liver plasma membranes and BRL 3A2 rat liver cells with an apparent molecular mass of 260,000 when reduced and 220,000 when nonreduced. In contrast, IGF-I binding was predominantly to sites of >300,000 molecular mass nonreduced and 130,000 when reduced. A more extensive study by Massague and Czech (M7), in which receptors in a variety of tissues were examined by affinity labeling, led to the definition of two distinct receptor types. Type-I receptors, with high affinity for IGF-I, are heterotetramers with a native molecular mass of 350,000 comprising two α- and two β-subunits of molecular masses 130,000 and 98,000, respectively. The larger appears to contain the peptide binding site, although weak affinity labeling is also seen on the smaller subunit. The subunits are thought to be linked by disulfide bridges in the order β-α-α-β. Quite a different structure is seen for type-II receptors, which have a high affinity for IGF-II: they appear by affinity labeling to consist of a single polypeptide chain of molecular mass 258,000 to 268,000 (M7). This has subsequently been confirmed in studies on type-II receptors purified to near homogeneity by affinity chromatography (O3, A9), although in one such study (A9) a 68,000-molecular-mass form was also identified.

A striking feature of the type-I growth factor receptor (B24, C6) is the close resemblance of its structure to that of the insulin receptor [see Massague and Czech (M7)]. To some extent this has been confirmed by immunochemical techniques, while at the same time some differences have emerged. Jonas *et al.* (J4) found that three of five sera containing autoantibodies against the human insulin receptor were also capable of precipitaing the SM-C/IGF-I receptor, whereas the other two showed little or no cross-reactivity. Similar findings were subsequently reported by Kasuga *et al.* (K4). In specificity studies of monoclonal antibodies raised against the insulin receptor, a range of cross-reactivities towards insulin and SM-C/IGF-I receptors has been reported, indicating both common and unique antigenic determinants on the two molecules (K17, R14). An important feature of the study of Kull *et al.* (K17) was the immunoprecipitation of receptors from solubilized surface-iodinated cell membrane preparations, allowing equal visualization by autoradiography of the α- and β-subunits regardless of the lack of hormone binding by the β-subunit. The α-subunit of the SM-C/IGF-I receptor from human placenta appeared to be slightly smaller than the corresponding subunit of the insulin receptor (K17), a finding confirmed by the recent study of Stuart *et al.* (S33), which indicated a mass difference of about 8000. It is not clear, however, whether the differences in mass and antigenicity between insulin and SM-C/IGF-I receptors reflect different

primary structures or merely differences in glycosylation or some other posttranslational modification. In support of the idea that the two receptors represent the same gene product, Massague *et al.* (M9) have described a mutant human fibroblast line in which the concentrations of both receptors are decreased in parallel, while IGF-II receptors are unaffected.

Another form of the insulin and SM-C/IGF-I receptors, precipitable by specific monoclonal antibodies, has been reported with a molecular mass of 180,000 (J1). It has been suggested that this may be a precursor form; this is supported by the demonstration that monensin, which inhibits posttranslational protein maturation, causes the accumulation of this larger single-chain receptor form at the expense of the α- and β-subunits (J1). These observations have led to speculation of a structural relationship between type-I and type-II receptors, the proposal being that type-II receptors might contain regions analogous to the α- and β-subunits of insulin and SM-C/IGF-I receptors, resistant to the proteolytic processing which results in the formation of the separate α- and β-chains (J1). Sequence studies on receptors of both types should reveal whether this relationship actually exists.

4.2. Distribution and Specificity

Over the past several years, a multiplicity of IGF receptor specificity studies has resulted in some confusion as to the specificity of binding of various radiolabeled ligands in different tissues. In retrospect, much of this confusion was due to the use of incompletely characterized ligands, a problem which is becoming less significant as the availability of purified peptides improves. One of the most widely studied sources of IGF receptors has been the placenta. When either labeled MSA or somatomedin-A is used as tracer for human placental membrane receptors, the unlabeled peptides are approximately equal in their displacing activity (R3). However, as shown in Fig. 2, with human IGF-II as tracer, IGF-II is some 8-fold more potent than SM-C/IGF-I, and insulin, though showing quite high cross-reactivity, only displaces about 60% of tracer, suggesting that IGF-II tracer binds partly to insulin-insensitive type-II receptors and partly to another, insulin-sensitive receptor type. Sheep placenta displays much more specific IGF-II binding, with only 1–2% cross-reaction by SM-C/IGF-I, and none by insulin (Fig. 2; see Section 5.4). Similarly, when IGF-II is used as tracer with rat placental membranes, only unlabeled IGF-II and MSA cause displacement, whereas SM-C/IGF-I is without effect (D5). The affinity of binding of IGF-II is approximately 0.3×10^9 liters/mol (D5).

In contrast, when SM-C (V3) or IGF-I (D5) preparations are used as tracer, the binding affinity is approximately 1×10^9 liters/mol (D5, J4), and somatomedin-A, IGF-II, and MSA all show relatively low potency compared

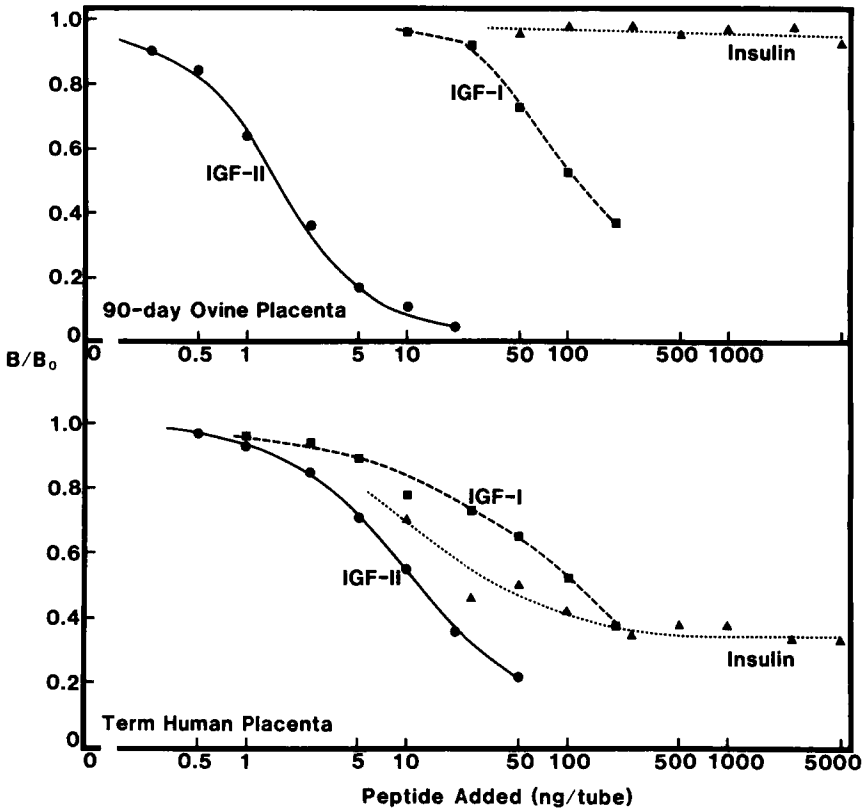

FIG. 2. Displacement of ^{125}I-labeled IGF-II (~50 pg, 200 Ci/g) from microsomal membranes isolated from 90-day ovine placenta (top) or term human placenta (bottom). Peptides tested were human IGF-I and IGF-II prepared in the author's laboratory, and porcine insulin. B_0 represents specific binding in the absence of added peptides: 25.9% of total tracer for ovine placenta (40 μg protein per tube) and 14.9% of total for human placenta (100 μg protein per tube). Incubations were for 2 hours at 20°C in 0.3 ml final volume of 30 mmol/liter sodium phosphate buffer pH 7.4 containing 0.25% bovine albumin.

with SM-C/IGF-I in causing displacement. These results are, in general, consistent with the existence of separate SM-C/IGF-I and MSA/IGF-II receptors in placenta, as has now been confirmed by cross-linking studies (Section 4.1); the relatively low activity of somatomedin-A at the type-I receptor is probably explained by the purity of the preparation used. One report apparently in conflict with this postulate describes MSA binding to human placental membranes which is displaced by IGF-I in preference to IGF-II (B36). It appears that the peptide termed MSA in this study, which was isolated from BRL 3A liver cell culture medium, is in fact an IGF-I

related peptide, and not the same as the MSA characterized as rat IGF-II by Marquardt *et al.* (M5).

Receptors for IGF peptides have also been described in a wide variety of other cells and tissue preparations, of which liver (K3, M13), adipocytes (Z1), chondrocytes (P4) and chondrosarcoma (A9), fibroblasts (A4), pituitary and neural tissues (G6, S7), erythrocytes (P12) and lymphocytes (R11), Sertoli cells (B31), and arterial smooth muscle (P3) represent a cross-section. In general it appears that the structurally defined (M7) type-I and type-II growth factor receptors fit satisfactorily into two functional classes. The type-I receptor has a high affinity for SM-C/IGF-I, a somewhat lower (but sometimes equal) affinity for IGF-II, and low affinity for insulin (0.1 to 1% of that for SM-C/IGF-I). The type-II receptor has a high affinity for IGF-II, low to moderate affinity for SM-C/IGF-I, and does not interact at all with insulin. A type-III receptor for IGF-II has recently been postulated to exist on IM-9 lymphocytes (H21), but whether this will eventually turn out to be a distinct structural class, or simply the result of interactions of IGF-II tracer with IGF-I, IGF-II and insulin receptors, remains to be established.

4.3. Receptor Ontogeny

An important approach to the question of the role of the IGF peptides in fetal and neonatal development is the study of the ontogeny of their receptors. The earliest study of this type was reported by D'Ercole *et al.* (D15), who examined SM-C/IGF-I receptors in membranes from fetal pig organs of a variety of gestational ages. Binding sites in fetal placenta increased in abundance and affinity as gestation progressed, whereas no change was seen in liver, kidney, heart, or maternal placenta. It was also noted that binding was higher in fetal lung membranes than in those from adult lungs. These results suggested involvement of SM-C/IGF-I in fetal development, perhaps with a special role in lung growth. In a more recent study of SM-C/IGF-I receptors in human placental membranes, no difference was seen in binding to membranes from placentas between 6 and 40 weeks gestational age, and affinity-labeling studies indicated no structural changes over this period (G11). Studies of ILAs binding (presumably to IGF-II receptors) in human placenta showed a different pattern, with higher binding being seen in early gestation (11–19 weeks) placentas than in term (38–42 week) placentas (D11), perhaps indicating a role for IGF-II in early placental development. The ontogeny of IGF receptors in human liver and brain has been studied by Sara *et al.* (S6) using a variety of tracer peptides of both the IGF-I and the IGF-II type. IGF-II receptors on liver membranes showed no change in fetuses over a range of gestational ages, an observation also made in the author's laboratory (B7). Figure 3 illustrates that, in liver membranes

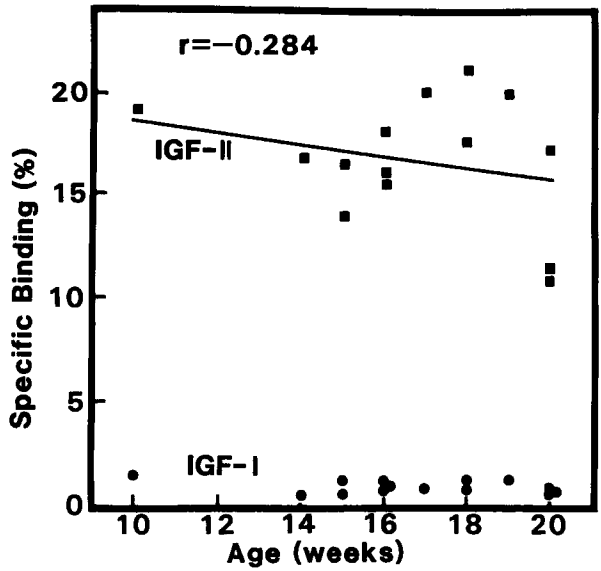

FIG. 3. Specific binding of ^{125}I-labeled IGF-II (~25 pg, 200 Ci/g) and IGF-I (~40 pg, 120 Ci/g) to microsomal membranes (100 μg membrane per tube) isolated from liver samples from human fetuses of between 10 and 20 weeks gestational age. The effect of age on binding was not significant for either tracer. Assay conditions were as described under Fig. 2.

from fetuses aged between 10 and 20 weeks of gestation, IGF-II binding showed an insignificant decrease with increasing age, and IGF-I binding remained at a barely detectable level. In the brain, IGF-II binding seemed to be favored early in gestation, while a high concentration, high affinity IGF-I receptor appeared later (S6). Although it remains to be proven that such receptor changes truly reflect changes in growth factor action throughout fetal development, the majority of these studies would appear to favor a role for IGF-II early in fetal life.

4.4. Receptor Regulation

The concentration and affinity of somatomedin receptors on intact cells and isolated membranes are subject to modulation by a variety of factors. In common with many other peptide hormone receptors, SM-C/IGF-I receptors on cultured IM-9 lymphocytes are down-regulated by exposure of the cells to SM-C/IGF-I. Insulin and other related peptides are also capable of causing receptor loss, with a potency proportional to their ability to bind to the SM-C/IGF-I receptor (R11). In contrast, binding sites for MSA tracer (i.e., type-II sites) on chondrosarcoma chondrocytes are reported to be un-

affected by exposure of the cells to either MSA or insulin, although insulin actually induces its own receptor in these cells (S30).

The apparent lack of effect of insulin on MSA receptors is surprising in view of its profound effect on IGF-II receptors in rat adipocytes, H-35 hepatoma cells, and mouse pancreatic acini. Several groups (K9, M8, O4, P15, S18) have described increases in IGF-II binding to these cell types following incubation with insulin. Studied most closely in adipocytes, the effect was initially ascribed to an affinity increase with no change in receptor number (O4). Despite the apparent constancy of receptor number seen in intact cells, receptor measurement in isolated membrane fractions indicated an insulin-induced redistribution of IGF-II receptors from low-density microsomal to plasma membranes (O4), analogous to that reported for the glucose transport protein (C23). Very recently, anti-IGF-II receptor antibodies have been used to confirm that insulin does increase the number of type II sites on the adipocyte cell surface (O2), and, by blocking receptor endocytosis with cyanide, it has been possible to show that these receptors come from a microsomal pool calculated to contain 82% of the total cell IGF-II receptors in the absence of insulin, and 68% after insulin stimulation (W2).

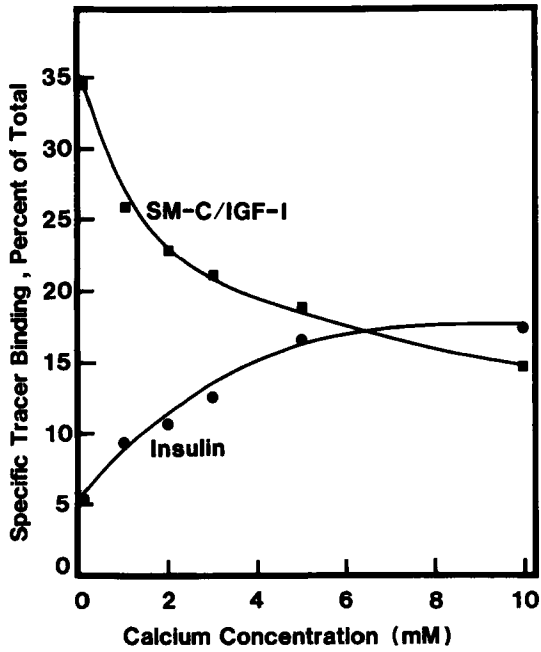

FIG. 4. Effect of increasing calcium concentrations on the binding of ^{125}I-labeled IGF-I or insulin to human placental microsomal membranes (60 μg protein). [From Baxter and Williams (B9) with permission.]

In pancreatic acini, the addition of the calcium ionophore A23187 has been shown to decrease the binding of IGF-II (P15), implicating calcium movements in the modulation of type-II receptor expression at the cell surface. Calcium also appears to have a role in regulating the SM-C/IGF-I receptor. Addition of calcium to human placental microsomal membranes causes a marked decrease in IGF-I binding, while increasing insulin binding (Fig. 4). The reduction of IGF-I binding is due to a decrease in the affinity of high-affinity sites, whereas insulin sites are increased in affinity by calcium (B9). The opposite effects of calcium on the two receptors provides another example of functional differences between these two structurally similar molecules, and suggests that calcium might be involved in regulating cell responses to insulin and the growth factors.

5. Analytical Methods

5.1. Units of Concentration

Reflecting the diverse origins of somatomedin research, three main concentration or potency units are currently in use to express results of somatomedin assays. In laboratories using cartilage bioassays, potency has been expressed in Units per milliliter, where 1 U was defined as the somatomedin activity in 1 ml of a pool of plasma from healthy humans. As these laboratories, and others, developed radioligand assays, this traditional unit was often retained, generally because pure peptides were not available for routine use as assay standards. Standardized in this way, a healthy population will have a mean value of about 1 U/ml, although the exact value will depend upon the plasma pool used for calibration. For example, in the SM-C/IGF-I RIA of Furlanetto *et al.* (F9), the mean ±1 SD for normal subjects was about 1.5 ± 0.5 U/ml. Since the specific activity of pure SM-C/IGF-I, measured under the same conditions, is about 10,000 U/mg (S34), this range represents about 150 ± 50 ng/ml.

In laboratories which use an NSILA bioassay, however, somatomedin activity has been expressed in units of insulinlike activity, generally μU/ml, a convention which has to some extent continued as radioligand assays were introduced. Expressed in these units, normal human samples have a mean value of 350 ± 66 μU/ml by competitive protein-binding assay (Z4). In the same laboratory, a mean normal value (±SD) of 148 ± 45 ng/ml was determined by IGF-I RIA (Z5). The close correspondence between reference values obtained by RIA in different laboratories, when expressed as ng/ml of SM-C or IGF-I, suggests that mass units, if uniformly adopted, would allow better comparison of results. With the increasing availability of highly pu-

rified peptides, expression of assay results in these units should rapidly become more widespread.

5.2. Bioassays

Any biological system in which a response to the addition of somatomedins can be demonstrated has the potential to be used as a bioassay. As the number of such systems increases, the potential for new bioassays is expanding constantly. However, as RIA methods for both SM-C/IGF-I and IGF-II become more readily available, most of these bioassay systems will not be put into routine use. In practice, the systems commonly used fall into three classes: (1) assays for cartilage-stimulating activity, including "sulfation factor" activity and stimulation of thymidine incorporation into cartilage (D6, G1, H1, V4), (2) assays for insulinlike activity (NSILA), generally using fat pads or isolated adipocytes (S16, F5), and (3) assays for mitogenic activity (MSA), commonly in chick embryo or human skin fibroblasts (P10, N3). Relative to radioligand assays, these bioassays may be difficult and time-consuming to perform, although their value in somatomedin research cannot be overemphasized. Interpretation of bioassay results may be complicated by the presence of inhibitors known to circulate in a variety of conditions (see Section 3.5). These problems, and the relatively high detection limit of bioassays, have led to the development of RIA and other radioligand methods.

5.3. Protein Binding Assays

As discussed in Section 3, the somatomedins circulate in the blood bound to carrier proteins. These have been used, after acid treatment to remove endogenous ligands, as a source of binding sites for competitive protein binding assays for somatomedin (S10, Z4). The acid treatment consists of chromatography of serum in dilute acetic acid on gel permeation columns. In different laboratories, binding activity is reported to emerge between 60 and 80% (Z10) or between 29 and 46% (S4) of the bed volume on Sephadex G-200. To separate somatomedin from endogenous binding protein in samples for assay, each specimen is passed through a Sephadex G-50 column under acid conditions; fractions emerging between about 50 and 80% of the bed volume are then pooled and assayed. Incubations are for 60–90 minutes at room temperature, after which bound and free ligand are separated using charcoal.

Using binding protein from human serum, this technique predominantly measures IGF-II. Tracer IGF-II is reported to show three times higher specific binding than tracer SM-C/IGF-I, and unlabeled IGF-II is more

potent in displacing either tracer from binding sites than SM-C/IGF-I (Z1). Similarly, in a binding system using carrier protein from rat serum, and labeled MSA as tracer, human IGF-II is more than ten times as potent as SM-C/IGF-I (R4). As expected for assays with relative specificity for IGF-II (see Section 7), the activity measured by this method shows relatively poor dependence on GH status (Z4). However, Binoux *et al.* (B26) have described a competitive binding assay utilizing a binding protein released by rat liver explants in organ culture, which preferentially measures SM-C/IGF-I although with 50% IGF-II cross-reactivity. This assay appears to reflect GH status well. With this exception, the protein binding assay is comparable in performance to the rat fat-pad NSILA bioassay (Z4).

5.4. Radioreceptor Assays

These assays take advantage of the fact that membrane preparations from a number of sources are rich in somatomedin receptors (see Section 4). Most commonly used is a crude microsomal preparation from human placenta, similar to that originally described by Cuatrecasas (C22) as a source of insulin receptors. Purified plasma membranes from rat liver, isolated by the technique of Neville (N2), have also been used. Marshall *et al.* (M6) first described the use of the human placental membrane RRA, which has subsequently been used in a number of laboratories (B12, B35, D4, G12, H24, T3). A typical assay protocol involves incubating 100–250 μg of membrane protein with tracer and standards or samples for 16 hours at 2°C, then separating the bound and free ligand by centrifugation. As discussed in Section 4.2, the specificity of this assay is determined by the peptide used as tracer, but, with either SM-C/IGF-I or IGF-II tracer, there is some cross-reactivity with the other peptide. In contrast, when rat placental membrane is used with IGF-II tracer, a high specificity for IGF-II has been reported (D7). In the author's laboratory, an IGF-II RRA has been established using ovine placental membranes, which appear to possess a typical type-II receptor (see Section 4.2). As illustrated in Fig. 2, the specificity of this assay for IGF-II compares very favorably with that of the human placental IGF-II RRA, and the sensitivity (half-maximal displacement by 1–2 ng of IGF-II per tube) is somewhat better than that reported for the rat placental membrane assay (D5).

Use of the rat liver plasma membrane RRA was introduced by Megyesi *et al.* (M11, M12). Although these membranes possess receptors for both IGF-I and IGF-II related peptides (K3), IGF-II receptors predominate (M7). The tracer used in the original studies (M11) was an iodinated preparation of NSILAs, a mixture containing both IGF-I and IGF-II (Z5), which might be expected to bind to both receptor types. Specificity studies with this tracer

showed strong competition for binding by unlabeled NSILAs and MSA, with somatomedin-A lower in potency and SM-C almost without effect (M13). This implies that the assay prefentially measured IGF-II related peptides.

5.5. Radioimmunoassays

The earliest somatomedin antiserum reported was that of Reber and Liske (R2), for which the antigen was a preparation of NSILAs judged to be more than 90% pure. A RIA using this antiserum, with radioiodinated NSILAs as tracer, was sensitive to about 30 pg of NSILAs per tube. The assay gave much lower values for specimens from healthy humans than those obtained by other methods (see Section 6), and it was unclear which peptides were being measured, as specificity data were not presented.

Furlanetto *et al.* (F9) described the first of several more completely characterized RIA methods. Using a specific antiserum against human SM-C/IGF-I, the assay shows half-maximal displacement of tracer by about 0.1 ng of SM-C/IGF-I, with somatomedin-A (presumably an impure preparation) less than 5% as reactive, and even lower cross-reactions by IGF-II and MSA (V3). This RIA has been used in many laboratories, as antiserum is distributed in the United States by the National Pituitary Agency, Baltimore, Maryland. Several other SM-C/IGF-I antisera (B2, B14, Z3) appear to resemble the Furlanetto antiserum in specificity, and, when compared directly, give similar analytical results (B14). In an innovative approach to the problem of maximizing specificity, two antisera have been raised against synthetic part-sequences of the SM-C/IGF-I molecule. The sequences selected represent the carboxy-terminal octapeptide (H17) and the 12 amino acid C-peptide region (H19), the two regions of greatest difference between IGF-I and IGF-II, and between the IGF peptides and proinsulin. Competitive binding studies indicate the unique specificities of these antisera. The one raised against the carboxy-terminal region, when used with intact SM-C/IGF-I tracer, has very low cross-reactivity by MSA and IGF-II (H17), while the C-peptide antibody shows no IGF-II cross-reactivity even at 1000 ng/ml (H19). Although both antibodies have rather low sensitivity for SM-C/IGF-I when compared to antisera raised against the intact molecule, with 50% displacement occurring at about 100 ng/ml, the C-peptide antibody has proved suitable for routine analytical use (H18).

Hall *et al.* (H3) have described a sensitive somatomedin-A RIA using an antiserum raised in a hen. Antibodies are bound to agarose beads, simplifying the separation of bound and free tracer. SM-C/IGF-I is actually 10 times more potent in this assay than the somatomedin-A preparation used, which was perhaps of lower potency than that most recently described (E3). IGF-II is only 10% as active as somatomedin-A, and MSA shows no cross-reactivity.

Half-maximal displacement of tracer is seen with about 1 ng/ml of somatomedin-A. Antisera have also been raised against IGF-II and MSA. In the IGF-II RIA described by Zapf *et al.* (Z5), IGF-I, somatomedin-A, and MSA (presumably not the same peptide characterized as rat IGF-II) have activities between 0.2 and 2% of that of IGF-II. In a more recent publication (Z3), SM-C/IGF-I and somatomedin-A cross-reactivities are stated to be about 10%. Hintz and Liu (H16) have also raised a highly specific IGF-II antiserum using synthetic IGF-II C-peptide octapeptide as antigen, and a MSA RIA has been described (M25) in which various MSA subfractions cause 50% displacement of tracer at concentrations between 1 and 10 ng/tube, while IGF-I, IGF-II and somatomedin-A show weak, nonparallel cross-reactivity.

Two laboratories have reported monoclonal antibodies suitable for somatomedin RIA. The SM-C/IGF-I antibody of Baxter *et al.* (B11) is useful for RIA in ascites fluid at 200,000-fold dilution. Its relatively high affinity for SM-C/IGF-I, 1.7×10^{10} liter/mol, allows detection of the peptide down to 0.05 ng/tube, with 50% displacement of tracer at 0.7 ng/tube. Assays using this antibody are in use in a number of laboratories, and have given results which correlate closely with those obtained using specific polyclonal antisera. A similar antibody has also been described by Laubli *et al.* (L3). Although it has a somewhat lower affinity, it still appears suitable for RIA. An interesting feature of both monoclonal antibodies is their particular sensitivity towards immunoreactive substances in guinea pig serum, which appears to have 5–7 times the potency of human serum, and their lack of cross-reactivity with rat and mouse somatomedins (B11, L3), a feature not seen in any rabbit antiserum raised against intact SM-C/IGF-I, although surprisingly similar to the IGF-I C-peptide and carboxy-terminal antisera (W7).

5.6. Sample Extraction and the Effect of Binding Protein

There are two theoretical reasons why somatomedin binding proteins should be removed from serum or plasma samples before somatomedin determination by RRA or RIA. First, when attached to larger proteins, the peptides might not be fully accessible to receptor or antibody binding sites, and second, unsaturated binding proteins might interfere in the assay by removing somatomedin tracer from the reaction mixture. In practice, opinions differ as to the necessity of treating samples before assay to remove carrier proteins. Placental membrane receptor assays using somatomedin-A (T3), SM-C/IGF-I (M6), and MSA (B35) as tracers have been performed on unextracted serum samples. In other laboratories, assays using ILAs (G12) or SM-C/IGF-I (D4, H24) as tracer have been performed on plasma extracts

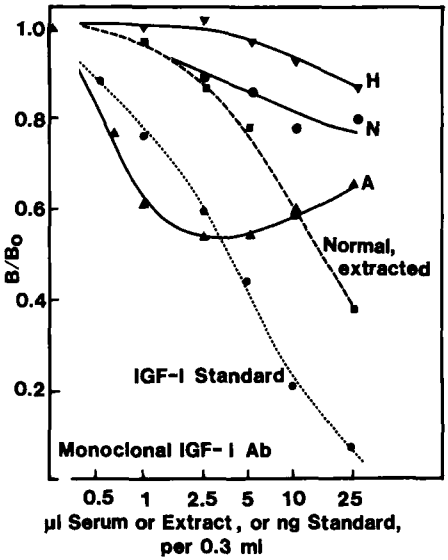

FIG. 5. Displacement of ^{125}I-labeled IGF-I from monoclonal IGF antibody by standard IGF-I (20% pure), unextracted serum samples from hyposomatotropic (H), normal (N), and acromegalic (A) subjects, and acid–ethanol extracted normal serum. After extraction, all samples show parallelism with standard. For assay details see Baxter *et al.* (B11).

prepared either by gel chromatography of each sample at low pH (H24) or by a simple acid–ethanol extraction technique (D4). In a study comparing the use of somatomedins-A and C as tracers for RRA, Horner *et al.* (H24) concluded that sample extraction was unnecessary when somatomedin-A tracer was used, but necessary to achieve meaningful results in assays using SM-C tracer. Given the supposed identity of these peptides (E3), the significance of this observation is not clear.

In radioimmunoassays, the presence of binding proteins may also cause problems. In an extreme case, such as with the monoclonal antibody of Baxter *et al.* (B11), unextracted samples are almost devoid of activity, even when containing high levels of antigen, and extraction is required before the antibody is able to detect the peptide (Fig. 5). This suggests that the antigenic determinant on the SM-C/IGF-I molecule is close to the protein-binding region. Other antibodies appear to tolerate the presence of binding protein, although a nonequilibrium incubation protocol, in which samples and standards are incubated with antibody for several days before addition of tracer, may be required to ensure parallelism between unextracted serum samples and purified standards (F9). It has been demonstrated, however, that this method may allow the detection of as little as 20% of the total SM-C/IGF-I in human serum (C5), and that the proportion detected may vary

depending upon the GH status of the patient (D4). Furthermore, the use of glass or polystyrene tubes, the presence of heparin, and the length of time the sample has stood at neutral or low pH, all influence the ability of the antibody to detect antigen in the presence of binding protein (B2, C5, F8).

For these reasons, many investigators choose to subject all samples for analysis to a routine separation procedure, generally either gel chromatography at low pH (Z3) or acid–ethanol extraction (B14, D4). Due to the ease of treating many samples at once, the latter procedure appears to be the method of choice. It should be noted, however, that it has been validated only for human serum, and may not be fully effective on other sample types, or in different species. For example, in the author's laboratory acid-ethanol extraction has been found not to remove all binding activity from human seminal plasma (B17) or from conditioned hepatocyte culture media (S22). In the latter case, the problem was overcome by adapting the acid gel chromatography technique to a high-pressure liquid chromatograph with automated sample injection. This method, which allows the processing of up to 60 samples per day with complete separation and quantitative recovery of binding protein and SM-C/IGF-I (S22), appears a suitable alternative to acid–ethanol extraction whenever that method proves unsatisfactory.

6. Circulating Levels Throughout Life

6.1. Levels in Healthy Adults

Since the concentration of SM-C/IGF-I has traditionally been expressed in U/ml, with 1 U/ml arbitrarily defined as the activity in a pool of normal adult plasma, it has been difficult to compare in absolute terms the values obtained in different laboratories. As purified peptides have become available, it is now possible to express both SM-C/IGF-I and IGF-II in mass units, generally ng/ml. In a number of laboratories which report in mass units or provide conversion factors between U/ml and ng/ml, the level of SM-C/IGF-I in apparently healthy adults is generally about 200–300 ng/ml, although some laboratories have reported different mean values in different studies. Some typical mean values and ranges (either presented as published or calculated from data provided) are shown in Table 2. In recent years reference ranges for IGF-II have also become established. As seen in Table 2, mean values are 2 to 3 times higher than for SM-C/IGF-I.

It is not possible to state with confidence the levels of somatomedins in the circulation of other species, as purified peptides are not yet available to use as standards. Thus, values can only be expressed in terms of the human peptides, and are entirely dependent upon the cross-reactivities of the assay

TABLE 2
ADULT REFERENCE VALUES OF SM-C/IGF-I AND IGF-II IN THE CIRCULATION

Peptide	Mean value (ng/ml)	Range or ±2 SD (ng/ml)	Reference
SM-C/IGF-I	193	77–309	Z3
	302	66–538	M17
	231[a]	134–449	H18
	360[b]	200–625	B2
	243[b]		B4
	232[c]	149–406	B14
IGF-II	647	395–899	Z3
	687	349–1025	H16
	571	351–928	H6
	580[d]	316–893	

[a] Converted assuming 1 U = 220 ng (H18).
[b] Converted assuming 1 U = 250 ng (B2).
[c] Converted assuming 1 U = 240 ng (author's data).
[d] Values obtained on acid–ethanol extracted samples from 100 healthy adults using the ovine placental membrane RRA established in the author's laboratory (see Section 5.4 and Fig. 2).

methods employed. For example, in a typical RIA using a SM-C/IGF-I antiserum to measure activity in acidified sera (B2), rat serum appears to have about 5 times the potency of human serum (that is, equivalent to about 1200 ng/ml of human SM-C/IGF-I), and rabbit serum about twice the potency (equivalent to 500 ng/ml). In contrast, measurements on acid–ethanol extracts using a monoclonal SM-C/IGF-I antibody (B11) suggest that rabbit serum has only 30% of the activity of human serum (equivalent to 75 ng/ml of human SM-C/IGF-I), and rat serum less than 10% of the human activity (i.e., <25 ng/ml). Furthermore, there may be major strain differences within some species; thus, SM-C/IGF-I levels in dogs, expressed in human equivalents, range from less than 40 ng/ml in spaniels to 280 ng/ml in German shepherds (E1). For these reasons, measurements in nonhuman species are not at present meaningful in absolute terms, and are perhaps best expressed in U/ml relative to a normal pool for each species (or strain) studied.

6.2. DIURNAL VARIATION

Studies in chronically cannulated rats in which blood was sampled every 15 minutes for 6 hours, have indicated significant pulsatility of immunoreac-

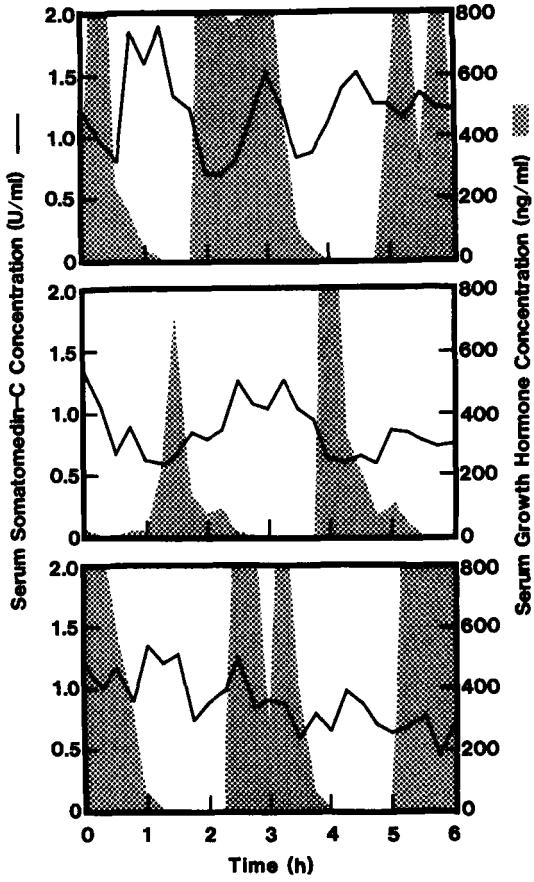

FIG. 6. Serum profiles of SM-C/IGF-I (lines) and growth hormone (shaded) in 3 rats sampled at 15 minute intervals over 6 hours. The mean peak value (±SD) for 10 animals was 1.21 ± 0.29 U/ml, and the mean trough value was 0.60 ± 0.20 U/ml, with a mean peak interval of 1.93 ± 0.47 hours. [From Baxter *et al.* (B19) with permission.]

tive SM-C/IGF-I, with a mean period of approximately 2 hours (B19). Typical GH and SM-C/IGF-I profiles in three rats are shown in Fig. 6. Integrated values over the test period showed a significant correlation with the GH secretory bursts occurring over the same time ($p = 0.0007$). Results in man have not clearly demonstrated a similar degree of pulsatility. Minuto *et al.* (M19) reported essentially stable values during waking hours, decreasing by 25% after the onset of sleep, and rising again in the morning. This may be compared with an earlier study using RRA, in which "a slight increase" was observed early in the morning (T1), and a study using a cartilage bioassay in which low values were seen between about midnight and 9 AM (P16). These

fluctuations were not confirmed by Horner *et al.* (H23), who found stable values over 24 hours. It is important to note, however, that in all the human studies, the relatively infrequent sampling times would have precluded the detection of pulsatility of the type observed in the rat (B19). Therefore the question of SM-C pulses in man is not yet resolved.

6.3. Effect of Age in Adults

Although the previous discussion of healthy adult reference values for somatomedins suggests that average levels remain constant throughout adulthood, this is not in fact the case. Determined by RRA, the serum somatomedin-A levels in adults were found to decrease slightly after the age of 40 (T1). Data for control subjects shown in Fig. 9 (Section 8) indicate that serum immunoreactive SM-C/IGF-I levels in healthy adults decline steadily with increasing age. By analysis of variance, the effect of age is highly significant ($p < 0.001$), and it is clear that a value of 0.4 U/ml, though very low for a young adult, would not be abnormal in a 60 year old. Other studies using SM-C/IGF-I RIA methods (B21, C10, H18) have shown similarly declining values with advancing years. Age appears to have the opposite effect on IGF-II levels. A group of 25 adults aged over 70 had a mean value by RRA 44% higher than the mean for a group aged 20–69 years (D7), although this increase was not confirmed by IGF-II RIA (B21).

6.4. Fetal and Childhood Levels

The observations that fetal tissues in culture produce somatomedins (Section 2.1), and that receptors for somatomedins (especially IGF-II) are abundant in membranes of fetal tissues (Section 4.3), both suggest that somatomedins may play a role in fetal development. This is supported by measurements of the peptides in the fetal circulation. While some early studies in this area are inconclusive due to the broad cross-reactivities of the assay methods employed, specific determination of SM-C/IGF-I and IGF-II levels has been possible in recent studies, although much of the fetal data is derived from nonhuman species.

In rat fetuses, the SM-C/IGF-I level at day 21 of gestation (i.e., just prior to birth) was 5 times higher than at day 18 postpartum, though not above the usual adult level (D8). IGF-II immediately prior to birth was almost four times higher than the adult level, and rose to over seven times the adult level five days later, only falling some three weeks postpartum (D8). Somewhat different patterns have been reported in lambs. Whereas SM-C/IGF-I levels rose from 30% of the adult value at 50–80 days of gestation to 80% of the adult value at term (approximately 150 days), they did not fall on delivery

as in the rat, but continued to rise to 2.5 times the adult level 3–7 days postpartum (G2). IGF-II levels remained almost 3-fold higher than the adult value between 50 and 140 days gestation, declining to the adult value in the week before delivery (G2).

In human umbilical cord blood, SM-C/IGF-I levels are approximately half of the adult values, and correlate with both birth weight and gestational age (B22, G3). However, there is disagreement about cord blood IGF-II levels: Bennett *et al.* (B22) have reported lower than adult levels, significantly correlating with birth weight and gestational age, while Gluckman *et al.* (G3) found levels similar to those in the adult, independent of weight or age. Thus while pre- and perinatal changes in SM-C/IGF-I and IGF-II in nonprimates suggest involvement with fetal or neonatal growth, it is not yet possible to reach a firm conclusion in humans, particularly with regard to IGF-II.

It is now well established that, in the human neonate, immunoreactive SM-C/IGF-I levels are extremely low, frequently near the limit of detection of the assays used (B4, K2). The values rise through childhood, remaining slightly higher in females than in males (B4), until by about 10 years of age, they are in the range of adult values. In contrast to the marked age dependence of SM-C/IGF-I, circulating levels of IGF-II, though reported to be low in the first year of life, are not significantly different from adult values after 1 year of age (L6, Z3). The very low SM-C/IGF-I values of early childhood are a serious impediment to the use of the SM-C/IGF-I RIA for diagnosis of hyposomatotropism in young children since, up to about 4 years of age, values in healthy subjects may be indistinguishable from those seen in GH deficiency.

6.5. Puberty and the Effect of Sex Steroids

In the original SM-C RIA studies of Furlanetto *et al.* (F9) and D'Ercole *et al.* (D17), values in the midpubertal age group did not appear higher than those seen in adults. In other laboratories, however, a marked rise in SM-C/IGF-I in puberty has been demonstrated, to levels 2 to 3 times those seen in adults (B4, H5, L6); such levels would, in postpubertal subjects, be found only in acromegaly (see Section 7). Interestingly, no such rise in IGF-II has been reported (L6, Z3). The rise in SM-C/IGF-I appears to occur 1 to 2 years earlier in females than in males (B4, H5), and may be related to the pubertal increase in sex steroids, as correlations with both estrogen and androgen levels have been found (R13). Until the age of peak pubertal growth velocity, there is a significant association between linear growth velocity and SM-C/IGF-I levels; for several years after this time, however, while growth velocity decreases, SM-C/IGF-I levels remain above adult values (R13). Treatment of hypogonadotropic patients with gonadotropin releasing hormone (GnRH) also causes a sharp rise in SM-C/IGF-I.

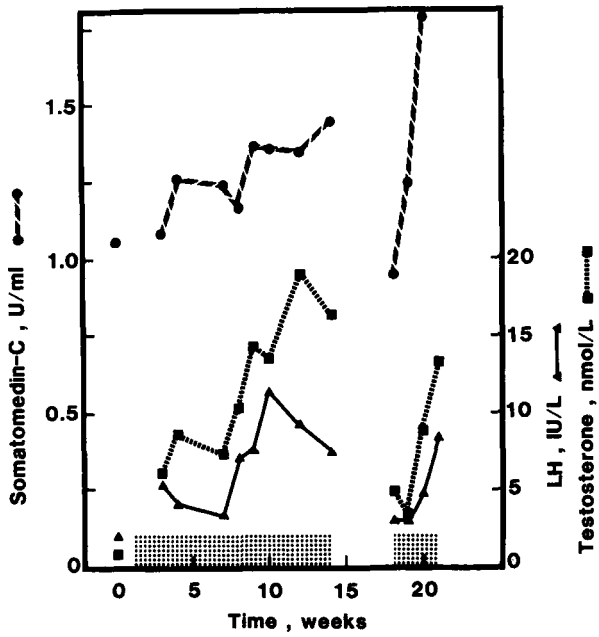

FIG. 7. Immunoreactive SM-C/IGF-I, LH, and testosterone profiles in a 22-year-old male with Kallman's syndrome, receiving pulsatile GnRH therapy, 5 μg sc at 90-minute intervals. Shaded bars indicate periods of continuous treatment. Note the almost 2-fold rise in SM-C/IGF-I between weeks 18 and 21. (Data of D. J. Handelsman and R. C. Baxter.)

Figure 7 illustrates LH, testosterone, and SM-C/IGF-I profiles in an adult male with hypogonadotropic hypogonadism, treated by pulsatile administration of GnRH. In the initial 14-week period, a rise in SM-C/IGF-I from the middle to the upper part of the reference range was seen, concomitantly with increases in testosterone and gonadotropins. Following a 4-week period without GnRH, during which a rapid fall in SM-C/IGF-I was seen, reinitiation of treatment caused a sharp rise in SM-C/IGF-I to values in the pubertal range.

The observation that testosterone treatment of boys with delayed puberty causes a marked increment in SM-C/IGF-I levels in subjects with normal GH secretion, but is without effect in GH-deficient boys whether or not they receive exogenous GH, has been interpreted to indicate that the effect of sex steroids may be at the level of pituitary GH secretion (P1). While it is true that GH secretion has been reported to increase in puberty (F1, M18), in one study (M18) the increase was not seen in girls; girls do, however, have a pubertal rise in SM-C/IGF-I. In other studies, overall GH secretion did not increase with developing puberty (S29, T11), implying that there must be another explanation for the rapid rise in SM-C/IGF-I. This is supported by

the observation in castrate female baboons that estradiol administration to achieve serum levels similar to those in healthy adult females, causes a 2-fold increase in SM-C/IGF-I levels without any effect on GH (C20). The balance of evidence, therefore, is against the pubertal rise in somatomedin being due simply to increased GH secretion. It has also been reported that sex steroids can stimulate long bone growth without an increase in circulating somatomedin levels: infusion of either testosterone or dihydrotestosterone in boys doubled ulnar growth velocity without significantly elevating SM-C/IGF-I (C2). The possibility cannot be excluded, however, that the steroids caused local increases in growth factors which were not reflected in peripheral levels.

6.6. Pregnancy

Concomitantly with many other hormonal changes, circulating levels of somatomedins are affected by pregnancy. In either cross-sectional (W8) or longitudinal (H6) studies, a modest increase in SM-C/IGF-I levels has been reported in the first trimester of pregnancy compared with nonpregnant controls, whereas Furlanetto *et al.* (F10) found a significant decrease over this period. A more clear-cut effect can be seen in the third trimester, where reported group mean values of 320 to 380 ng/ml (H6, W8) or 2.2 U/ml (F10) are 50–130% higher than for corresponding control groups. At term, the levels appear to remain 80% higher than in nonpregnant women (B4), but a significant drop to nonpregnant values is seen postpartum (W8). IGF-II does not show as clear a relationship to gestational stage as does IGF-I, although Wilson (W8) reported slightly elevated levels in the third trimester falling rapidly after delivery to values below the reference range, changes not seen in the study of Hall *et al.* (6).

The elevated somatomedin levels of the third trimester do not appear to depend upon the continued presence of GH. Daughaday and Kapadia (D2) demonstrated that hypophysectomy of pregnant rats did not significantly reduce their serum somatomedin bioactivity until after delivery, when somatomedin levels fell rapidly to the range expected in hypophysectomized animals. It was postulated from this study that placental lactogen secretion by the fetal-placental unit was capable of maintaining somatomedin levels in the absence of GH. In a later report of pregnancy in a GH-deficient dwarf (M15), IGF-I and IGF-II concentrations were found to be in the normal range in the thirty-fifth week, although both had been at levels typical of hypopituitarism (see Section 7) prior to pregnancy. Within 48 hours postpartum, both peptides were again in the range seen in hypopituitarism. Hall *et al.* (H6) have recently confirmed these findings in three GH-deficient women. In view of the significant correlation demonstrated between the rising

levels of placental lactogen and SM-C/IGF-I during pregnancy in healthy women (F10), and the demonstration in hypophysectomized rats that placental lactogen increases SM-C/IGF-I in the circulation (H27), it seems likely that the greatly elevated placental lactogen levels of late pregnancy can stimulate somatomedin generation in states of GH deficiency.

7. Circulating Levels in Disorders of Growth

7.1. Growth Hormone Deficiency

Because dependence upon circulating GH levels is one of the definitive characteristics of the somatomedins (see Section 1.2), new assays have generally been characterized by their ability to distinguish somatomedin levels in patients with GH disorders from those in healthy subjects. Several studies have compared values in GH-deficient children with those in healthy adults and, while an assay method that could not make this distinction would be invalid, dependence upon GH cannot be established by such comparisons, since somatomedin levels (particularly SM-C/IGF-I) in children without GH deficiency are lower than adult values.

It is, however, well established that after about 5 years of age for SM-C/IGF-I and 1 year of age for IGF-II (the ages below which very low levels are seen in healthy individuals), GH deficiency is associated with reduced levels of both peptides. This is demonstrated most easily by RIA methods, which clearly separate GH-deficient from healthy subjects. By SM-C/IGF-I RIA, the mean value in hyposomatotropism is 15–25% of that in healthy subjects (B14, D12, Z5), while the corresponding figure for somatomedin-A RIA is 21% (H3). In GH-deficient children, the degree of growth retardation, expressed as the ratio of bone age to chronological age, is significantly correlated with the serum SM-C/IGF-I level (S11). The effect of GH deficiency on IGF-II levels is not as severe: mean values of 39 (Z3) and 34% (H16) of the normal adult mean have been reported, the latter figure being for children. In the relatively specific IGF-II RRA of Daughaday *et al.* (D7), the average value in GH deficient children was 68% of that seen in healthy subjects.

In studies directly comparing different assay methods, RIA methods invariably show somewhat better discrimination than do other radioligand methods or bioassays. In one such study (B14), 5 out of 13 samples from hyposomatotropic subjects, all of which were clearly low by SM-C/IGF-I RIA, were not distinguishable from samples from healthy subjects by placental membrane RRA using SM-C/IGF-I tracer. The rather poor correlation (r=0.836) between RIA and RRA results for 69 healthy, GH-deficient, or

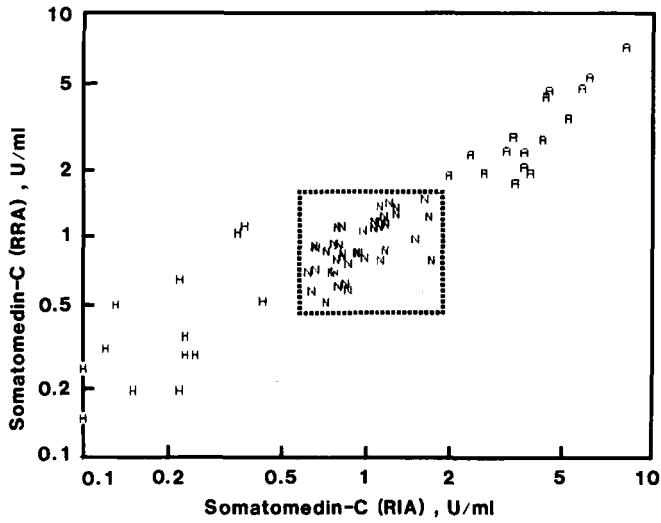

FIG. 8. Comparison of SM-C/IGF-I levels measured by placental membrane RRA or RIA (B14) in 13 hyposomatotropic (H), 40 normal (N), and 16 acromegalic (A) subjects. The dotted lines enclose values for healthy subjects measured by both assays. Note the values in the normal range for some GH-deficients subjects, when measured by RRA.

acromegalic subjects is shown in Fig. 8. The comparable correlation coefficient between two RIA methods was 0.988. Similarly, in comparisons of somatomedin-A RIA and RRA methods (H3), GH deficiency resulted in a lower value in most samples by RIA than RRA. An extensive study by Zapf *et al.* (Z5) showed that in a group of hypopituitary dwarfs with a mean immunoreactive SM-C/IGF-I value 15% of normal, activities determined by two bioassays for insulinlike activity and by a protein-binding assay were 40–50% of normal. While it is clear that reactivity towards IGF-II in methods other than SM-C/IGF-I immunoassays will inevitably give higher values in GH deficiency, the reason for some of these patients having RRA values in the range expected for healthy subjects is not understood.

Treatment of GH-deficient subjects with human GH (hGH) generally causes a prompt rise in immunoreactive SM-C/IGF-I levels, detectable within a few hours and reaching a peak value approximately 24 hours after administration of the hormone (C19). Similar to SM-C/IGF-I in the serum of healthy subjects, SM-C/IGF-I induced by acute hGH treatment is found mainly in the 150,000-molecular-mass fraction in serum (C19). Rosenfeld *et al.* (R12) have reported that the rise in serum SM-C/IGF-I in GH-deficient children measured over the first 5 days of hGH treatment is highly predictive of the growth factor level determined after 6 months of regular hGH administration. However, there is disagreement as to the relationship be-

tween serum SM-C/IGF-I levels after chronic hGH therapy and the growth rate in the treated subjects. Although in some GH-deficient children on long-term hGH therapy there is a significant correlation between changes in height and their serum SM-C/IGF-I levels over 1–2 years (R18, S11), Rosenfeld *et al.* (R12) found that neither baseline levels, nor those after either acute or chronic hGH administration, were related to the growth rate after a 6-month treatment period. Similarly, in a collaborative Canadian study involving 177 children, many discrepancies between SM-C/IGF-I and growth responses to 6 months of hGH treatment were found (D12).

There are many reasons why discordant results could have been obtained in different trials. For example, the duration of hGH treatment in the studies in which good correlations between growth rates and growth factor levels were found, was much longer than in those in which no association was seen. In addition, differences in the time between hGH administration and blood sampling for SM-C/IGF-I determination could profoundly influence the results, since levels of the peptide following hGH treatment rise and fall with a sharp peak and follow a different time course in different subjects (C19). Thus, with blood sampled 12–14 hours after hGH administration in one study (R12) and at least 24 hours after in another (S11), SM-C/IGF-I levels might be as much as 50% below their peak values in some patients. Finally, samples were treated to remove binding proteins in some studies but not in others. If the time course of the binding protein response to hGH treatment is different from that of SM-C/IGF-I, and if it differs markedly in different patients, then variable interference in the SM-C/IGF-I RIA by binding protein could greatly distort the results. Since the influence of these factors on the results obtained in various studies cannot be quantitated, it cannot yet be stated with certainty whether or not acute SM-C/IGF-I responses to hGH therapy might potentially be of value in predicting eventual improvement in the growth rate.

7.2. Growth Failure with Normal Growth Hormone

Broadly speaking, there are two groups of patients in whom growth retardation and low SM-C/IGF-I levels are seen despite normal or even high hGH levels. The first group, subjects who do not respond to exogenous hGH, was originally described by Laron (L2). In Laron dwarfs, hGH treatment fails to elicit an increase in either growth rate or bioactive somatomedin levels. Immunoreactive SM-C/IGF-I levels are in the low hypopituitary range, and somatomedin measured by protein binding assay, which largely reflects IGF-II levels, is also greatly decreased (Z5). Erythropoietic stem cells of these patients have been found not to respond to the normal actions of GH, perhaps indicating a more widespread GH resistance in other tissues (G5). This may

be explained by the recent observation of Eshet *et al.* (E4) that membranes prepared from liver samples from Laron dwarfs are devoid of the normal binding sites for GH, despite showing insulin binding. This suggests that an isolated deficiency of GH receptors may be sufficient to cause low levels of SM-C/IGF-I.

The second group of growth-retarded patients deficient in SM-C/IGF-I but not hGH are those in whom exogenous hGH causes an increase in growth rate and somatomedin levels. Kowarski *et al.* (K15), and subsequently other investigators (F6, R17), found that the circulating GH in a group of such patients had an unusually low potency when measured by radioreceptor assay compared to RIA. This has led to the postulate that reduced biological activity of the endogenous GH accounts for its failure to elicit somatomedin production or usual growth (H10, K15, R17). An alternative explanation (B34), based on the observation that the average concentration of administered hGH in these patients was significantly higher than that of their endogenous hormone, is that the condition is due to an abnormally high tissue threshhold for GH action, which might be due to a defect either in GH receptors, or at an intracellular site.

In a 6-month treatment trial of a group of these patients with hGH, Rudman *et al.* (17) found that their acute (10-day) and chronic (6-month) SM-C/IGF-I responses were highly correlated, and that both responses showed a very high association with the increased growth velocity at the end of the test period. Patients with this condition might normally be denied GH therapy as they do not satisfy criteria for GH deficiency, but it appears from these studies that GH treatment would be of benefit and, importantly, that a short-term trial of GH administration, monitoring SM-C/IGF-I levels, would differentiate this group from Laron dwarfs or other nonresponders.

7.3. Acromegaly

Measured by RIA for SM-C/IGF-I or somatomedin-A, somatomedin values in acromegaly are significantly higher than in any healthy subjects except children in puberty. Mean SM-C/IGF-I levels are typically elevated about 4-fold above normal (B14, F9, Z5), while somatomedin-A is reported to be 7.6 times normal (H3). In general, random SM-C/IGF-I levels, whether determined by RIA or RRA (see Fig. 8), show excellent discrimination between healthy subjects and those with active acromegaly. Thus this test has the potential to replace the expensive and time-consuming dynamic testing currently used to diagnose acromegaly. In contrast, immunoreactive IGF-II levels in acromegaly are not significantly different from those found in healthy adults by RIA (H16, Z3) or RRA (D7).

Highly significant correlations have been reported between somatomedin levels in acromegalic patients and the clinical activity of the disease, assessed by a variety of parameters (R7). Treatment by estradiol administration results in a decrease in SM-C/IGF-I which parallels the improvements in metabolic activity as indicated by the phosphate : creatinine clearance ratio and urinary hydroxyproline excretion (C15). In bromocriptine-treated acromegalics, Wass *et al.* (W3) found that clinical improvement was associated with a fall in SM-C/IGF-I values of at least 30% in all patients, while GH levels did not always show a concomitant decrease. This contrasts with a study by Carlson *et al.* (C1), in which neither GH nor somatomedin levels accurately reflected improved clinical status. These studies indicate that, at least in some patients, bromocriptine improves acromegaly by a mechanism other than a simple decrease in GH secretion and somatomedin generation. An unclear relationship between SM-C/IGF-I and clinical response has also been reported for patients treated by surgery and irradiation. Although it has been clearly shown that such treatment significantly lowers somatomedin levels, frequently to within reference values (R7), Stonesifer *et al.* (S32) found poor correlations between symptomatic improvement and both SM-C/IGF-I and GH levels, and were of the opinion that SM-C/IGF-I measurement in acromegaly might offer no advantages over GH.

8. Circulating Levels in Other Disease States

8.1. Nutritional Deprivation

It is becoming increasingly clear that, of the factors apart from GH which influence circulating somatomedin levels, nutritional status is among the most important. Bioassay measurements have shown reduced somatomedin activity in children with protein-calorie malnutrition (G8, H22), and in rats either acutely (P6) or chronically (S24) undernourished. The interpretation of these findings, however, is complicated by the presence of bioassay inhibitors in fasting serum specimens (see Section 3.5), raising uncertainty as to whether the concentrations of somatomedin peptides were actually decreased.

This question has been answered by RRA and RIA studies showing profound falls in somatomedin peptides in malnourished rats and humans. Takano *et al.* (T2) reported that, in fasting rats, an 80% loss of receptor-reactive somatomedin-A occurred within the first 24 hours. Similar decreases have been seen using SM-C/IGF-I RRA or RIA methods on extracted or unextracted serum samples, in fasted adult rats (B16, M2), or chronically mal-

nourished rat pups (M3). In studies on humans involving radioligand assays to measure somatomedins, Takano (T1) reported a 40% decrease by somatomedin-A RRA in three healthy subjects fasted for 72 hours. More recently, immunoreactive SM-C/IGF-I was reported to fall by about 75% in seven obese patients fasted for 10 days (C16), with partial restoration on 3 days of refeeding. The percentage decrease in urinary urea nitrogen excretion closely paralleled that in plasma SM-C/IGF-I levels. Like SM-C/IGF-I, IGF-II has also been reported to fall during a 72-hour fast in normal subjects, although the observed decrease of only 27% suggests that this peptide is less closely regulated by nutritional factors than is SM-C/IGF-I (M16).

Studies involving the refeeding of fasted subjects have yielded valuable information about the relative importance of various dietary components in maintaining serum somatomedin levels. Isley *et al.* (I2) found that, after fasting normal adults for 5 days, refeeding with a protein-deficient diet caused a significantly smaller increase in SM-C/IGF-I levels than did a normal diet, while a diet deficient in both protein and energy was even less effective in raising SM-C/IGF-I. This confirmed earlier studies in rats which indicated the importance of protein in restoring somatomedin levels depleted by fasting (P8, T4). As in the study of Clemmons *et al.* (C16), a high correlation was seen between the change in SM-C/IGF-I over the fasting and refeeding periods, and the mean daily nitrogen balance (I2). In a further investigation of the relative importance of energy and protein, Isley *et al.* (I3) observed that there was a threshold energy requirement below which optimal protein was unable to restore SM-C/IGF-I levels. Above this threshold, the maximal level attained seemed to show direct dependence both on the energy intake and on the amount of protein. These studies suggest that measurement of SM-C/IGF-I levels may be useful in monitoring the nutritional status of patients who have absorptive diseases or require nutritional support for other reasons.

In fasting GH-deficient subjects, SM-C/IGF-I levels have been shown to respond very poorly to GH administration compared to the response in the fed state, suggesting resistance to the action of this hormone during fasting (M16). In another recent study in fasting obese subjects, SM-C/IGF-I levels showed positive correlation with fasting insulin, but not GH, levels (C3). These observations might both be explained by the finding that starvation in rats causes severe depletion of liver receptors for GH (B16). The GH receptor numbers in fasted animals were found to correlate closely with circulating insulin levels, consistent with the postulate that this receptor is regulated by insulin (B15, B16). Thus the decreased insulin in fasting might lower GH receptors, leading to the inability of GH to stimulate somatomedin generation by the liver.

8.2. Diabetes and Hyperinsulinemia

In rats with experimentally induced diabetes, circulating somatomedin levels are very low compared with values in control animals (F4, P4). As with the investigations of the effects of undernutrition, this was originally observed using bioassay methods to measure somatomedin levels and, similarly, the discovery of inhibitors of somatomedin bioactivity in the circulation of diabetic animals (see Section 3.5) raised the possibility that the apparently reduced somatomedin levels might simply be due to interference of these substances in the bioassays. More recently, RRA (B12) and RIA (M1) studies have confirmed that the concentration of somatomedin peptides is indeed reduced in experimental diabetes. This is perhaps not surprising, since both circulating GH levels (T7), and the concentration of GH receptors on liver membranes (B13, B15), are very low in these animals.

Results in diabetic humans are less clear-cut. Although Yde demonstrated that diabetes in man was associated with a mild reduction in somatomedin bioactivity (Y1), other studies using radioligand assays have failed to show values different from those seen in healthy subjects (H23, L1). Of particular interest are two recent studies, using both bioassay (A7) and RIA (M17) methods, in which insulin dependent diabetics with severe proliferative retinopathy had serum SM-C/IGF-I levels up to twice as high as controls, while levels in patients in whom retinopathy was mild or absent were normal. Non-insulin-dependent diabetics have also been reported to show an exaggerated SM-C/IGF-I response to GH infusion (G9). In contrast, data from the author's laboratory indicate significantly reduced levels of immunoreactive SM-C/IGF-I in adults aged from 20 to 70 years with either insulin-dependent (Fig. 9) or non-insulin-dependent (data not shown) diabetes. Low values have also been found by RIA in neonatal diabetes (B28), and in a study of nine adolescent type-I diabetics subjected to intensive insulin treatment either by pump or multiple injections, a significant increase in growth velocity resulting from intensive treatment was accompanied by a doubling of plasma SM-C/IGF-I (R19). Since GH levels in diabetic humans are elevated (H8), rather than reduced as in the rat, a finding of low SM-C/IGF-I levels would suggest the possibility of hepatic resistance to GH in these subjects. This may yet prove to be associated with a loss of GH receptors since, in contrast to the rat in which GH receptors are induced by high GH levels (B10), these receptors in human cells appear to be down-regulated by GH (M27).

Because of reports of decreased somatomedin levels in insulin deficiency, as well as direct demonstrations in the rat that insulin increases somatomedin output by perfused livers (K13) or cultured hepatocytes (K14), there

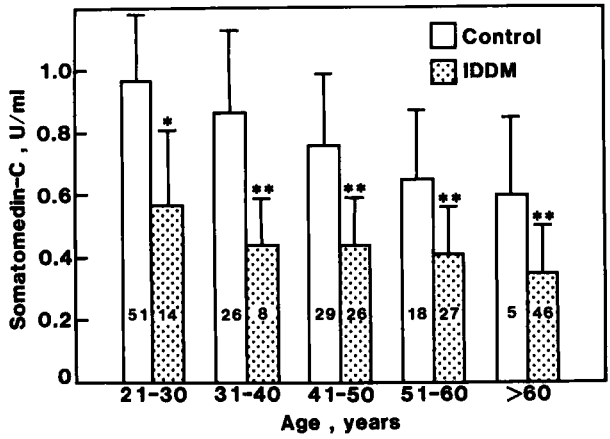

FIG. 9. SM-C/IGF-I levels measured by RIA (B14) in acid–ethanol extracted serum from healthy or insulin-dependent diabetic (IDDM) subjects, classified according to age. The number in each group is shown within the bars. Vertical lines indicate 1 SD. Comparison (multiple-range test) of normal and diabetic groups in each age range: $*p<0.05$; $**p<0.01$. (Data of K. Mintohadi and R. C. Baxter.)

has been interest in the possibility that hyperinsulinemia might be associated with elevated somatomedin levels. Hyperinsulinemic children with GH deficiency following removal of a craniopharygioma have been found to have normal somatomedin levels (B37), leading to the suggestion that the high insulin levels might be able to stimulate somatomedin generation even in the absence of GH. However in a study of hyperinsulinemic children with normal GH secretion, neither SM-C/IGF-I nor IGF-II levels were found to be elevated (B29). This is consistent with a study in rats which indicated that, whereas decreased serum insulin (below 18 μU/ml) was significantly correlated with low receptor-reactive somatomedin levels, higher insulin levels were not accompanied by an increase in somatomedin above the normal value of 1 U/ml (B13).

8.3. Nonpancreatic Tumor Hypoglycemia

There has been some controversy over the possibility that hypoglycemia associated with nonpancreatic tumors might be associated with elevated growth factor levels. Both Megyesi *et al.* (M11) and, more recently, Daughaday *et al.* (D7), have found, using RRA methods, that NSILA or IGF-II levels in about half of patients with these tumors are indeed elevated. In contrast, the RIA studies from the laboratory of Zapf and Froesch (W5, Z3) have failed to show an increase in IGF-II levels, and indicate slightly reduced IGF-I levels, in these subjects. Although these discrepancies have

been ascribed to methodological differences between the different laboratories, it is still not entirely clear why conflicting results were obtained. The balance of evidence, however, seems to favor the conclusion that IGF-I and IGF-II are not elevated in cases of nonislet tumor hypoglycemia, but the possibility cannot be excluded that a related peptide, with IGF-II RRA activity but no immunoreactivity in the RIA methods used, is secreted by these tumors.

8.4. Hypothyroidism

Primary hypothyroidism in children is accompanied by significant growth retardation, even though GH secretion may not always be diminished (C7, K6). Bioassay (M4) and competitive protein-binding assay (D19) results have suggested low serum somatomedin values as an explanation, whereas two studies using RRA methods on unextracted samples have failed to show differences between healthy and hypothyroid subjects (D17, T3). After acid–ethanol extraction, low values were seen by SM-C/IGF-I RIA using two different antibodies, while a placental membrane RRA gave even lower results, with a mean value for 10 patients almost 60% below normal (B14). Since this RRA is known to give higher values than the RIA methods in GH-deficient subjects, this argues against the low SM-C/IGF-I in hypothyroidism being due primarily to deficient GH secretion (B14). Chernausek *et al.* (C7) have shown that 1 to 9 months of thyroid hormone therapy increases SM-C/IGF-I levels back to within reference values. A single GH injection also caused a dramatic increase in SM-C/IGF-I, indicating that there was no defect in the ability of these patients to respond to GH, and suggesting that hypothyroidism itself probably does not inhibit somatomedin synthesis (C7). Thus the exact mechanism by which a deficiency in thyroid hormones lowers somatomedin levels remains unclear.

8.5. Hyperprolactinemia

In hyperprolactinemia, it appears that the elevated prolactin levels may be active in stimulating somatomedin generation. Clemmons *et al.* (C14) have described 20 patients with prolactin-secreting tumors but no measurable GH response to provocative stimuli, in whom the serum somatomedin-C level was in the normal adult range, i.e., significantly higher than in GH-deficient subjects without hyperprolactinemia. Similarly, in children with craniopharygioma who have GH deficiency and hyperprolactinemia but no hyperinsulinemia, normal growth rates and IGF-I levels in the first postoperative year appear to be due to their prolactin hypersecretion. These effects in humans are consistent with the previously described elevation of somato-

medin following prolactin administration to hypophysectomized rats (B3). Paradoxically, in acromegalic patients with hyperprolactinemia, immunoreactive somatomedin-A levels have been reported to be lower than in acromegalics with normal prolactin levels, even after surgery which lowered GH levels to similar values in both groups (H26). These results might be explained if prolactin acted as a low-potency agonist at the GH receptor, stimulating somatomedin production in the absence of GH but causing a relative inhibition of production by competing for GH binding sites in the presence of normal to elevated GH levels.

8.6. Hypercortisolism

Because growth retardation is well known in Cushing's syndrome, a number of studies have investigated the possibility that corticosteroid excess might be associated with somatomedin deficiency. Low levels have indeed been found by bioassay (E2, G10), but the interpretation of these results is difficult due to direct inhibition of cartilage sulfation by glucocorticoids (T10). Immunoassays for somatomedin-C (F9) and somatomedin-A (T12) have indicated normal or slightly elevated values in Cushing's disease, and Gourmelen *et al.* (G7), using a protein-binding assay, found low values in only 9 out of 39 subjects, with the group mean identical to that for healthy subjects. In this study carrier protein levels were marginally reduced. The administration of hGH to patients with hypercortisolism results in the usual rise in somatomedin-A, indicating that corticosteroids do not inhibit the somatomedin-generating process (T12). The conclusion from these studies must be that factors other than a deficiency of somatomedin peptides are responsible for the growth deficiency seen in these conditions.

8.7. Renal Failure

In patients with chronic renal failure, serum somatomedin bioactivity is reported to be low, rising after transplantation (S1, T6). This contrasts with results obtained by RRA using somatomedin-A (T3) or ILAs (S12) as tracer, and by RIA using somatomedin-A as tracer (T6), where levels up to 3 times higher than normal have been observed. The discordance between bioassay and radioligand assay results has been taken as evidence that uremic serum contains bioactivity inhibitors, similar to those found in diabetes or fasting (see Section 3.5). However, a more recent study using a SM-C/IGF-I RIA on acid–ethanol extracted plasma found levels significantly below normal in predialysis samples, rising to within reference values immediately after dialysis (B14). As shown in Fig. 10, this differs from the results found by SM-

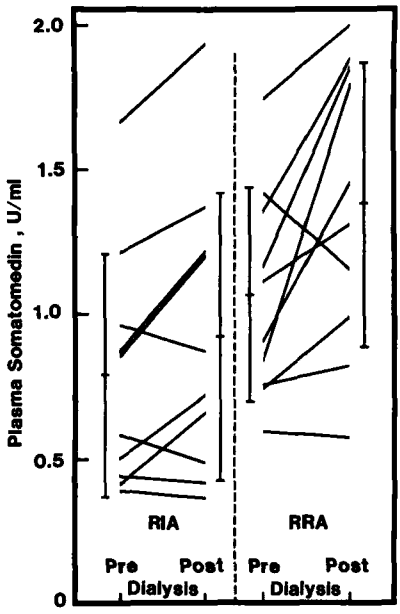

FIG. 10. SM-C/IGF-I levels measured by placental membrane RRA or RIA (B14) in acid–ethanol extracted serum from 10 patients with chronic renal failure, sampled immediately pre- and postdialysis. By paired t test the increase on dialysis was significant in both assays ($p<0.05$). Predialysis mean values are lower than normal by RIA ($p<0.05$), but normal by RRA. Postdialysis values are normal by RIA, but higher than normal by RRA ($p<0.001$).

C/IGF-I RRA, in which values were within the reference range before dialysis and significantly elevated afterwards.

An explanation for the conflict between RIA and RRA results was provided by Goldberg *et al.* (G4). They confirmed the low immunoreactive SM-C/IGF-I levels in acid–ethanol extracted uremic samples, but found that IGF-II was markedly elevated, and that the somatomedin binding capacity of uremic serum was also much higher than normal and of a different molecular mass distribution. It seems most likely that the high values reported earlier using ILAs RRA were principally reflecting the high IGF-II levels. Although increased IGF-II would also contribute to the high values found using somatomedin-A RRA, another major factor affecting this assay and the somatomedin-A RIA would be interference by binding protein, since in neither assay were samples treated to remove binding protein. Differences between healthy and uremic serum samples in the competition with antibodies or receptors for tracer by unsaturated or exchangeable binding sites would inevitably lead to apparently elevated results in the uremic samples.

8.8. Liver Disease

Consistent with a major role of the liver in maintaining normal somatomedin levels in the circulation, low levels of somatomedin-A activity have been found in unextracted plasma of patients with cirrhosis of the liver and chronic hepatitis (S14, T5). Significant correlations were seen between somatomedin-A and albumin, cholinesterase, and other indicators of liver function (T5). The decrease measured in this RRA appears to be due to low levels of both somatomedins and binding protein, since Zapf *et al.* (Z5) have shown an 89% decrease in immunoreactive IGF-I, a 74% decrease in "total IGF" by protein binding assay (which preferentially measures IGF-II), and a 57% decrease in specific binding of somatomedin tracer to stripped serum, in patients with cirrhosis.

9. Concluding Remarks

The past few years have seen many major advances in the understanding of insulinlike growth factor action, culminating in direct demonstrations of growth effects in laboratory animals. Inevitably, however, some of the most important questions are still unanswered. Remaining areas of controversy span all facets of somatomedin research, among which the following may be included.

1. What forms do the peptides take? Although the existence of a separate fetal form of somatomedin has been postulated, only SM-C/IGF-I and IGF-II, both of which are found in the fetus as well as postnatally, have been characterized to date.
2. Where are they made? IGF-II is 3 times more abundant in the adult human circulation than SM-C/IGF-I, yet its origin is entirely unknown.
3. How are they delivered to cells? Free somatomedins are not detectable in the circulation, but the extreme sensitivity of some cells to small amounts of the peptides leaves open the possibility that very low concentrations of unbound peptide might be responsible for major biological effects. If this is not the case, how does the binding protein complex release the growth factors at their target cells? Where is the 150,000-molecular-mass complex produced, and what is its relationship to the various 40–60,000 molecular mass species?
4. Do somatomedins principally act locally, or at a location distant from their site of synthesis? The evidence for local action is indirect, being based largely on observations of growth factor production by many different cells and tissue explants in culture. Is the secretion of growth factors an adapta-

tion to tissue culture, or does it reflect a normal function of whole tissues *in vivo*?

5. How are their intracellular effects mediated? The sequence of events between the interaction of somatomedins with their receptors, and the subsequent stimulation of anabolic or mitogenic effects, remain to be elucidated.

6. What is the significance of their levels in the circulation? The many questions in this area include: Why do some children have normal growth despite very low SM-C/IGF-I levels; can acute responses to GH administration in hyposomatotropic children predict the outcome of chronic therapy; is there any diagnostic value in IGF-II measurements, for example in patients with malignancies?

With such a diverse array of unresolved problems awaiting answers, it seems probable that the increasing interest in somatomedin research seen in the recent past will not diminish, but will continue to expand. This will be aided by the greater availability of pure somatomedin peptides resulting from their synthesis by recombinant DNA techniques. Thus it seems probable that an exciting and fruitful period of research into the somatomedins will follow over the next few years.

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SYNTHETIC PEPTIDE SUBSTRATE ASSAYS FOR HEMOSTASIS TESTING

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1. Introduction

In 1972, Svendsen and co-workers introduced an improved substrate for monitoring thrombin activity (S26). Their approach used the earlier discovery of Blomback's group at the Karolinska Institute that a critical tripeptide structure (Phe-Val-Arg) was recognized by thrombin during the digestion of its natural substrate, fibrinogen (B13). In addition, they utilized the earlier observation of Erlanger and co-workers that the coupling of the chromogenic moiety, *p*-nitroaniline (pNA), to a synthetic substrate would enhance the monitoring of enzymatic activity (E1). Therefore, the new synthetic peptide substrate had improved affinity for a specific proteolytic enzyme and could be monitored by a simple spectrophotometer. The enzyme concentration was proportional to changes in absorbance per unit time resulting from the release of pNA from the substrate structure.

From the appearance of this report, a rapid development of additional synthetic peptide structures has occurred worldwide with a number of applications to hemostatic testing. To many, this 1972 paper by Svendsen and co-workers has created a revolution in hemostasis similar to that experienced 37 years earlier when A. J. Quick and his associates introduced a simple reliable clot-endpoint test to quantify the concentration of prothrombin in plasma (Q2).

This review will emphasize those areas where this new test procedure has reached its potential and those where more work is needed so that these substrates can be accepted in routine laboratory hemostasis testing. The list of literature presented is up to late 1984. The reader is referred to several excellent reviews on chromogenic and fluorogenic peptide substrates that have been previously published (F3, F5, F6, H5, S9, S16, T10).

2. A Brief Review of Hemostasis

Before discussing the design, applications, and advantages of the synthetic peptide substrates, it is useful to review briefly the molecular events of hemostasis—a complex process by which blood remains fluid in the vascular system of mammals. Our knowledge of this subject has progressively expanded, based on almost 50 years of worldwide studies. Forty-two years of pioneering research work by Dr. Walter Seegers played a major role in the process (O6). The subject of hemostasis has recently been extensively reviewed (H4, J1, O1, O4).

Most of the factors involved in blood coagulation and fibrinolysis systems are proteolytic enzymes. They are members of a class of catalysis known as serine proteases and share a common basic mechanism of action. These

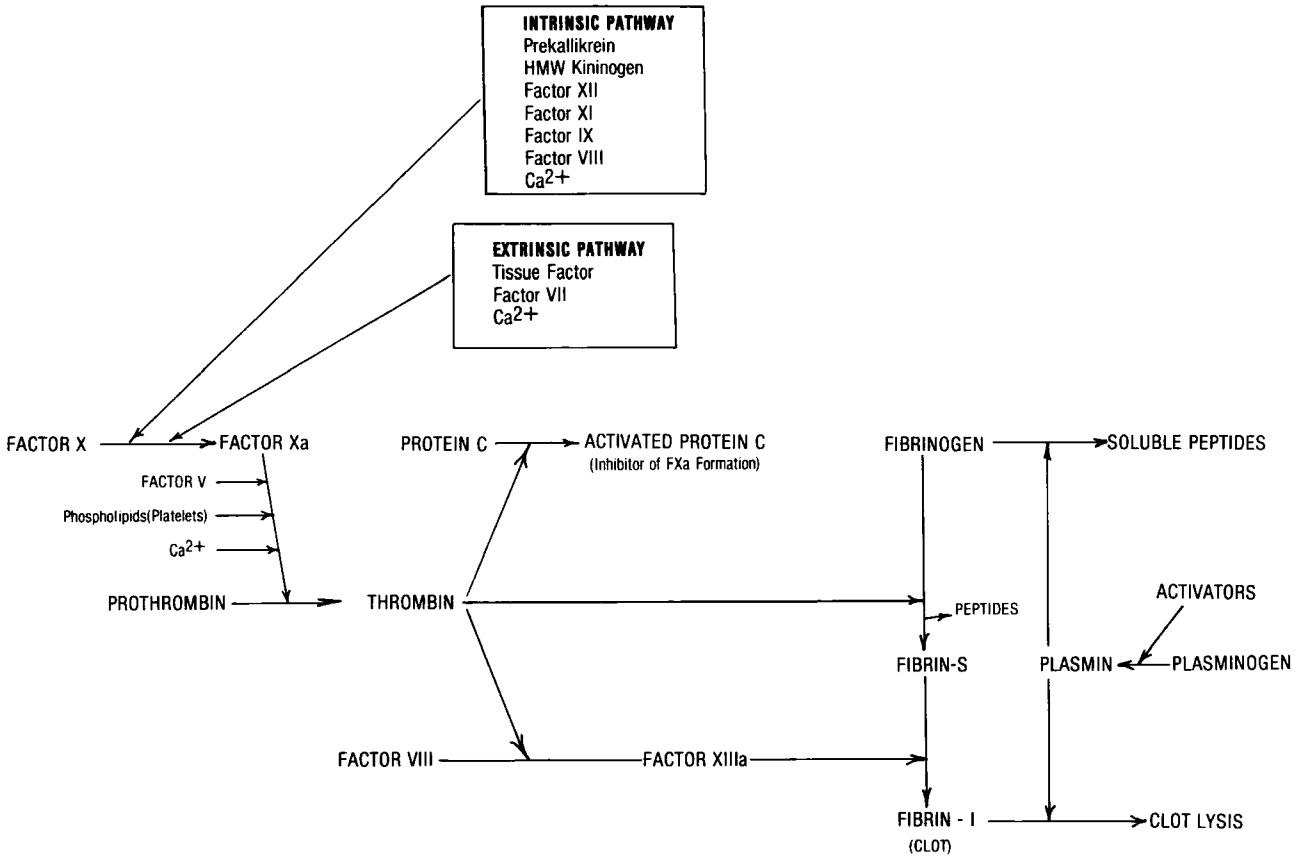


FIG. 1. Generalized scheme of the hemostasis system.

TABLE 1.

Factor	Molecular weight	Migration in electrophoresis	Structure of zymogen	Structure active enzyme	Enzyme activity
Fibrinogen	340,000	β-Globulin	Symmetrical with 6 subunits		
Prothrombin	69,000	α- or β-Globulin	Single polypeptide chain	2 Subunits bound by disulfide bond	Serine protease
Factor V	290,000–400,000	β-Globulin	?	?	Cofactor
Factor VII	63,000	α-Globulin	Single polypeptide chain	Two-chain form	Serine protease
Factor VIII	2,000,000	β-Globulin	?	?	Cofactor
Factor IX	57,000	α_1-Globulin	Single polypeptide chain	2 Subunits bound by disulfide bond	Serine protease
Factor X	58,900	From α to prealbumin	2 Polypeptide chains	2 Polypeptide chains	Serine protease
Factor XI	160,000	As β- or γ-globulin	2 Subunits linked by disulfide bond	Cleavage of internal bond of each subunit	Serine protease
Factor XII	76,000	β-Globulin	Single polypeptide chain	Single chain, molecular weight 28,000	Serine protease
Prekallikrein	85,000	Fast γ-globulin	Single polypeptide chain	2 Chains held together by disulfide bond	Serine protease
High-molecular-weight kininogen	110,000	α-Globulin	Single chain		Cofactor
Factor XIII	320,000	α_2-Globulin	2 α (75,000) and 2 β (88,000) chains	2 α Chains	Transamidase
Plasminogen	92,000	β-Globulin	Single polypeptide chain		Serine protease
Protein C	62,000	—	—	—	—
Protein S	69,000	—	—	—	—

[a] From Triplett (T9), with permission. Copyright CRC Press, Inc., Boca Raton, Florida.

PROPERTIES OF COAGULATION PROTEINS[a]

Biological half-life	Carbohydrate content (%)	Site of production	Dependent on vitamin K	Concentration in plasma (mg/dl)	Presence in $BaSO_4$ adsorbed plasma	Presence in serum
90 hr	4.5	Liver	No	300–400	Yes	No
60 hr	2–10	Liver	Yes	10–15	No	No
12–36 hr	?	Liver	No	0.5–1.0%	Yes	No
4–6 hr	50	Liver	Yes	0.1	No	Yes
12 hr	5.8	Portion produced in endothelial cells	No	< 1	Yes	No
24 hr	26	Liver	Yes	0.75	No	Yes
40 hr	10	Liver	Yes	1.2	No	Yes
48–52 hr		Liver	No	0.4	Yes, 1/3	Yes
48–52 hr	3.3	Liver	No	0.29	Yes	Yes
		Liver	No	0.15–0.45	Yes	Yes
6.5 days	12.6	Liver	No	0.70	Yes	Yes
3–5 days	4.9	Liver	No	2.5	Yes	No
		Liver, kidney eosinophil	No	10–15	Yes	
—	—	—	Yes	0.5	No	Yes
—	—	—	Yes	—	No	Yes

particular enzymes exist in mammalian blood as zymogens along with their activators, inhibitors, and natural substrates. This system is accurately balanced by interdependent positive and negative feedback reactions. Depending upon the extent of the perturbation, the equilibrium between clot formation and dissolution is shifted accordingly. The complex sequence of reactions is shown in Fig. 1. Temporary overpowering of the negative feedback forces involves three principal events: (1) the formation of Factor Xa; (2) the formation of Factor IIa (thrombin); and (3) the formation of fibrin (S10). The components which accelerate the formation of Factor Xa and, hence, thrombin, come from separate anatomic compartments, platelets, plasma, and tissues. With the onset of injury these factors are brought together and the clotting process is initiated. Interestingly, many of the same forces which drive the clotting event will also institute the formation of the enzyme plasmin which acts as a counterforce to thrombin and causes lysis of the clot network (A4). Listed in Table 1 are the characteristics of many of the proteins involved in this complex system. Crucial to the understanding of these pathways is the recognition that several of these enzymes can participate in an intricate feedback system to help regulate this potent system; thus, thrombin, for example, helps to increase its own formation by activating the cofactors, Factor V and Factor VIII (N3). However, thrombin's role in producing activated protein C (autoprothrombin IIa) limits its additional formation and helps to induce clot lysis (C10, Z2).

One must remember that the suggested series of events depicted in Fig. 1 has been determined in laboratory *in vitro* tests and that the *in vivo* sequence could be significantly different. For example, patients with deficiencies of prekallikrein (Fletcher factor), high-molecular-weight kininogen (Fitzgerald factor), or Factor XII (Hageman factor) will have abnormal laboratory test results although clinically they are asymptomatic. Likewise, earlier *in vitro* studies have suggested that two separate pathways, intrinsic and extrinsic, exist for the formation of the key enzyme, Factor Xa. Recent results have shown, however, that Factor VIIa can be activated by components of the intrinsic pathway and, likewise, Factor VIIa can participate in the formation of Factor IXa (O5, M9).

Accepting the above limitations of our knowledge of the true *in vivo* reaction sequence, a series of clot-endpoint tests has been used for many years to determine the hemostatic balance in any patient. The first, known as the thrombin time test, can be used to measure the concentration of fibrinogen (B15). The second assay, the activated partial thromboplastin time test (APTT), measures the components of the intrinsic pathway (M4). These include the serine proteases (Factors XII, XI, X, and II), the cofactors (Factors VIII and V) and again, fibrinogen. Most of the hereditary deficiencies

such as hemophilia A and B will affect the APTT results. Also, because factors of this pathway are influenced by heparin, the APTT assay is used as a presurgical screen and for monitoring patient heparin therapy (S19). The third test, the prothrombin time test (PT), determines the status of the coagulation factors of the extrinsic pathway, Factors VII, X, V, II, and fibrinogen (Q1). This test is also used to monitor the effectiveness of oral anticoagulant therapy since the synthesis of Factors II, VII, and X is inhibited by this drug (F10). The activity of plasmin is monitored either by its impact on prolonging the thrombin time test or by measuring the time for clot lysis to occur (C1).

In theory, at least, all of the above serine proteases can be assayed by selective specific tests employing synthetic peptide substrates. While these new tests are becoming routine research procedures (S16), the key question for the clinician and laboratory head is how to interpret these results in light of the extensive knowledge of patient testing with the above nonspecific clot-endpoint tests like the PT and APTT. This point will be examined more fully later.

3. Natural Substrates versus Synthetic Substrates

3.1. Bioassays Compared with Chemical Assays

A simple example of a chemical assay is the titration of an unknown amount of base with a volume of an acid solution. One mole of base will be neutralized by exactly one mole of acid. In contrast, the relationship of the components in a bioassay, such as the PT or the APTT, is complex and generally unknown. In this test, the assay system is a black box and the input and output characteristics must be determined. Thus, the quantification of a certain output will allow conclusions about the input. The output of a coagulation assay is a clotting time, and this result is used to estimate concentrations of the various coagulation factors, which are the input. With the advent of fluorogenic or chromogenic substrates many had hoped to see the conversion of the clot-endpoint bioassays into chemical assays. However, as Hemker has pointed out, this is not automatically true (H3). The same black box now yields a yellow color rather than the old clotting time results; yet the use of the synthetic substrate in the bioassay offers several advantages. This procedure is more comfortable for the clinical chemist, can be easily automated and offers the potential for using less specialized personnel. On the other hand, it requires a basic knowledge of enzyme kinetics. This point will be expanded in the assay design section below.

3.2. First Generation of Synthetic Substrates

The development of synthetic substrates as replacements for the natural substrates of the clotting and fibrinolytic systems began with the observation of Schwert and co-workers that amino acids were sensitive substrates for serine proteases, chymotrypsin, and trypsin (S4). Later on, Troll and his associate developed a small nonprotein thrombin substrate, tosyl-arginine methyl ester (TAMe) (T11). A series of these compounds using either arginine or lysine as the target site was tested during the 1950s to study coagulation and fibrinolytic enzymes (S15). The endpoint for these reactions was the measurement of either the released alcohol or the free amino acid. Some of the problems with these early substrates were a low affinity for the enzyme, lack of specificity and a significant rate of substrate hydrolysis in the absence of the enzyme (T10). These substrates are still commercially available.[1] In 1961, a paranitroanilide derivative of benzoyl arginine (BAPNA) was introduced (E1). This type of substrate has an improved selectivity since an amide bond is not split by esterases and the rate of hydrolysis is greatly reduced. This change also allowed direct endpoint measurement in a spectrophotometer since a chromogenic amine (*p*-nitroaniline) was released during the reaction. Still, none of these one-amino-acid based substrates was specific or sensitive enough to be seriously considered outside the research laboratory.

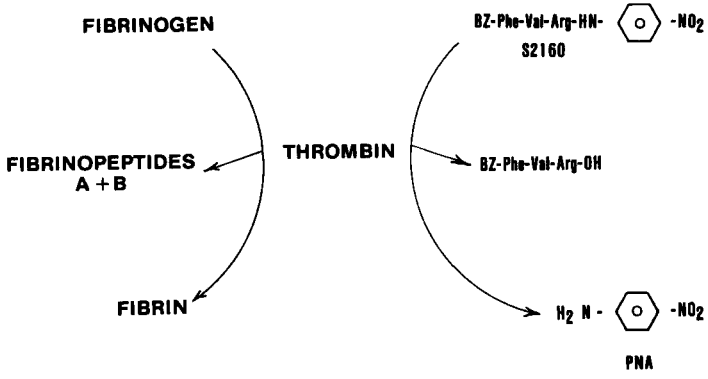
3.3. Further Developments of Synthetic Substrates

During the 1960s, a greater understanding of the molecular structure of serine proteases, their substrates, activators, and inhibitors occurred and it was possible to prepare polypeptides that resembled the cleavage sites of the natural substrates. It has long been known that recognition of both the selectivity of these proteases and their catalytic efficiency depend on interactions with amino acid sequences around the cleavage site of the natural substrate. A synthetic substrate containing several amino acids was needed to increase the selectivity factor. All of the serine proteases of the coagulation and fibrinolytic systems are trypsinlike in their primary specificity (arginine or lysine) and, therefore, influences from secondary binding-site interactions are essential for an improved synthetic substrate. Leading the effort to understand the effect of the amino acid sequence in fibrinogen on the selectivity of thrombin was Blomback and his co-workers from Sweden. This team studied, from various animal species, the 16-amino-acid sequence of a fragment which is released from fibrinogen during the early stage of thrombin's attack. This fragment is called fibrinopeptide A (B12). They

[1] Sigma Chemical Company, St. Louis, Missouri.

found a high level of conservation of this amino acid sequence and the same three amino acids were always in position 9, 2, and 1 from the carboxyl end of this fragment, phenylalanine, valine, and arginine, respectively. This observation suggested that the action of thrombin on this large substrate is strongly influenced by this 3-amino-acid sequence.

With the above knowledge, the researchers at the Bofors Company, now a part of A.B. Kabi in Stockholm, began synthesizing oligopeptides which mimicked the amino acid sequence around the cleavage sites in fibrinogen. Interestingly, the initial focus of this work was not to develop synthetic substrates but to explore the use of these compounds as anticoagulants. In laboratory experiments the peptide which had the maximal anticoagulant effect was one with the amino acid sequence of phenylalanine-valine-arginine; this sequence was noted above from the earlier work with fibrinopeptide A. When these experiments were tried *in vivo* they were less successful because of the short half-life of this small compound in the circulation; so this approach was not pursued. However, the Bofors group next turned their attention to using these unique peptides and the earlier idea of Enlanger concerning the advantages of a chromophore group like pNA to develop a new generation of synthetic substrates for detecting serine proteases. The first commercial product designed to detect thrombin was based on the familiar sequence of Phe-Val-Arg (see Fig. 2).



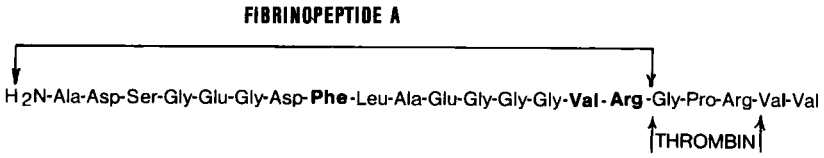
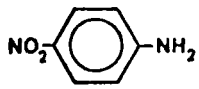
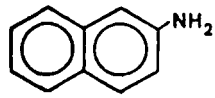
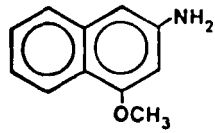
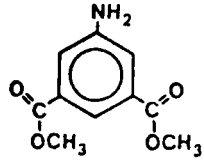
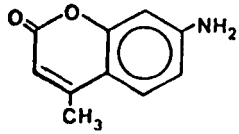
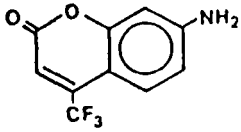


FIG. 2. Structure of fibrinopeptide A and reaction products formed from thrombin attack on its natural and synthetic substrate.

TABLE 2

COMMON DETECTOR GROUPS USED FOR SYNTHETIC SUBSTRATE TECHNOLOGY[a]

Detector group[b]	Spectral characteristics
p-Nitroaniline (pNA)  (colorimetric)	Yellow color when cleaved from peptide (405 nm). May also be coupled with *p*-dimethylamino-cinnamaldehyde to yield a colored Schiff base compound (570 nm)
2-Naphthylamine (BNA)  (fluorometric or colorimetric)	Fluorometric when cleaved from peptide (excitation 340 nm/emission 425 nm) or azo coupling reaction with fast blue B as red product (525 nm)
4-Methoxy-2-naphthylamine (4 MNA)  (fluorometric or colorimetric)	Fluorometric when cleaved from peptide (excitation 340 nm/emission 425 nm) or azo coupling fast blue B as red product (525 nm)
5-Aminoisophthalic acid (AIE) dimethyl ester  (fluorometric or colorimetric)	Fluorometric when cleaved from peptide (excitation 335 nm/emission 430 nm) or coupling via Bratton–Marshall reaction to yield colored product
7-Amino-4-methylcoumarin (MCA)  (fluorometric)	Fluorometric when cleaved from peptide (excitation at 380 nm/emission 460 nm)
7-Amino-4-trifluoromethylcoumarin (AFC)  (colorimetric or fluorometric)	Yellow color when cleaved from peptide (380 nm) or fluorometric (excitation 400 nm/emission 505 nm)

[a] From Huseby and Smith (H9), with permission.

[b] General structure of peptide B–$(AA)_n$–D, where B (blocking group), $(AA)_n$ (peptide), n (1 to 4 amino acids), and D (detector group).

From 1972 to 1977, numerous papers on the testing of tripeptide or tetrapeptide substrates, all employing either arginine or lysine as the carboxyl terminal amino acid and pNA as the reporter or leaving group, were published (F6). Crucial to this research were the discoveries concerning the amino acid sequences around the active centers of these serine proteases. A leading worker in this area was Magnusson. For example, he and his coworkers (M2) showed that in bovine prothrombin the two bonds split by Factor Xa are preceded by the same amino acid sequence: Ile-Glu-Gly-Arg. Again, the Bofors group used this knowledge to design a specific substrate for the detection of Factor Xa (A5). In 1977, reports appeared in which a new type of reporter group was employed which used fluorescence to measure the enzymatic action. A comparison of the two types of reporter groups is shown in Table 2. The switch to fluorogenic substrates was claimed to have increased the detecting sensitivity of the same oligopeptide sequence by a magnitude of one to two times (H9). However, this advantage was counterbalanced by claim of a lower k_{cat} and lower solubility for the fluorescent substrates than the corresponding pNA derivatives (F6). To overcome the limitation that a fluorometer is less available in the clinical laboratories than a spectrophotometer, systems were made available to utilize azo dye coupling reactions so these new reporting groups could indicate their responses on a spectrophotometer like pNA (S18). Recently, it was reported that an improved chromogenic reporting group had been found (N1). This group is 3-carboxy-4-hydroxyanilide (CHA) and replaces pNA at the carboxyl end of the synthetic substrate. The workers found greater solubility and specificity with the new substrates in tests with thrombin, plasmin, Factor Xa, kallikrein, urokinase, and trypsin.

In 1982, a new series of synthetic substrates was made available (S27). These substrates utilized synthetic amino acids such as cyclohexylglycine (CHG), cyclohexyltyrosine (CHT), and norleucine (NLeu) to impart information about the tertiary structure of the natural substrate into the synthetic one. Svendsen, at a recent meeting, reported that this new generation of substrates used for human Factor Xa, thrombin, and plasmin, when compared with the second-generation substrates for the above enzymes, were found to be more soluble in physiologic buffers, to be less susceptible to autoamidolysis at reaction conditions and not to produce opacity of the test solution in final concentrations of greater than 5×10^{-4} M. (S23). A contrasting view was reported by Aurell and co-workers based on a comparative study of the two types of substrates (A9).

Another interesting development has been the efforts to measure these serine proteases by electrochemical activity principals. A. B. Kabi has made available modified chromogenic substrates for this application. This subject will not be covered in this review but the reader is referred to the series of papers on this topic (C12).

TABLE 3
AVAILABLE SYNTHETIC PEPTIDE SUBSTRATES

Name	Chemical structure	Enzyme	Source[a]
I. Chromogenic Substrates			
S-2160	-Bz-Phe-Val-Arg-pNA-	Thrombin	1
S-2238	-H-D-Phe-Pip-Arg-pNA-	Thrombin	1
S-2366	-Pyro-Glu-Pro-Arg-pNA-	Thrombin	1
Chromozym-TH	-Tos-Gly-Pro-Arg-pNA- -CBz-Gly-Pro-Arg-pNA-	Thrombin	2
Spectrozyme-TH	-H-D-CHT-Ala-Arg-pNA-	Thrombin	3
PS-915	-H-D-Phe-Pro-Arg-CHA-	Thrombin	4
CBS-34-47	-H-D-CHG-But-Arg-pNA-	Thrombin	5
S-2222	-Bz-Ile-Glu-(αOR-)Gly-Arg-pNA-	Factor Xa	1
S-2237	-Bz-Ile-Glu-(γ-*o*-Piperidyl-)Gly-Arg-pNA-	Factor Xa	1
Spectrozyme-Xa	-CH_3-O-CO-D-CHG-Gly-Arg-pNA-	Factor Xa	3
S-2302	-H-D-Pro-Phe-Arg-pNA-	Factor XII, kallikrein	1
Chromozym PK	-Bz-Pro-Phe-Arg-pNA-	Factor XII, kallikrein	2
Spectrozyme PK	-H-D-Pro-CHT-Arg-pNA-	Factor XII, kallikrein	3
CBS 33.27	-H-D-Phe-Gly-Phe-Arg-pNA-	Factor XII, kallikrein	5
S-2251	-H-D-Val-Leu-Lys-pNA-	Plasmin	1
Chromozym P	-Tos-Gly-Pro-Lys-pNA-	Plasmin	2
Spectrozyme PL	-H-D-Nleu-CHT-Lys-Arg-pNA-	Plasmin	3
PS-994	-H-Lys-(Tos-)Phe-Lys-CHA-	Plasmin	4
CBS 30.41	-H-D-But-CHA-Lys-pNA-	Plasmin	5
S-2444	-Pyro-Glu-Gly-Arg-pNA-	Urokinase	1
Chromozym-UK	-Bz-Val-Gly-Arg-pNA-	Urokinase	2
II. Fluorogenic Substrates			
Protopath	-H-D-Phe-Pro-Arg-Aie-	Thrombin	6
—	-BOC-Val-Pro-Arg-MCA-	Thrombin	7
—	-CBZ-Gly-Pro-Arg-BNA-	Thrombin	7
—	-BOC-Ile-Glu-Gly-Ars-MCA-	Factor Xa	7
—	-Z-Phe-Arg-MCA-	Factor XII, kallikrein	7
—	-Pro-Phe-Arg-MCA-	Factor XII, kallikrein	7
Protopath	-H-D-Val-Leu-Lys-Aie-	Plasmin	6
—	-BOC-Gly-Leu-Lys-MCA-	Plasmin	
—	-Glu-Gly-Arg-MCA	Urokinase	7

[a] (1) A. B. Kabi, Stockholm, Sweden or Helena Lab, Beaumont, Texas; (2) Pentapharm AB, Basil, Switzerland; (3) American Diagnostia, Greenwich, Connecticut; (4) Nitto Boseki, Fukuyama, Japan; (5) Diagnostica Stago, Asnieres, France; (6) American Dade, Miami, Florida; (7) Peptide Institute, Minoh Osaka, Japan; (8) Additional substrates for thrombin, plasminogen, and Factor Xa detection are available from Wellcome Diagnostics, Research Triangle Park, North Carolina.

3.4. Current Offerings of Chromogenic and Fluorogenic Peptide Substrates

Today over 100 synthetic peptide substrates are available for testing coagulation and fibrinolytic enzymes. In Table 3 one can find a listing of some of these substrates including their structure, type of reporting group, enzyme designed for, and source.

3.5. Recommended Selection Principles

The above experience with synthetic peptide substrates has generated some guidelines on how to select suitable candidates and what features are most desirable. As can be expected, some workers have initiated efforts to design a computer program to optimize this process (H9). Obviously, one selection approach is to use trial and error. Thus, many combinations of amino acids are synthesized in an attempt to find that sequence which best fits a particular enzyme. To help increase the chance of success certain characteristics of enzyme actions are used. For example, designing a substrate for a serine protease calls for the placement of either arginine or lysine at the residue site preceding the cleavage point (site S_1). If the enzyme in question has a metal cofactor requirement the amino acid in S_1 should be alanine if zinc or cobalt is required or a proline if manganese is the cofactor (H9). A second approach, which should be more efficient, utilizes information on the amino acid sequence around the cleavage sites in the natural substrate. The sequence for various serine proteases is known (S27) and some selected ones are shown in Table 4. Even this approach does not guarantee success, as found when synthetic substrates for urokinase and Factor IX were under development (B11, S20).

TABLE 4
Cleavage Sequences of Various Serine Proteases[a]

Serine protease	Natural substrate	Cleavage sequence
α-Thrombin (human)	Fibrinogen (human)	-Phe-$(X)_7$-Val-Arg-Gly-Pro-Arg-
	Factor XIII (bovine)	-Leu-Val-Pro-Arg-Gly-Phe-Asx-
	Prothrombin (bovine)	-Val-Ile-Pro-Arg-Ser-Gly-Gly-
Factor Xa (bovine)	Prothrombin (bovine)	-Ile-Glu-Gly-Arg-Thr-Ser-Glu-
Factor IXa (bovine)	Factor X (bovine)	-Gln-Val-Val-Arg-Ile-Val-Gly-
Factor XIa (bovine)	Factor IX (bovine)	-Lys-Leu-Thr-Arg-Ala-Glu-Thr-
	Factor IX (bovine)	-Glu-Phe-Ser-Arg-Val-Val-Gly-
Kallikrein (bovine)	HMW kininogen (bovine)	-Ser-Leu-Met-Lys-Arg-Pro-Pro
Urokinase (human)	Plasminogen (human)	-Cys-Pro-Gly-Arg-Val-Val-Gly-
Plasmin (bovine)	Plasminogen (human)	-Phe-Glu-Lys-Lys-Val-Tyr-Leu
	Fibrinogen (human)	-Gly-Tyr-Lys-Ala-Arg-Pro-

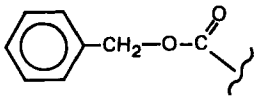
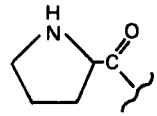
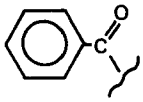
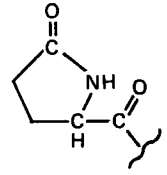
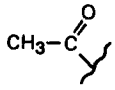
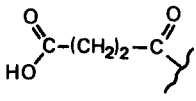
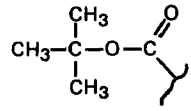
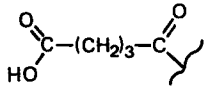
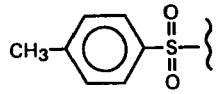
[a] Reference source for above sequences is (S27).

In designing and preparing synthetic peptide substrates, several items beyond that of active-site modeling must be considered (F6, H9, L10, T10). The length of the sequence should not exceed six amino acids to avoid cleavage at more than one site. The inclusion of proline in the sequence helps to encourage the desired single-site cleavage. The enzymatic hydrolysis of the substrate must obey Michaelis–Menten kinetics. The hydrolysis must be at a reasonable rate and liberate a product at a concentration that can be easily measured. As mentioned before, an amide bond is preferred over a substrate with an esterase linkage. The latter can easily be attacked by esterolytic enzymes which are readily found in blood or crude preparations from it. For chromogenic substrates, the released colored product should absorb at a wavelength where the substrate itself does not. The molecular extinction coefficient of the reporter group should be high to allow detection of low concentration of the enzyme. The pNA chromophore meets this requirement due to its high molar extinction coefficient, 9800 liters mol^{-1} cm^{-1} (S16). The solubility of the substrate in water should be at least 5 times its Michaelis constant (K_m) and excess substrate should not inhibit the action of the enzyme.

The selection of the right reporter group is obviously also very important to this design. This compound occupies the position of the neighboring amino acids following the cleavage point and consideration must be given to the impact of this group on the enzymatic action. There are at least three points to consider. The first is that the compound must not interfere with the reaction either by product build-up or by reacting with an activator for the enzyme in question. Second, the acylamide bond linking this agent to the amino acid in position S_1 must be stable to spontaneous hydrolysis, yet not resist enzymatic attack. The third is that the product from the enzymatic hydrolysis must remain in solution to allow spectrophotometric or fluorometric detection. Also, one must consider that while the sensitivity of some reporter groups can be enhanced through common azo coupling reactions (H9), these methods require endpoint analysis and therefore the desired automated kinetic methods cannot be used; a balance must be made in this area. Radioimmunoassays are considered the most sensitive method of analysis. Interestingly, results from fluorescence analysis for serine enzymes has shown detection limits comparable to those of radioimmunoassay (H9).

Finally, the impact of the blocking group at the amine terminal of the synthetic peptide substrate must be considered. One function of the blocking group is to prevent any exopeptidase attack on this end of the substrate. Also, the presence of this group has the effect of extending the length of the amino acid sequence and helps to provide additional recognition by the enzyme to the substrate. The blocking group may also be used to influence the water solubility of the substrate. In Table 5, the structures of ten com-

TABLE 5
COMMON BLOCKING GROUPS USED IN SYNTHETIC OLIGOPEPTIDE SUBSTRATES[a]

Blocking group[b]	Usefulness	Blocking group	Usefulness
N-α-Benzyloxycarbonyl 	Temporary protecting group in peptide synthesis. Provides an aromatic binding site for some enzymes. Chemical symbol: CBZ	Proline 	Blocking group particularly useful for excluding exopeptidase activity. Provides unique geometry within peptide structure, hydrophobic in nature. Chemical symbol: Pro
N-α-Benzoyl 	Stable blocking group provides aromatic binding sites for enzymes. Chemical symbol: Bz	Pyroglutamic 	Anhydrous form of glutamic acid found naturally in some proteins. Resistant to exopeptidase activity. Chemical symbol: Pyroglu
Acetyl 	Aliphatic analog of the benzoyl blocking group. Good stability. Chemical symbol: Ac	D amino acids D isomers of naturally occurring L amino acids.	Resistant to exopeptidase activity. Provides increased solubility. Chemical symbol: D amino acid
Succinyl 	Blocking group used for increased aqueous solubility. Chemical symbol: Suc	*t*-Butyloxycarbonyl 	Useful as a temporary protecting group but with limited solubility characteristics. Chemical symbol: Boc
Glutaryl 	Blocking group used to increase aqueous solubility. Allows for additional spacing in secondary binding sites. Chemical symbol: Glut	*p*-Toluenesulfonyl 	Very stable blocking group. Increases solubility. Also used as a pseudoaromatic binding site for enzymes. Chemical symbol: Tos

[a] From Huseby and Smith (H9), with permisssion.

[b] General structure of peptide B–$(AA)_n$–D, where B (blocking group), $(AA)_n$ (peptide), n (1 to 4 amino acids), and D (detector group).

mon blocking groups are shown. *N*-α-benzoyl is the most commonly used (H9). Proline is often considered because it prevents exopeptidase activity and it helps to mimic more closely the natural substrate. Of interest, is the use of the D isomer instead of the L form of the natural amino acids to again block exopeptidase activity and also to increase the solubility of the synthetic substrate.

One can use all of the above information to design the synthetic peptide substrate for maximal usefulness in these serine protease assays.

3.6. Practical Aspects of Assays with Synthetic Peptide Substrates

There are at least three areas of concern that each laboratory head faces as he or she considers switching from the current bioassays using the natural substrates to ones employing synthetic peptide substrates. The first is how does one report results so that the physicians will be able to relate to them. The second is the observation that different results have been reported on the stability of standard enzyme preparations when tested by both procedures. The third is the quality control programs for the synthetic substrates.

Results from a substrate assay may be reported in several different ways. For example, six different ways to report the level of the thrombin inhibitor, antithrombin III, have been used (S25). One system familiar to biochemists would be nanokatals (nkat) per unit of volume. A nkat is defined as the amount of enzyme which converts one nanomole of substrate per second. The second system, which would be more familiar to the coagulationist, reports results in terms of percent of normal or plasma equivalent units (PEU), one PEU being the amount of a substance present in 1 ml of a standard laboratory plasma pool drawn from normal donors. This question on reporting units becomes more important when the substrate assays are substituted in the global assays, PT and PTT. The latter clot-endpoint tests measure the total effect of many factors and it is known that the PT and PTT are insensitive to changes in some of the factors over a wide range, i.e., 60–120%. This point will be discussed later in Sections 6 and 7.

Important to the performance of substrate assays is the problem of availability of well-defined enzymes for standardization purposes. The noted variability within and among the currently available commercial reagents for thrombin, Factor Xa, and kallikrein as well as the observation that denatured or altered enzymes lose more of their biological clotting activity than their amidase potency illustrates this problem. Hemker has pointed out two examples of this latter concern where an apparently false higher level of activity was indicated by the substrate assay method (H3). With thrombin it

has been known that the native enzyme, α-thrombin, can degrade on storage to altered forms, β- and γ-thrombin (S11). It is the presence of the latter forms in standard thrombin solutions which will be detected by substrate assays but not by the clot-endpoint test (G1). One must take these concerns into account before standardization procedures can be established. Progress has been made on these questions through the work of the synthetic substrate committee of the International Society of Thrombosis and Hemostasis. The recent handbook from Hemker was designed to answer many of these questions (H5).

Various reports have been published concerning the area of quality control for these substrates. Friberger has outlined the extensive program employed by A. B. Kabi to ensure consistent quality (F5). Nine different tests are performed before a lot of substrate is released. The key test is the functional assay employing the enzyme for which the substrate is intended to be used. Stability claims (L5) for these reagents are 3 years in the dry state when stored at 2–8°C or 2 years if stored at 20–25°C. The stock solutions are stable for a maximum of 6 months if stored at 2–8°C and only 2 months if stored at 20–25°C. Contamination of the solution with enzyme or bacteria when pipetting or exposure to strong light must be avoided. The first sign of decomposition of these substrates is the appearance of a yellow color which may be visible to the naked eye at concentrations below 1% in both dry and liquid products. It should be kept in mind that, because the synthetic substrates are hydroscopic and often packed with inert fillers (manitol) stock solutions should be prepared spectrophotometrically rather than by weighing. Siverberg recommends making up solutions initially more concentrated than desired and then using the measured absorbance to calculate the dilution required to achieve the correct concentration (S16).

4. Assay Design

4.1. Principles of the Assay

Before discussing the various assays which have developed using synthetic peptide substrates, one should review briefly the basic principles for these procedures. In order to introduce substrate assays into a laboratory, a thorough knowledge of enzyme kinetics is desirable. Several excellent reviews on this subject are available (C4, S22, W4).

It has been shown that the relationship between substrate concentration and reaction velocity is hyperbolic and that the parameters of this curve are given by two factors, K_m and V_{max} (see Fig. 3). K_m is the concentration at which half-maximal reaction velocity is achieved. The smaller K_m value is for

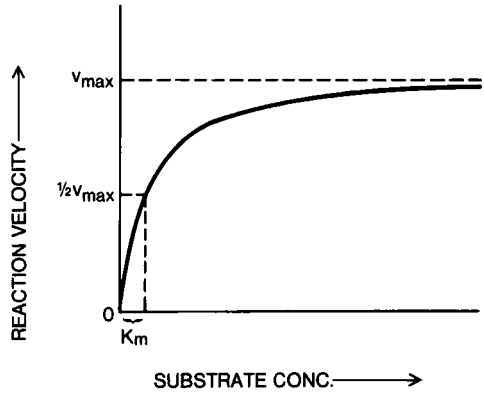


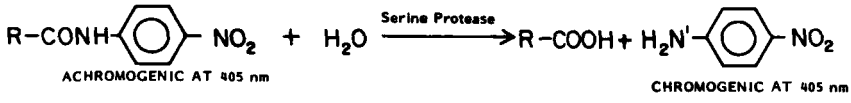
FIG. 3. Dependence of reaction velocity on substrate concentration.

a particular enzyme substrate system, the less substrate will be required for saturation and, hence, the greater affinity of the substrate for the enzyme. V_{max} is the maximal reaction velocity obtained at an infinite substrate concentration. The V_{max} term is proportional to enzyme concentration and another constant, k_{cat}, is the proportionality factor. An equation describing this hyperbolic relationship is given by the following formula derived from the Michaelis-Menten Equation (S16).

$$V = \frac{k_{cat}\, E\, S}{(K_m + S)}$$

At any given concentration, the initial velocity rate (V_0) is proportional to the enzyme concentration so the term V_0 can be used as a measure of the enzyme concentration provided certain precautions are taken. One should be aware of the variability of these reaction constants with pH, temperature, and ionic strength. Likewise, conditions for substrate concentration should be selected to guarantee no interference with the reaction rate. Usually, the concentration of the substrate is chosen to be well above the K_m value to ensure acceptable conditions (H3). As a side issue, it should be noted that substrates with a free N-terminal are 10 to 100 times more soluble than the corresponding benzoyl derivatives (T10). Finally, one should always report the conditions under which the assay was run to allow comparison with other laboratories.

In Fig. 4, one can see the familiar UV absorbance curve for an intact chromogenic substrate and of the free pNA at the same concentration. By monitoring the formation of pNA at 405 nm one is ensured minimal interference from the reactants (F5). A linear relationship exists between the concentration of the enzyme in the assay system and the release of either the



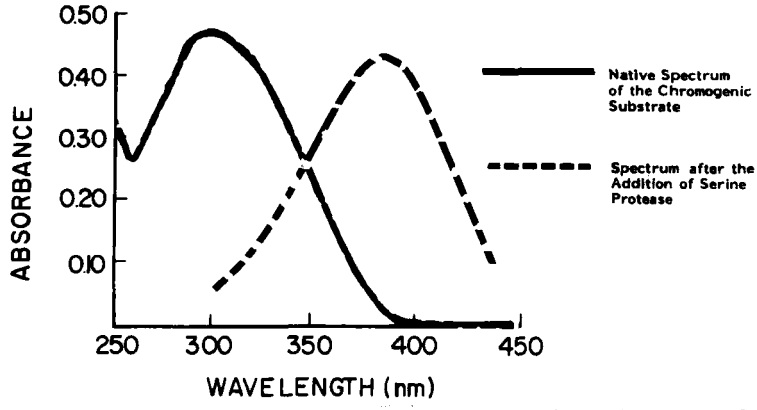


FIG. 4. Amidolysis of a synthetic chromogenic substrate and the relative spectral changes. [Reprinted with permission from Fareed *et al.* (F3). Copyright CRC Press, Inc., Boca Raton, Florida.]

chromogenic or fluorogenic indicator group over a specified time limit. Based on these principles, a standard curve can be prepared using pooled normal plasma and values of unknown samples can be read off this curve. For proenzymes and enzymes a direct relationship exists between concentration and response from reporter group, while for inhibitors there is an inverse relationship between these two parameters.

4.2. Equipment Needs and Automation Options

The basic equipment needed to perform the substrate assays is listed below:

1. Appropriate spectrophotometer or fluorometer which is equipped with thermostated cuvette housing and a recorder if the kinetic method is used.
2. Thermostated incubator.
3. Stop watch.
4. Automatic pipettes with disposable tips and disposal test tubes.

The response change such as the release of pNA can be followed by two methods. The first is called an endpoint method and measures the absor-

bance at 405 nm after a predetermined time following the addition of a reagent like acetic acid or citric acid to stop the reaction. The developed color is usually stable and the samples can be read at any convenient point up to 4 hours. The second method is a kinetic or initial rate method by which the released reporter group can be followed on a recorder or by using repeated readings at regular time intervals such as every minute.

There are several factors encouraging the development of automated sub-

TABLE 6
AVAILABLE AUTOMATED INSTRUMENTS AND APPLICATIONS[a]

Type of automated instrument	Number of samples/run	Application
I. Centrifugal analysis		
GEMENI	20	Factor X
CENTRIFICHEM	30	Factor X, antithrombin III, and prekallikrein
GEMSAEC	15	Factor X, antithrombin III, and heparin
Cobas Bio	30	Factor X, antithrombin III, heparin, prekallikrein, and α_2-antiplasmin
Multistat III	20	Factor X, antithrombin III, heparin, and α_2-antiplasmin
II. Multichannel analyzer		
Olli 3000	24	Factor X
III. Selective/batch/random access analyzer		
Technion RA-1000	30	Factor X
Vitatron XYP	128	Prothrombin, Factor X, antithrombin III, plasminogen, and α_2-antiplasmin
IV. Others		
KEN-O-MAT	32	Factor X, antithrombin III, prekallikrein plasminogen, and α_2-antiplasmin
Abbott VP	32	Antithrombin III and heparin
LKB 2086	—	Prothrombin, Factor X, antithrombin III, and α_2-antiplasmin
LKB 8600	—	Prothrombin, antithrombin III, prekallikrein, and α_2-antiplasmin
Gilford 3500	Flow thru cuvette	Prothrombin, Factor X, antithrombin III, plasminogen, and α_2-antiplasmin
Vitatron PA800 and ACES	100	Prothrombin, Factor X, antithrombin III, heparin, plasminogen, and α_2-antiplasmin

[a] Source of above data is reference (F6).

strate assays: increased precision, more efficient operation, need for smaller plasma samples, and lower assay costs (F2, F5, H3, S9). This type of equipment is designed around the central part of the substrates assay system, the photometric reaction rate response. These instruments usually have a sample dilution unit in which small volumes, 5 to 50 μl, are dispensed and diluted with a large volume of buffer. The temperature of the mixture is then adjusted and controlled prior to the reagent addition. The instruments are usually programmed to carry out these actions for each sample simultaneously. The number of samples tested per cycle can vary from 20 to 150 depending on the instrument design (F6). Some equipment like the LKB 2086 test each sample continuously while others of the centrifugal analyzer type perform a large number of discrete readings of each sample. Two features which are attractive are units which automatically report out values in enzyme activity units or percent of normal plasma and those which allow random access so a choice of assays can be done. While automation has its advantages there are potential problems to be aware of. Since these serine proteases are hydrophobic they will adhere to surfaces. Also, exposure to glass must be avoided to prevent surface activation of these enzymes. One must also take precautions to minimize opportunities for bacteria contamination in the delivery and storage components. Table 6 lists some of the available automated instruments used for these assays.

Aiach and co-workers have reported on the adoption of five different substrate assays onto an automated discrete analyzer (A2). They observed excellent within-run precision coefficient of variation (CV) values ranging from 1 to 3%, as well as satisfactory day-to-day precision. Ito and Statland similarly evaluated and compared the DuPont aca and Kabi/CentrifiChem automated method for measuring antithrombin III and plasminogen (I3).

4.3. Optimal Assay Conditions

There are many factors that one must consider to optimize the substrate reaction method. Starting with the biological test sample, such as plasma, one must usually adequately dilute the specimen to minimize interference by substances like bilirubin and hemoglobin on the photometric response as well as prevent clotting of the sample due to the presence of the natural substrate, fibrinogen. Most assay systems minimally dilute the starting material 1 : 100 to avoid these interferences. Frequently the buffer used for this dilution is tris(hydroxymethyl)aminomethane at a concentration of 0.05 to 0.10 *M*, pH of 7.5 to 8.5. In a comprehensive study, Lottenberg and coworkers studied the effect of pH and buffer composition in a thrombin assay system with two different synthetic peptide substrates (L13). Large differences in the k_{cat}/K_m ratio were observed. The specificity of these synthet-

TABLE 7
CROSS-REACTIVITY OF CERTAIN SUBSTRATES[a]

Substrate	Target enzyme	Activity (k_{cat}/mole of enzyme)			
		α-Thrombin	Factor Xa	Plasmin	Trypsin
S-2160	Thrombin	36.690	0.035	1.180	20.850
S-2238	Thrombin	241.500	0.104	2.040	23.730
CBZ-Chromozym TH	Thrombin	178.800	0.094	4.980	83.300
Tos-Chromozym TH	Thrombin	308.200	11.100	10.890	155.900
S-2222	Factor Xa	0.470	13.200	1.810	89.730

[a] Source of data is Reference (B2).

ic substrates is quite impressive as evidenced by the finding that S-2238 was more specific for thrombin than the natural substrate, fibrinogen (C6, S8). Still, certain precautions should be taken to minimize the well-known interferences from other related enzymes in the test fluid (C2). The magnitude of this cross-reactivity for some selected substrates is shown in Table 7. Two methods for blocking a second enzyme are the addition of specific inhibitor and the adjustment of the pH and ionic strength of the assay buffer (L5, L10). These precautions are obviously needed in a complex enzyme system like clotting where the various proenzymes and enzymes interact to activate each other.

In order to perform a meaningful substrate assay, it is necessary to select conditions that allow the accurate determination of the initial reaction rate, V_0. For the kinetic method with chromogenic substrates the change in absorbance per minute should be between 0.01 and 0.10. At that rate, there should be a minimum consumption of the substrate and the reaction is slow enough to be recorded accurately. It has been reported that an enzyme concentration of 0.02–0.20 μkat/liter will generate that response (F5). For the endpoint method, the same worker recommends a target absorbance range of 0.2–0.8 for maximum accuracy. For an assay with a 2-minute incubation period, an enzyme concentration of approximately 0.4 μkat/liter is recommended. Experience has shown that it is important to prevent adsorption of the enzyme to surfaces such as test tubes, cuvettes, or pipettes (F5, S16). As expected, this problem is more pronounced with pure enzyme solutions. The workers from A. B. Kabi have found that the addition of certain surfactants like Carbowax 6000 or Triton X-100 or proteins like heat-treated albumin or γ-globulin in a concentration of 0.1 to 0.5% act to prevent this adsorption (F6).

Figure 3 indicates that it is advantageous to work at a substrate concentration that is higher than K_m. The rate measurement will then be close to

maximum and in an area relatively insensitive to substrate concentration (F5, H3, S16). This approach offers the advantages of reducing sensitivity to error in the preparation of the substrate solution, reducing the effect of substrate exhaustion on the linearity of the assay and minimizing product inhibition. Since several different substrates are available for detecting a particular enzyme (see Table 3), the selection of the one with the lowest K_m value helps to optimize the assay and generally offers a lower per-test cost (S16). The specific activity observed depends on the k_{cat} value. Several factors can be manipulated to increase k_{cat}. Some of the parameters having this effect are ionic strength, pH and composition of the assay buffer, and the reaction temperature. An excellent reference to supply kinetic data useful for substrate assay design was reported by Lottenberg and co-workers (L13). For every 10°C increase, the reaction rate will generally double. An assay temperature of 37°C is usually preferred since higher temperatures can cause denaturation of the enzymes. However, some workers prefer either 30°C or even 25°C. Friberger has reported better assay reproducibility when the reaction temperature is accurately controlled (F5).

With the work of the last 12 years, adequate guidelines are available to ensure that accurate and precise substrate assays can be designed as long as certain good laboratory practices are followed (L5). The next sections will discuss some applications to detect the serine proteases of the coagulation and fibrinolytic systems. The reader is referred to the excellent handbook by Hemker which lists assay details for many of the procedures discussed below (H5).

5. Measurement of Specific Factors

5.1. Thrombin and Prothrombin

Studies by many workers have revealed two peptide sequences which are very selective for thrombin: -Phe-Val-Arg- and -X-Pro-Arg- (C5, S24, S26). As can be seen in Table 3, these two sequences have been widely used to design the various synthetic substrates to detect thrombin. This measurement can be done directly by incubating this enzyme with the appropriate substrate. Latallo has recommended adding an agent like aprotinin or soybean trypsin inhibitor to prevent attack by kallikrein, Factor Xa, or plasmin on these thrombin substrates (L5). The use of the original substrate, S-2160, has sharply declined due to its lower sensitivity, specificity, and solubility when compared to substrates like S-2238 and Chromozyn TH. Today, S-2160 is no longer commercially available.

A direct assay cannot be used to measure the level of prothrombin in plasma. This zymogen must first be converted to thrombin and then the

synthetic substrates designed for thrombin can be used. There are several types of prothrombin activators; nonphysiological ones like *Echis carinatus* venom, trypsin, or staphylocoagulase and the natural one, prothrombinase (S21). The latter consists of Factor Xa bound to a phospholipid surface in conjunction with Factor Va (H4). The methods used for prothrombin measurement differ mainly in choice of activator and synthetic substrate. As early as 1974, Bergstrom and Blomback had designed a chromogenic assay based on Factor Xa conversion of prothrombin (B6). Since then, many assays using different activators have been published (B5, L6, W4).

The reasons for measuring plasma prothrombin concentration are to screen for genetic deficients and, more important, frequently to monitor the anticoagulant state of a patient on vitamin K anticoagulant drugs. While the frequency of congenital dysprothrombinemia is rare, at least 11 cases worldwide have been studied following the initial discovery by Shapiro and coworkers in 1969 (B10, G5, O7, S14, W1). In his review, Triplett has listed data showing that for all 11 prothrombin variants the clotting activity generated with prothrombinase was uniformly abnormally low (T9). Mixed results were obtained if the nonphysiological activators were used or if immunological assays were performed. Girolami and co-workers reported that for two of these variants, prothrombin Molise and prothrombin Padua, normal level of activity was observed with a chromogenic substrate assay and therefore these deficiencies would have been missed (G6). It appears that, as noted in Section 3, there will be occasions where these two assay systems, clotting and synthetic substrate, will yield different results. This problem is also a concern in monitoring patients on vitamin K anticoagulant drugs who have both forms, decarboxylated and carboxylated, of prothrombin as well as Factors VII, IX, and X in their circulation (F10, L11). In Section 6, the similarities and differences seen with these patients when tested by these two assay systems will be discussed in more detail.

5.2. Factors X and Xa

Based on the work of Magnusson and co-workers with bovine prothrombin, the sequence of -Ile-Glu-Gly-Arg- has been identified as being selective for Factor Xa (M2). The commercial substrates intended to detect this enzyme therefore contain the sequence or a close derivative (A6, A7, M1). Two areas of concern in attempting to measure Factor Xa activity in biological samples are the interference from any generated thrombin and the inhibition by antithrombin III and heparin. Because of the low concentration of Factor X in plasma, only 1/10 that of prothrombin (F6, L5), the test sample can only be diluted 40- to 50-fold and one must be more concerned over fibrin formation. Van Wijk and co-workers have recommended the use of the

inhibitor hirudin to reduce this problem (V4), while Latallo and co-workers used the addition of KCl to the buffers to block secondary thrombin and hence fibrin formation (L8). Inhibition by antithrombin III–heparin complex is obviously more of a problem with those patients on heparin therapy. The addition of polybrene in low concentration appears sufficient to block this inhibition of Factor Xa (B7, V4). It has also been shown that calcium can inhibit the amidolytic activity of normal Factor Xa but not that of the decarboxylated species (L11).

As with the case of prothrombin, Factor X can be activated by both physiological and nonphysiological activators. For substrate assays, both types of activators have been employed. An example of the former is the use of thromboplastin (A6, V5); an example of the latter is the employment of Russell's viper venom (RVV) in the presence of calcium ions (A1, B7, V4). In general, the latter activator appears to be favored because it does not require phospholipids for activation; hence thrombin generation is retarded (A6, D1), and decarboxylated Factor X is not activated by RVV. The most frequently used substrate has been S-2222, but S-2337, which has a lower K_m, may become more widely used (A8, S16).

There are a number of potential clinical applications for monitoring plasma Factor X or Factor Xa levels. It is suggested that small amounts of Factor Xa may exist in the proposed "hypercoagulable state" (V9). Also, the measurement of Factor X levels in oral anticoagulant patients has been under evaluation since 1978 (B7, E2, G7, I1, R3). Finally, this functional assay may be useful for detecting hereditary Factor X deficiency. In 1971, Prydz and co-workers reported finding this condition using both immunologic as well as clot-endpoint assays (P7). Girolami and co-workers had earlier discovered a group of patients in the Friuli district of northern Italy whose Factor X was slowly or not at all activated by the extrinsic pathway (G4). It is now claimed that Factor X Friuli represents the major Factor X variant and that either the substrate assay using S-2222 or the standard clot-endpoint assay can be used to detect it (G8).

5.3. Factor VII

Factor VII is the only coagulation factor in the circulating blood which has enzymatic activity in its native form (O3). While Factor VII has esterase activity, it must interact with tissue thromboplastin to form a potent activator of Factor IX or Factor X (O5). An assay which could detect the presence of Factor VIIa in the circulation could help to screen for those patients at a high risk for cardiovascular disease.

As of now, there is no specific synthetic peptide substrate for measuring Factor VIIa. Factor VII is indirectly determined by coupling the Factor VII

activation of Factor X to Xa and then measuring the generating Factor Xa activity by using substrates like S-2222 and S-2337 (A10, S13). This assay system requires a mixture of purified Factor X, tissue factor, and calcium (T10). Certain precautions must be followed to optimize an indirect assay system (S16). It is desirable to have as few steps as possible between the component of interest and the generated enzyme to minimize interferences.

In general, the Factor VII activity estimated by a substrate assay correlated well with Factor VII activity determined by a clot-endpoint assay (P4, S13). However, some differences have been seen. Activation of Factor VII by kaolin or by exposure to cold temperatures (2–8°C) increased the level of clotting Factor VII by 4–7 times but had no effect on the amount detected by the substrate assay (S9, T10). It has been postulated that the partially carboxylated molecules of Factor VII present in patients' plasma during anticoagulant therapy are detected by a substrate assay but not in a clot-endpoint test (P4). These differences between the two assay systems have been used to screen for hereditary Factor VII deficients and to detect the presence of Factor VIIa in thrombotic disorders and in components for transfusion therapy (S13).

5.4. Prekallikrein and Factor XII

The activation of Factor XII to XIIa is the initiation step in the intrinsic pathway (see Fig. 1). The event is frequently referred to as the contact activation system since the zymogen will undergo conversion to Factor XIIa on exposure to negatively charged surfaces like glass test tubes (H8). This system is not only important to coagulation but also plays a role in fibrinolysis, complement activation, generation of kinin, and regulation of blood pressure (C8). There is a circular activation relationship between two of the major components of the contact activation system, prekallikrein–kallikrein, and Factors XII and XIIa. First, Factor XIIa can catalyze the conversion of prekallikrein to the enzyme kallikrein. Next, the latter can either cause the formation of Factor XIIa or digest the latter single-chain structure into a degraded species. Interestingly, this is another example of the feedback control mechanism of the entire hemostasis system discussed in Section 2, since kallikrein's action leads to the production of plasmin and the degraded form of Factor XIIa loses its ability to activate Factor XI (C8, M5).

Assays for plasma prekallikrein have been available for over 10 years. Since the immunologic assays fail to distinguish between prekallikrein, kallikrein, and kallikrein-inhibitor complexes, a functional assay (clot-endpoint or synthetic substrate), is preferred to monitor the patient status (F4). Functional assays of prekallikrein, however, have encountered technical problems. To measure prekallikrein in plasma, it must be quantitatively converted to kallikrein. As stated above, this transformation is dependent on the

presence of Factor XIIa and a sufficient concentration, >10% of normal, of the protein cofactor, high-molecular-weight (HMW) kininogen (M5). Also, once kallikrein is formed in plasma, the enzyme is rapidly inhibited by the action of C_1-inhibitor and α_2-macroglobulin. Thus, the concentration of inhibitors influences the assay of prekallikrein and must be controlled by proper dilution, use of cold temperature activation or employment of certain organic solvents, like acetone (F4, K2). Another factor for consideration is the selection of the best activator. In general, ellagic acid or dextrose sulfate are preferred over kaolin because of the need for an optically clear medium (K2, L2). Since calcium ion is not required for activation of prekallikrein, citrated blood can be used directly with minimal subsequent interference by Factor Xa and thrombin.

Several manual and automated procedures using chromogenic or fluorogenic substrates to measure prekallikrein levels have been published (G2, I2, I4, M7). The amino acid sequence of -Ser-Pro-Phe-Arg- is commonly used based on the two peptide-bond cleavage sites favored by kallikrein in HMW kininogen. The assay has been used to monitor for endotoxemia, liver disease, and hypertension.

To date, none of the sequences cleaved by Factor XIIa in its natural substrates has been reported. This enzyme can cleave substrates S-2302 (H-D-Pro-Phe-Arg-pNA) and S-2222 (BZ-Ile-Glu-Gly-Arg-pNA) but at a rate much less than the enzymes for which these substrates were designed, kallikrein and Factor Xa, respectively (S16). In 1979, Vinazzer published a chromogenic substrate procedure for Factor XIIa which utilized S-2302 and employed epsilon aminocaproic acid to control the cross-reactivity of plasmin (V7). This method was an indirect procedure based on the ability of Factor XIIa to convert prekallikrein to kallikrein and good correlation was found with the APTT clot-endpoint assay. This investigator found the generated Factor XIIa to have a significantly longer half-life than kallikrein and this observation can be used to set up a two-stage assay employing an external source of prekallikrein rather than utilizing the sample source (V8). Recently, Kluft and coworkers tested over 80 synthetic substrates for their specificity for Factor XIIa (K3). They identified 10 that were sensitive for both Factor XIIa and kallikrein. The amino acid sequence of -Leu-Gly-Arg- had the highest activity ratio for Factor XIIa over kallikrein.

Specific assays for Factor XII have been recommended as an indication for intravascular clotting activity and transplant rejections (V8).

5.5. Factors XI and IX

Factor XI is another component of the contact activation system but it is the only factor whose absence may result in a hemorrhagic disorder. Factor XI in plasma is activated *in vitro* by Factor XIIa and the co-factor, HMW

kininogen (S5). Traditionally, Factor XI has been assayed by a modified APTT clotting assay. In 1979, Iwanaga and co-workers reported a fluorogenic substrate assay for this factor (I4).

The major plasma inhibitor of Factor XIa is α_1-antitrypsin while antithrombin III, in the presence or absence of heparin, plays a minor role in controlling this enzyme (S5). Interestingly, plant inhibitors like soybean, corn, and lima bean trypsin inhibitors are able to block Factor XIIa and kallikrein but only mildly inhibit Factor XI. Thus, one can use these agents to minimize cross-reactivity in an indirect synthetic substrate assay for Factor XIa.

Recently, Scott and co-workers reported on the development of a chromogenic substrate assay for Factor XI in plasma (S6). The substrate used had an amino acid sequence of -Glp-Pro-Arg-, S-2366 from A. B. Kabi.[2] A complex series of steps was needed to ensure precision and accuracy of this indirect assay. First $CHCl_3$ was added to neutralize plasma protease inhibitors, especially α_1-antitrypsin. Next, kaolin was used to activate Factor XIIa which then converted Factor XI to XIa. Soybean trypsin inhibitor and corn trypsin inhibitors were added to block the activity of kallikrein and Factor XIIA after the desired activation of Factor XI had occurred. The method required the presence of Factor XII and HMW kininogen. Prekallikrein was not an absolute requirement, but its presence helped to accelerate the overall reaction. The intra-assay CV was only 3.4%, and good correlation with coagulant activity for Factor XI was reported by this group. However, they recommend a finding of low patient Factor XI activity be confirmed by a separate testing for Factor XII and HMW kininogen.

Factor IX (antihemophilia Factor B) participates in the intrinsic pathway as the enzyme that activates Factor X with the help of a cofactor, Factor VIIIa, phospholipids, and calcium ions. From studies with purified bovine and human Factor IX, we have learned how this zymogen undergoes activation. Factor IX can be converted by Factor XIa in the presence of calcium ions. During this activation, two internal bonds, Arg^{146}-Ala^{147} and Arg^{181}-Val^{182}, are cleaved and a carbohydrate-rich activation fragment of molecular weight 10,000 is released (L12). The complex of tissue factor and Factor VIIa also activates Factor IX, possibly by cleavage of the same two bonds (O5). Several methods have been used to determine Factor IX (T3): a clotting assay using Factor IX-deficient patient plasma, an esterase assay using radiolabeled esters (like benzoyl-L-arginine ethyl ester), detection of the released carbohydrate-rich fragments, and active site titration with pNPGB. Only the clotting assay can be used to determine Factor IX in plasma.

In 1982, Tans and co-workers reported an indirect synthetic substrate

[2] Glp refers to a modified form of glutamic acid, pyroglutamic, whose structure is shown in Table 5.

assay using S-2222 to detect this zymogen in plasma (T3). Earlier work by Kabi to develop a direct test based on the amino acid sequence at the cleavage site of bovine Factor X proved nonspecific for Factor IXa (S20). The former method added purified Factor XIa to the test plasma sample. The generated Factor IXa was then measured by adding an aliquot of this plasma to a mixture of Factor X, calcium ion, and phospholipids. The subsequent generated Factor Xa then digested its specific substrate, S-2222. The workers found that this method was proportional to the level of Factor IX in plasma and that decarboxylated forms of this zymogen did not participate in the reaction. Hence good agreement with the Factor IX level in anticoagulated patients was observed in both the standard clot-endpoint test and this synthetic substrate assay.

One additional area where synthetic substrate assays may prove useful is in the characterization of the abnormal variant Factor IX molecules. Previously, Bertina and Veltkamp proposed seven other factors to test with (B8). The future development of a substrate more specific for Factor IXa activity could be very helpful.

5.6. Factors V and VIII

The roles of Factors V and VIII in the reaction sequence leading to thrombin formation have slowly become known. Both these proteins are very large in size, serve as cofactors in this cascade reaction sequence, and increase by approximately 100 times their cofactor activity upon exposure to minimal concentrations of thrombin. Likewise, both factors are inactivated by a similar mechanism when attacked by activated protein C (F11). Tans and coworkers utilized synthetic substrate assays specific for Factor Xa and thrombin to demonstrate that both these factors act predominantly by increasing k_{cat} (T2).

Since both these proteins are cofactors and not proteases, the assay system for synthetic substrates involves a complicated method that requires the availability of purified coagulation factors. For Factor VIII, the assay is based on the rate of Factor X activation, and the conditions must be chosen so as to make the test proportional only to the Factor $VIII_c$ activity. In 1979, Segatchian reported on a two-stage test method that utilized the chromogenic substrate S-2222 (S12). In this assay the inhibitor hirudin is added to block thrombin's activity so the generation of Factor Xa is the critical parameter. Recently Rosen has reported on a modified assay (R4).

Clinically, disorders of Factor VIII are the most common inherited abnormalities of hemostasis. Classical hemophilia A, Factor $VIII_c$ deficiency, occurs in approximately 1 out of 20,000 live births in the United States.

For Factor V, the assay is based on the formation of thrombin. Niesheim and Mann using the original thrombin synthetic substrate, S-2160, reported

in 1979 on an indirect assay to detect Factor V levels (N2). In 1983, two additional reports appeared from different laboratories (H1, K1).

5.7. Protein C

Protein C, like Factors II, VII, IX, and X, is a vitamin K–dependent protein. Activated protein C (PCa) can be derived by limited proteolysis of this zymogen by thrombin, trypsin, or by Russell's viper venom (V1). PCa, in contrast to the other vitamin K–activated factors, exerts an anticoagulant activity by inhibiting Factors Va and VIIIa (F11). A second activity identified for PCa is the release of plasminogen activator based on animal *in vivo* studies (C10, Z2). These biochemical observations and the discovery of patients with recurrent thrombosis who are congenitally deficient in protein C make PCa a candidate for a central role in hemostasis (B17).

Assays for protein C have had several problems to overcome: low concentration in plasma, endogenous inhibitors for PCa, and lack of specific activation methods. The zymogen has been monitored by immunoassay procedures (B9, M3). PCa possesses amidolytic activity and can be measured using synthetic substrates. Early work with both chromogenic and fluorogenic systems explored the amino acid sequence -Leu-Ser-Thr- as the target substrate (O2). Recently, Sala and co-workers reported an improved three-step method utilizing the chromogenic substrate S-2266, H-D-Val-Leu-Arg-pNA (S1). In the first step, the vitamin K–dependent factors are removed from plasma by a barium citrate adsorption. The protein C freed from its natural inhibitors is next activated to PCa by a complex of thrombin and its endothelial cell cofactor, thrombomodulin. Finally, the generated PCa is monitored by its attack on the substrate S-2266. For this complex system to work, one must also add antithrombin III and heparin to control thrombin and any other enzymes generated during the incubation period. These workers claimed a good within-assay precision (3.5%) and high correlation with other published immunologic and clot-endpoint assays.

5.8. Platelet Factors 3 and 4

Intact platelets do not show procoagulant phospholipids on their exterior. These phospholipids are located on the inside leaf of the bilayer membrane (H6). They become accessible when the platelets are disrupted or by a process initiated by small amounts of collagen and thrombin that shifts these procoagulant agents from the inside to the outside. Both in prothrombin and intrinsic Factor X activation, phospholipids cause a sharp decrease in the K_m for prothrombin and Factor X, respectively (T2). The procoagulant activity of the platelets is usually called platelet factor 3 (PF3). In the circulating blood

the level of the free PF3 is believed to be very low under normal conditions. The activity in plasma has usually been determined by a clot-endpoint test which is based on the activation of Factors V and X by Russell's viper venom. This assay is easy to perform but has some limitations. It has low sensitivity, is rather nonspecific and cannot be used if the anticoagulant solution contains ETDA.

A PF3 indirect assay using a synthetic substrate was developed in 1979 by Sandberg and Anderson (S2). This test monitored thrombin production with the substrate S-2238 and claimed to be 10 times more sensitive than the above assay and to be free of EDTA interference. Later Harsfalvi and co-workers claimed to improve the technique by adding soybean trypsin inhibitor to better control the reaction (H2). These workers believe this assay can be used for diagnosis of platelet disorders.

Test systems to monitor a second platelet release product, platelet factor 4 (PF4) are also under study. PF4 is a protein which has heparin-neutralizing properties. Radioimmunoassays have been reported for PF4 which are able to measure this component in the nanogram range much lower than that possible with a clot-endpoint test (L9). A functional assay for PF4 is based on neutralizing endogenous Factor Xa or thrombin in the presence of an inhibitor complex of antithrombin III and heparin. If increasing amounts of PF4 are present in the assay, heparin will be neutralized and either of the above enzymes will react at an increased rate. Synthetic substrate assays have been reported using either S-2222 for Factor Xa monitoring or S-2238 for thrombin detection (V6, W6).

The choice of which enzyme to monitor is important. Some workers prefer to select Factor Xa because it does not react with fibrinogen or cause the platelet release reaction as thrombin does. Wohl and co-workers prefer to test for thrombin activity since they found S-2238 to be more sensitive than S-2222 (W6). This objection could be overcome by using a substrate (S-2337) which is claimed to be more sensitive for Factor Xa (S16). As of now, the synthetic substrate assay for PF4 is unlikely to replace the radioimmunoassay because of lower sensitivity.

5.9. Plasminogen and Plasmin

The determination of plasminogen levels and the enzyme, plasmin, derived from this zymogen has been considered difficult because of the presence of potent inhibitors in human plasma. The older methods, utilizing natural substrates such as casein, lack the desired sensitivity, are lengthy and involve procedures not easily adapted for the routine laboratory or for interlaboratory comparison (F3). Because of the above concerns and the general interest in the fibrinolytic system, substantial research has been devoted to develop a synthetic substrate method for these two components.

TABLE 8
RELATIVE SENSITIVITY AND SELECTIVITY OF VARIOUS PLASMIN SUBSTRATES[a]

Peptide sequence	Plasmin	Thrombin	Kallikrein	Urokinase
BZ-Phe-Val-Arg	100	100	100	100
BZ-Ile-Leu-Arg	300	2	500	200
BZ-Ile-Leu-Lys	600	2	50	30
D-Val-Leu-Lys	500	1	70	20

[a] Adapted from Huseby and Smith (H9).

As early as 1975, Friberger and co-workers had attempted to use the chromogenic substrate S-2160 for this purpose (F7). The lack of specificity and relatively poor water solubility of S-2160 led researchers to search for a better substrate. Guiding this design was the knowledge of the amino acid sequences and activation sites within the plasminogen molecule (W2). Clavin and co-workers prepared a fluorogenic substrate using the amino acid sequence of -Ala-Ala-Lys- which markedly improved the specificity toward plasmin (C7). These workers found that if arginine is present in place of lysine at the C-terminal position, the specificity of the substrate for plasmin is greatly reduced. Additional substitution of more hydrophobic groups in place of alanine increased the sensitivity even more. Friberger *et al.* utilized the above to design a highly specific plasmin substrate known as S-2251 from Kabi (F8). This chromogenic substrate had the peptide sequence of H-D-Val-Leu-Lys-pNA. Pochron and co-workers used the same three amino acid sequence but switched reporter groups to that of a fluorogenic type, AIE (P3). In Table 8, one can compare the relative sensitivity of some synthetic substrates for plasmin and other enzymes.

There are several ways in which plasminogen can be activated to plasmin and the method chosen will influence the design of a synthetic substrate assay. Most normal tissues, except those of the liver, contain tissue plasminogen activators (t-PA). These activators are mainly localized in the lysosomal fraction of the cells and, until recently, have presented a challenge to isolation in pure form. The amount of t-PA present in plasma is usually quite low but it can be increased 50-fold by exhaustive exercise or by a 15-minute venous occlusion (W3). When a clot is formed, t-PA exhibits a high affinity for fibrin; so its content in serum is extremely low. The t-PA extracted from pig heart, pig ovaries, and human uterus is a single-chain serine protease with a molecular weight of 64,000 (F5). The vascular plasminogen activator (VPA) has been isolated from cadaver limbs and found to behave identically to t-PA in immunological studies and its affinity for fibrin. Current theory calls for t-PA to be synthesized in the vascular wall and then

released into the circulating blood (R2). Interestingly, polylysine can substitute for fibrin and markedly increase the activity of VPA in assay systems (A3). Urokinase (UK) is a two-chain trypsin-like serine protease which can be isolated from urine or from kidney cell cultures. Two forms of UK have been found in urine, a high-molecular-weight species of 54,000 (native) and a low-molecular-weight form of 32,000 (degraded). From both immunochemical and substrate specificity studies, it has been found that UK differs from most tissue or vascular plasminogen activators. Another source of a natural activator comes from the contact activation system generation of kallikrein. The latter enzyme has a rather low plasminogen activating activity compared to UK at the same molarity but the relatively high plasma concentration of prekallikrein suggests this activation must be considered. Likewise, bradykinin, released by the presence of kallikrein in blood, can potentiate the release of t-PA (F5). Finally, there are exogenous plasminogen activators of which streptokinase (SK) has been most studied since the first report on this phenomenon over 50 years ago by Tillett and Garner. SK is a protein product of certain strains of streptococci and has a molecular weight of 47,000. SK has no enzymatic activity by itself but it readily forms 1 : 1 stoichiometric complexes with either plasminogen or plasmin, which do possess both protease and amidolytic activity. For those interested, a review of the SK–plasminogen interactions has been published (R1). SK has an advantage over activators like UK in that the SK–plasminogen or SK–plasmin are not susceptible to neutralization by plasmin inhibitors like α_2-antiplasmin or exogenous ones like soybean trypsin inhibitor. However, one must consider that patient to patient differences could exist over different amounts of anti-SK titers due to streptococcal infections.

In plasma, plasminogen exists in two forms. A native specie is known as Glu-plasminogen, a single-chain protein with a molecular weight of approximately 90,000. A partially degraded form, also a single chain with a molecular weight of approximately 83,000, is known as Lys-plasminogen. Plasmin can be derived from both zymogens but UK can activate Lys-plasminogen at a faster rate and the latter has a higher affinity for the fibrin matrix. In a recent report a synthetic substrate has been used to help study the activation of these two forms of plasminogen (T1).

From the above, one can appreciate the complexities in designing a synthetic substrate assay for plasminogen and plasmin. Friberger has recommended certain guidelines in his recent review (F5). Most workers prefer SK over the other available activators. Latallo reported a total evaluation of the fibrinolytic system in patients (L7). This profile included the measurement of free plasmin activity and the concentration of plasminogen, its activators and inhibitors. A fully automated assay for plasminogens on a clinical analyzer has also been reported (P1). Comparison of the synthetic substrate

methods for plasminogen with immunologic or caseinolytic assays have shown a high correlation (F6).

Assays to monitor the fibrinolytic system have been recommended to help monitor disseminated intravascular coagulation, thrombolytic therapy, and oncogenic transformation. (F6, H9).

5.10. Inhibitors

The principle of assaying inhibitors by synthetic substrate methodology is simple. In the presence of an excess of the enzyme under study, the enzymatic activity remaining after reaction with the inhibitor is inversely related to the concentration of the inhibitor. Because reliable and easy to perform techniques with natural substrates have not been available, synthetic substrate assays for inhibitors have gained wide acceptance and commercial kits for some are available. Some of the inhibitors which are of interest are antithrombin III, α_2-antiplasmin, α_1-antitrypsin, C1 esterase inhibitor, and α_2-macroglobulin.

Antithrombin III (AT-III) is a plasma protein which is also known as heparin cofactor and Factor Xa inhibitor. The molecular weight of this protein is approximately 65,000 and it circulates in blood in a form which allows progressive inhibition with a series of serine proteases such as thrombin, Factors IXa, Xa, XIa, XIIa, and plasmin. It is, however, believed that the main role of AT-III is to block the action of Factor Xa and thrombin. The rate of this inhibition of these two enzymes is greatly potentiated in the presence of heparin. The therapeutic efficacy of heparin is greatly dependent on the AT-III level and those individuals lacking this inhibitor fail to respond to heparin therapy. A positive correlation between diminished AT-III concentration and an increase of thromboembolytic complications has been suggested by several studies (T6). In 1970 Fagerhol and Abildgaard published a definitive report on the effect of sex and age on the level of this inhibitor in humans (F1).

AT-III levels can either be measured by standard immunological methods or by a functional assay employing either a clot-endpoint or a synthetic substrate procedure (B4, C11, Z3). Since the immunological test can only determine concentration, not biological activity, most workers prefer a functional assay. In 1982 Philo and Gaffney compared the two types of functional assays (P2). They concluded that for assays without heparin good agreement was found, while in the presence of heparin a difference between clotting and amidolytic methods was observed. The latter was attributed to the need for a defibrination step in the clotting assay.

The principles of the AT-III assay performed in the presence of heparin is shown below (S25).

1. AT-III + heparin → [AT-III–heparin]
2. [AT-III–heparin] + thrombin(excess) → [AT-III–heparin–thrombin] + thrombin–residual
3. Peptide–X + thrombin(residual) → peptide + X
   X = pNA or AIE

The amount of thrombin inhibited reflects the amount of AT-III in the sample. The addition of heparin reduces interference by other plasma inhibitors like α_2-macroglobulin. It has been reported that the species of thrombin, bovine or human, used in the assay can influence abnormally low AT-III values. Friberger and co-workers in a 1982 report recommended bovine thrombin to insure better agreement with immunological AT-III values (F9).

The occurrence of AT-III deficiency is quite common. It has been estimated that in the Norwegian population, 1 in 5000 individuals have low levels of this inhibitor. Bauer and co-workers, in a recent review, compared the characteristics of five different types of abnormal AT-III molecules isolated from various patients (B4). The AT-III synthetic substrate assay was one of the parameters used to study these variants.

In 1976, Collen described a fast-acting inhibitor of plasmin activity with physical properties very different from other protease inhibitors (C9). This inhibitor is known as α_2-antiplasmin (α_2-AP). Friberger has recently reviewed the literature on this inhibitor (F5). In addition to forming a complex with plasmin, α_2-AP can also bind to fibrin and protect the clot from rapid lysis. Heparin has no effect on the rate of reaction with its substrates. The basic principles of the α_2-AP assay utilizing synthetic substrates are similar to those of the AT-III assay. To improve the specificity of this assay, it has been suggested to incorporate methylamine in the buffer to control the interference of another inhibitor, α_2-macroglobulin (F6). Other workers have suggested employing an extremely short incubation period, 20 seconds, to also increase the assay specificity (L10). Many reports have appeared where both manual and automated synthetic substrate assays have been used to measure the concentration of this important inhibitor (F5, J2, L4, L10).

α_1-antitrypsin is a nonspecific serine protease inhibitor with a broad spectrum of inhibitor activities. It is present in plasma at the highest concentration of any other serine protease inhibitor. While the protein has not been identified as a major inhibitor of the enzymes involved in coagulation and fibrinolysis, it is known to inactivate thrombin, plasmin, kallikrein, and Factor XIa. A deficiency in the circulating levels of this inhibitor has been strongly associated with the development of pulmonary disease. Several manual and automated synthetic substrate procedures for α_1-antitrypsin have been published using the first-generation substrate, BAPNA (M6).

C1 esterase inhibitor is a plasma component that exhibits a broad specificity for certain enzymes of the complement, coagulation, kinin, and fibrinolytic systems, including the $C\overline{1r}$ and $C\overline{1s}$ subunits of C1, kallikrein, plasmin, Factors XIa and XIIa. Deficiency of this inhibitor is usually associated with hereditary angioedema. A simple assay based on immunodiffusion has been reported (Z1). This assay does, however, require a 48-hour incubation period. In 1982, Schapira and co-workers published a functional assay for this inhibitor which could be performed in less than 3 hours and used the synthetic substrate S-2302 which is specific for kallikrein (S3). The assay is based on the knowledge that this inhibitor and α_2-macroglobulin account for over 90% of inactivation of kallikrein in normal plasma. These workers utilized assay conditions which selectively inactivated α_2-macroglobulin so that the observed inhibition was dependent on the concentration of C1 esterase inhibitor. Recently, M. Blomback and co-workers reported on results for this inhibitor using a new substrate, S-2314, from A. B. Kabi (B14).

α_2-macroglobulin, like α_1-antitrypsin, is a secondary inhibitor for many proteases such as thrombin, plasmin, kallikrein, urokinase, trypsin, and chymotrypsin. It is present in plasma in a relatively high concentration and has a very large molecular weight, approximately 800,000. When the primary inhibitors, such as AT-III or α_2-AP, are saturated and depleted, α_2-macroglobulin is thought to progressively neutralize the residual enzyme activity. It is generally accepted that the α_2-macroglobulin–enzyme complex is formed in two stages. First, there is a proteolytic cleavage of this inhibitor by the enzyme and this is followed by a conformational change in the modified inhibitor which traps the enzyme. Due to steric hindrance, access of high-molecular-weight natural substrates to the trapped enzyme is prevented. However, the complex retains enzymatic activity for small synthetic substrates. Likewise, the inhibitor–enzyme complex is not inhibited by smaller specific inhibitors so an indirect assay can be designed to measure the concentration of α_2-macroglobulin as shown below (W5).

1. Trypsin + α_2-M $\rightarrow$ [α_2-M–Trypsin] + residual trypsin
2. Residual trypsin + aprotinin $\rightarrow$ trypsin–aprotinin
3. Peptide–pNA + [α_2-M–trypsin] $\rightarrow$ peptide + pNA

Latallo had developed a similar assay using the enzyme plasmin and soybean trypsin inhibitor in place of aprotinin (L4).

6. Monitoring of Oral Anticoagulant Therapy

Synthesis of the clotting Factors II (prothrombin), VII, IX, and X is vitamin K-dependent and activities of these factors in plasma rapidly diminish

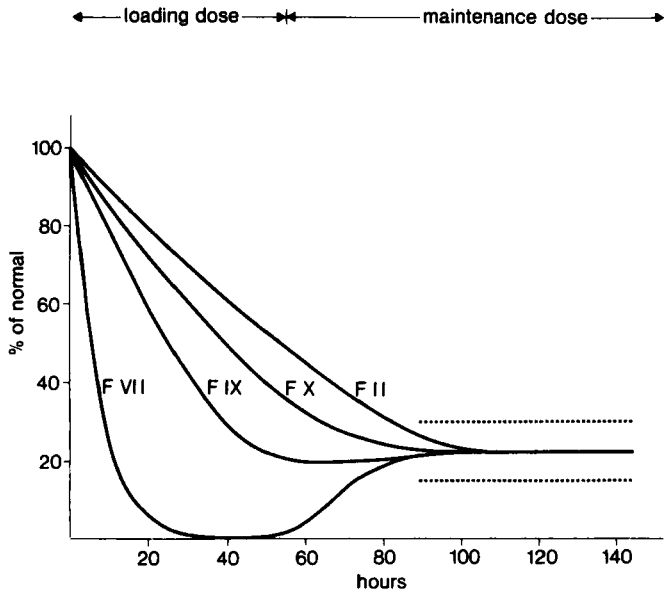


FIG. 5. The rate of decrease for the different vitamin K-dependent coagulation factors during oral anticoagulant treatment. Reprinted with permission from Frigerger (F6).

when oral anticoagulant drugs are administered (F10, V2). Immediately after initiation of therapy with these drugs, the biological activities of these four factors decline at different rates depending on their half-lives (see Fig. 5). Factor VII disappears the fastest while Factor II declines the slowest. In general for patients on long-term treatment with these drugs, the clotting activities of these four factors are reduced and held at approximately 20% of normal. Current medical practices have defined therapeutic levels of treatment in terms of prolongation of the prothrombin-time (PT) clotting assay. The latter method is especially sensitive to the concentration of Factor VII but as mentioned in Section 2, the PT method reflects the levels of five factors among which only three are vitamin K dependent. A key component of the PT assay is the tissue thromboplastin reagent. Many workers have proposed improving the standardization of this reagent to better normalize the therapeutic limits for monitoring this therapy. Readers are referred to a recent review by Poller and Thompson on this subject (P5). The development of synthetic substrate assays offered the possibility of another method for monitoring oral anticoagulant therapy. However, with the present potential for specific factor assays, the key question is which factor is preferred and to which level should this factor be reduced to ensure proper therapy without causing bleeding risks.

Since the first report in 1974, there have been many reports on the

potential of using the synthetic substrate assay system to replace the PT method (B6, B7, E2, F2, G6, G7, I1, L8, R3, V2, V4). Most of the investigators chose to monitor Factor X levels using Russell viper venom as the activator. Some of the reasons favoring this selection are the following: (1) the belief that this factor might reflect more closely than the other vitamin K–dependent factors, both the risk of bleeding complications and the thromboprophylactic effect of these drugs; (2) of the available synthetic substrates the ones for Factor Xa are the most specific; and (3) that the presence of decarboxylated Factor Xa molecules are activated too slowly by RVV to interfere with the results. Results from a recent study by Robijns and co-workers will serve as representative of the general results others have observed (R3). These workers used the chromogenic substrate, S-2337 from A. B. Kabi and compared PT values and amidolytic Factor X results from 44 patients on oral anticoagulant therapy. A reasonable correlation was observed (correlation coefficient 0.73), and fully concordant results were only obtained in 73% of the samples. The therapeutic range was between 15 and 25% with the PT and was 27 to 39% for the amidolytic method. The percent concordant was markedly different for two different drugs, 88% for fenprocoumon but only 40% for acenocoumarol.

In 1981 Latallo and co-workers had studied the effectiveness of synthetic substrate assays for Factors II, VII, and X separately and Factors II and X in combination (L8). They found the highest percent agreement, 87.5%, with the Factor VII test. In spite of this encouraging result, these workers called for confirmation by a large controlled clinical trial.

Van Dieijen-Visser and co-workers were the first to report on studying the amidolytic activity of all four vitamin K–dependent factors (V2). They concluded that for monitoring patients receiving long-term drug therapy, Factor II or X was preferred. For the short-term patients they recommended following Factor IX levels. Interestingly, they observed in testing 33 healthy subjects, that the smallest biological variations were for Factor IX (10%) and the largest for Factor VII (22%). Therefore they concluded that monitoring the latter factor was not recommended.

At this time, after more than 10 years of study, the question of whether the synthetic substrate assays are a viable substitute for the PT method monitoring remains unanswered. Most workers agree with the above request for a randomized, multicenter, blind clinical study to supply the necessary data. All results to date indicate, as expected, that there will not be a one-to-one correlation between these two functional assay systems and that new therapeutic levels will have to be carefully set for the specific amidolytic assay methods. Also there is broad agreement that the cost of the synthetic substrate test will be much higher than the current PT method (F2, G2, R3). The conversion to automated assay formats should help reduce the cost-per-

test differential. While most investigators believe the amidolytic tests are more precise than the clot-endpoint assays, the recently introduced automated instrument has markedly improved the precision of the PT method (M8). It should also be noted that some recent results with an immunoassay for the native prothrombin antigen offer an alternative to the above functional assays for monitoring these patients. Furie and co-workers, in a comparative study of 391 patients, found the immunoassay to correlate better with bleeding complications than the PT methods (F12).

7. Monitoring of Heparin Therapy

Heparin is a general name for a variety of mucopolysaccharides found in vertebrate tissue with a molecular weight range of 4,000 to 40,000. The subunit monosaccharides are glucose with alternating iduronic and glucuronic acids arranged in straight chains. The chemical heterogeneity of heparin can influence the results of any assay for this compound.

Studies have shown that heparin is able to exert several different biological activities. In addition to the one of interest for this review, an anticoagulant effect, heparin has the ability to increase plasma lipoprotein activity, both inhibit and stimulate the alternate pathway complement activation, and enhance the release of collagenase from bone in tissue culture (S17). It has also been associated with thrombocytopenia. Several recent reviews on heparin are available (C13, T7, T8).

As noted previously, for heparin to exert its anticoagulant effect, a plasma cofactor, antithrombin III, is needed. It has been proposed that heparin acts as a catalyst to cause a marked increase in the rate of interaction between AT-III and serine proteases like thrombin and Factor Xa (B4). Some uncertainty still exists as to whether the binding of heparin to the inhibitor or the enzyme or to both is the key step (P6).

It has been shown that in commercial heparin preparation two-thirds of the product has a low affinity for AT-III and little or no anticoagulant activity, while the remaining one-third has a high affinity and anticoagulant activity (L1). High-molecular-weight heparin fractions prepared by gel filtration are more potent in promoting platelet aggregation but may be less effective as anticoagulants than lower molecular weight fractions (S17). It then can be postulated that heparin fractions of low molecular weight and exhibiting high binding affinity of AT-III should be a more specific agent for therapeutic anticoagulation than the crude product currently available to the physician (H7).

Clinical use of heparin is made in three ways: (1) low dosage by subcutaneous injection for the prophylaxis of venous thromboembolism; (2)

standard dosage intravenously for treatment of established thrombosis; and (3) high dosage for maintaining the fluidity of blood in extracorporeal circulation (S7). Despite this wide application, heparin is a drug for which there is uncertainty about optimum clinical dosage.

Since heparin can inhibit fibrin formation at many points in both the intrinsic and extrinsic clotting cascade, many assays have been developed to detect its effect: whole-blood clotting time, whole-blood recalcification time, thrombin-time, and the APTT. The three commonly used clinical screening tests, thrombin-time, PT, and APTT, vary in their sensitivity to heparin (S17). The PT is essentially insensitive, while the thrombin time is extremely responsive to changes in this drug's level. However, a modification of the standard procedure for the latter assay, involving the addition of calcium chloride to the thrombin reagent, does lower the sensitivity to heparin (T4). The APTT exhibits intermediate sensitivity to the drug, and this allows the test to be essentially linear over the range of heparin recommended for clinical use. Currently most investigators in the United States prefer to monitor this drug's efficacy with the APTT assay (B1). This selection is based on a demonstrable level of anticoagulation once a prolonged patient clotting time has been found. This assay is routinely done and therapeutic rationale has been developed using this test. Balancing this view are the observations that the APTT assay can vary depending upon which reagents are used and that the test is relatively insensitive to low levels of heparin (<0.1 U/ml). There also exist intersubject variabilities; so markedly different responses to the same level of heparin can be found (V3). Some unpublished results from our laboratory are shown in Table 9 to illustrate this problem. Some workers propose that heparin monitoring be carried out with reference to the patient's own plasma before the start of therapy. Bleeding problems due to overdosage of patients being monitored by the APTT assay range from 8 to 20% (B16).

In light of the above, it has been proposed to replace the APTT method for heparin with an amidolytic assay that measures the actual concentration of the drug in the treated patient. Results from a recent CAP study found 13% of the laboratories using such a procedure (S7). Several reports have appeared using either chromogenic or fluorogenic substrates that are sensitive to Factor Xa or thrombin inhibition (B3, C3, G3, L3, T5). Scully, in his recent review, has listed the details of an automated amidolytic assay for heparin which uses the Factor Xa specific substrate, S-2222 (S7). As long as antithrombin III is present in excess, then a linear relationship is found between absorbancy and heparin concentration up to 0.6 U/ml of the latter (H5). Bilirubin levels of greater than 60 μg/ml in the test plasma can interfere with the above results unless individual blanks are employed. Also, interindividual variations like those found with clotting assays have been

TABLE 9

INDIVIDUAL VARIATIONS IN APTT DUE TO PRESENCE OF HEPARIN

Individuals[a]	Sex	APTT clot endpoint (seconds)[b] Heparin[c] units per ml (zero)	Heparin units per ml (0.2)	Heparin units per ml (0.4)
1	M	30.30	39.55	62.35
2	M	32.05	60.60	88.60
3	F	32.45	63.45	84.95
4	F	34.20	74.50	127.40[d]
5	M	35.60	68.00	122.40[d]
6	F	31.10	41.55	79.80
7	M	32.20	55.10	68.90
8	F	35.00	88.50	109.70[d]
9	M	35.95	46.35	52.45[d]
10	M	35.90	46.80	69.55
11	M	34.10	54.70	92.20
12	F	29.65	34.00	56.90[d]
13	F	28.15	46.05	56.75
14	M	33.20	53.70	88.50
15	F	31.35	38.55	59.90
16	M	28.35	42.35	48.90[d]
17	F	32.00	44.30	72.60
18	F	29.50	43.60	60.10
19	M	28.90	51.90	81.70
20	F	26.10	37.00	43.20[d]
21	M	26.30	52.30	65.30
22	F	33.40	65.90	93.60
23	M	29.90	31.45	50.10[d]
24	F	32.20	52.60	70.30
25	F	34.90	60.50	96.75
26	M	30.90	40.80	70.60
27	M	35.30	71.00	99.95[d]
28	F	31.90	36.00	45.80[d]
29	F	27.60	31.25	41.00[d]
Mean		31.69	50.77	74.49
Standard deviation		2.85	13.95	22.97
Minimum		26.10	31.25	41.00
Maximum		35.90	88.50	127.40

[a] The individuals tested were normal adults, aged 25–65, who were not on any medication.

[b] Clotting time was determined on an optical endpoint instrument using commercially available reagents.

[c] Sodium heparin (Wyeth Lab, Philadelphia, Pennsylvania) was added from a stock solution individually to the fresh platelet-poor plasma samples just prior to testing. This heparin is derived from porcine intestinal mucosa.

[d] Response in this person significantly greater or less than anticipated from average reaction.

reported (S7, V3). The reasons for these differences are believed to be variations in amounts of PF4 and/or AT-III. The former is influenced by blood collection procedures. It is recommended that the centrifugation of blood to obtain plasma should be done at 4°C rather than room temperature and that platelet-poor plasma be prepared by centrifuging the plasma at least 20 minutes at 13,600 *g*. Variation in AT-III to levels is believed to be minor when measuring heparin concentrations above 0.2 U/ml. Some procedures add purified human AT-III to the assay to overcome this variation (S17).

On the positive side, studies have found the amidolytic assay for heparin to be as precise as the APTT method and to be capable of measuring levels as low as 0.025 U/ml (H5, S17). However, balancing these findings is the need to show, through a large multicenter clinical trial, the true relationship of the amidolytic assay value and the anticoagulant effect in the patient and to reduce the large difference in assay cost of this method and the APTT assay.

8. Conclusions

Over the last decade, a new era in hemostasis testing has occurred with the introduction of synthetic substrate procedures. Clearly the progress during this period in the better understanding of the fibrinolysis, kallikrein–kinin, and protease inhibitor systems has been greatly assisted by the availability of specific amidolytic assays. Likewise in this time, there has been an encouraging evolution from manual amidolytic methods to automated procedures on dedicated instruments. The latter has allowed these tests to be done in greater output at a lower cost and with less trained personnel. In spite of the above, there has been a less than expected usage of these useful probes. Specifically, in the area of global assays, the clinician still prefers to employ the clotting assays, PT and PTT, to monitor his patients. Some of the reasons why the amidolytic methods are not yet done routinely are the following:

1. A much higher cost per test.
2. Concern over standardization procedures.
3. Lack of education programs.
4. Need for clinical trial confirmation of equivalence with current methods.
5. The need for rare, expensive, and highly purified assay cofactors.
6. Too many synthetic substrates for Factor Xa and thrombin and a lack of ones for Factor VIIa and IXa.

So, 12 years after the appearance of the revolutionary paper by Svendsen and co-workers (S26), it does not appear that the amidolytic assays will

replace the current clot-endpoint methods in the near future. One can appreciate the need, however, for both these assay systems. Together they will continue to support the efforts started almost 50 years ago by Drs. Quick and Seegers to unravel the mysteries of the hemostasis system.

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BILE ACIDS

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1. Introduction

Bile first attracted Man's interest long ago. Around the time of the sixth century BC, the Greeks developed the doctrine of the four humors or body fluids. These humors were yellow bile, black bile, blood, and phlegm. Bile

was therefore regarded as being very important for good health, since disease was thought to result from an imbalance in the humors. The reason why bile was prominent in early thinking on the cause of disease may have been because of its strong, dark color and intensely bitter taste (H11). This bitter taste is due to the presence of bile acids.

In later generations, around the fourth century BC, opinion dramatically changed as to the importance of bile. From this time until over 2000 years later, it was regarded solely as a waste product. Aristotle described bile as "a mere excrement and of no other use than by its acrimony to promote the excretion of the Guts" (G3). Eventually, it was suggested in the eighteenth century, and confirmed in the nineteenth century, that bile plays a role in the digestion of dietary fat. Studies on the chemical nature of bile commenced in the nineteenth century. The major solid in bile was identified as an acid component and named *bile acid* by Liebig (L8). It is of interest that Liebig's textbook of biochemistry, *Animal Chemistry*, which was published in 1843, devoted more space to bile than to blood (H11). The elucidation of the structure of the bile acids was carried out by Wieland and his co-workers beginning in 1912 (W8). An excellent historical account of early studies of bile and the discovery of bile acids has been written by Heaton (H11).

Bile is not a body fluid which is analyzed routinely in clinical chemistry laboratories today. However, major advances have been made in the last 20 years in understanding the physical and chemical properties of bile and bile acids, and the role of bile acids in health and various diseases. The purpose of this review is to discuss the present knowledge of the biosynthesis of bile acids in the liver and to describe how bile acids circulate within the body while carrying out their physiological functions. Because numerous methods for the measurement of bile acids are now available, the biochemical features of each method will be reviewed. Finally, application of bile acid measurement to clinical medicine will be described, along with the use of certain bile acids as pharmacological agents for the treatment of gallstone disease.

2. Physicochemical Properties of Bile Acids

2.1. Chemical Structure

Bile acids are C-24 to C-28 carboxylic acids with a steroid nucleus containing hydroxylic substituents and part or all of the side chain of 5β-cholestane (Fig. 1). The A/B ring junction is cis (5β hydrogen) in the bile acids of most higher vertebrates, although A/B trans (allo) bile acids (5α hydrogen) occur in lower vertebrates (H10). Bile acids do not occur in invertebrates. Most unconjugated bile acids have 24 carbon atoms with the basic structure of 5β-

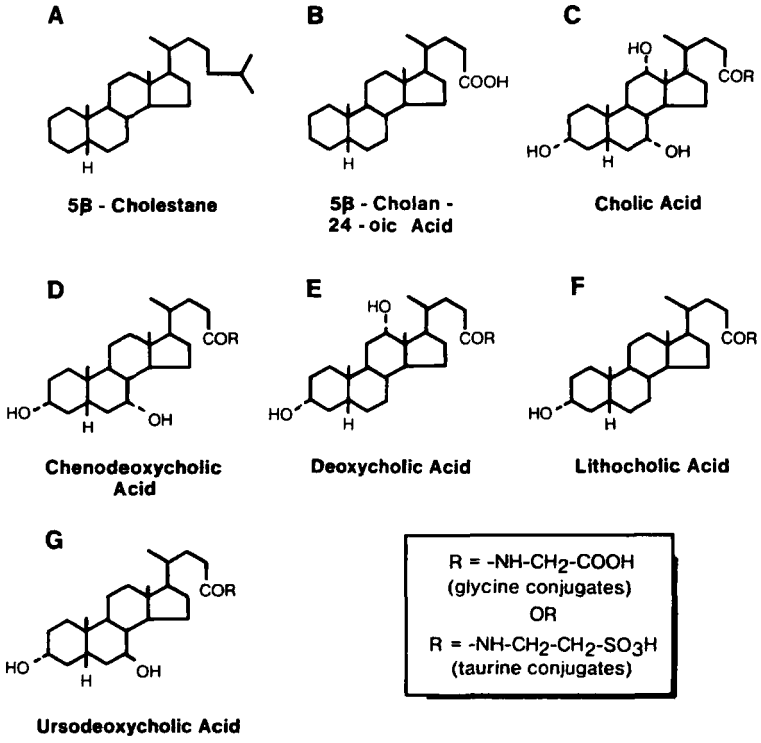

FIG. 1. Chemical structures of 5β-cholestane (A), 5β-cholan-24-oic acid (B), and bile acids found in human gallbladder bile (C–G).

cholan-24-oic acid (Fig. 1). This type is virtually the only bile acid in man and laboratory animals. However, the chemical structure of bile acids appears to have been modified through evolution, as C-27 bile alcohol sulfates are found in primitive vertebrates and taurine conjugates of C-27 and C-28 bile acids have been isolated from some reptiles and amphibians (H10, M18).

Bile acids contain hydroxyl groups, which are usually substituted at positions, C-3, C-7, or C-12 of the steroid nucleus. The three major bile acids found in man are 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid; 3α,7α-dihydroxy-5β-cholan-24-oic acid; and 3α,12α-dihydroxy-5β-cholan-24-oic acid. Because of the complexities of steroid nomenclature, bile acids are nearly always referred to by trivial names. Thus, the three major human bile acids are named cholic acid, chenodeoxycholic acid, and deoxycholic acid, respectively, and their chemical structures are shown in Fig. 1. Human bile does, however, contain small amounts of other bile acids, such as lithocholic acid (3α-hydroxy-5β-cholan-24-oic acid) and ursodeoxycholic acid (3α,7β-dihydroxy-5β-cholan-24-oic acid) (see Fig. 1).

There is a wide variety in the types of bile acids found in different animal species. Some species have unique bile acids, such as α-muricholic acid (3α,6β,7α-trihydroxy-5β-cholan-24-oic acid) and β-muricholic acid (3α,6β,7β-trihydroxy-5β-cholan-24-oic acid) in rats and mice, and hyodeoxycholic acid (3α,6α-dihydroxy-5β-cholan-24-oic acid) in pigs. Haslewood (H9) has studied the distribution of bile acids in the animal kingdom and has suggested that the C-24 acids, which are common to most advanced animal forms, can be regarded as the present endpoints in the evolution of the chemical structure of bile acids.

Free bile acids are not normally present in human bile, but are enzymatically conjugated with either of the amino acids glycine or taurine by peptide linkage at the C-24 position. Thus, six major bile acids exist in man, namely the glycine and taurine conjugates of cholic acid, chenodeoxycholic acid, and deoxycholic acid (see Fig. 1). The ratio of glycine-conjugated to taurine-conjugated bile acids is variable, but in normal subjects is around 3 : 1 (S32). In nonmammalian species, taurine is the only amino acid used for conjugation. Among mammals, carnivores tend to produce only taurine conjugates of trihydroxy bile acids (cholic acid), while herbivores and omnivores produce mainly glycine conjugates of dihydroxy bile acids (H9). Vessey has found that there is only one enzyme in liver for conjugation and that the supply of glycine or taurine and different enzyme affinities for glycine and taurine explain the patterns of conjugation observed in different animal species (V7).

The conjugation of bile acids with glycine or taurine has several important effects on their physicochemical properties. Because of the peptide bond, the polarity of the side chain is increased and the pK_a values of the major bile acids are lowered from around 6 to about 4 for glycoconjugates and 2 for tauroconjugates. Bile acid solubility in acid solutions is increased by conjugation. At pH 6.5 the sodium salts of cholate, chenodeoxycholate, and deoxycholate tend to precipitate from solution in acid (HA) form. However, the glycine conjugates of these bile acids remain in solution to about pH 4.5, while their taurine conjugates are fully soluble, whether the sulfonic acid group is ionized or not (H20).

Another type of conjugation occurring with bile acids involves the hydroxyl groups. These may form sulfate esters or conjugates with glucuronic acid, usually at the 3α position. The formation of sulfate esters was first demonstrated as a possible bile acid metabolic pathway in man by Palmer and Bolt (P3). These bile acids can be detected as excretion products in the urine (A6).

It should be pointed out that in this review no distinction is made between the terms *bile acid* and *bile salt*. These terms are used interchangeably, although in a strict sense un-ionized forms should be called bile acids, while

ionized forms should be called bile salts. To add to the confusion, sometimes unconjugated forms have been called bile acids and conjugated forms termed bile salts. At physiological pH values in healthy subjects, most bile acids are ionized and conjugated.

2.2. Micelle Formation

The stereochemistry of bile acids has been investigated by constructing molecular models. These models indicate that the bile acid molecule has a rigid disklike shape with one side being hydrophobic while the other is hydrophilic, containing the hydroxyl groups and terminal, highly polar, carboxyl or sulfonyl group. The 5β configuration of the junction between rings A and B of the steroid nucleus bends the molecule into an L shape and enhances the separation of polar and nonpolar regions of the molecule. Bile acids therefore are amphiphilic and have detergent properties (H20, S36). Above a certain concentration, bile acid molecules aggregate to form small particles called micelles. This concentration is called the critical micellar concentration (CMC) and is dependent on temperature, the chemical structure of the bile acid, electrolyte concentration, and the presence of other amphiphilic molecules which interact with the micelle (H20, M21). A diagramatic representation of a simple primary bile acid micelle is shown in Fig. 2A. The molecular arrangement is such that hydrophobic portions of the bile acid molecule form the interior of the particle, while polar groups are at the surface and are free to interact with the aqueous environment.

Micellar solutions of bile acids are optically clear but can be studied by light scattering or equilibrium ultracentrifugation methods (C6). Original estimates of the radii of bile acid micelles were in the range 10 to 25 Å, corresponding to aggregation numbers of 4 to 6 for cholate and its conjugates, around 12 for the dihydroxy bile acids chenodeoxycholic and deoxycholic acids, and approximately 20 for their tauro- and glycoconjugates (H20). Micelle size, however, is influenced by cation concentration and temperature, as well as the molecular structure of the bile acid itself (C6, H20). More recent studies have utilized quasi-elastic laser light scattering spectroscopy to deduce the hydrodynamic radius, shape, aggregation number, and polydispersity of bile acid micelles (M21). These studies have suggested that bile acid concentration can influence micellar size, particularly at high electrolyte concentrations, and that large secondary micelles (radius 15 to 60 Å) may form by aggregation of smaller primary micelles (Fig. 2A). The shape of large micelles was deduced to be elongated or rodlike, rather than globular as for small micelles (M21).

Studies of pure bile acid micelles have little physiological relevance, since

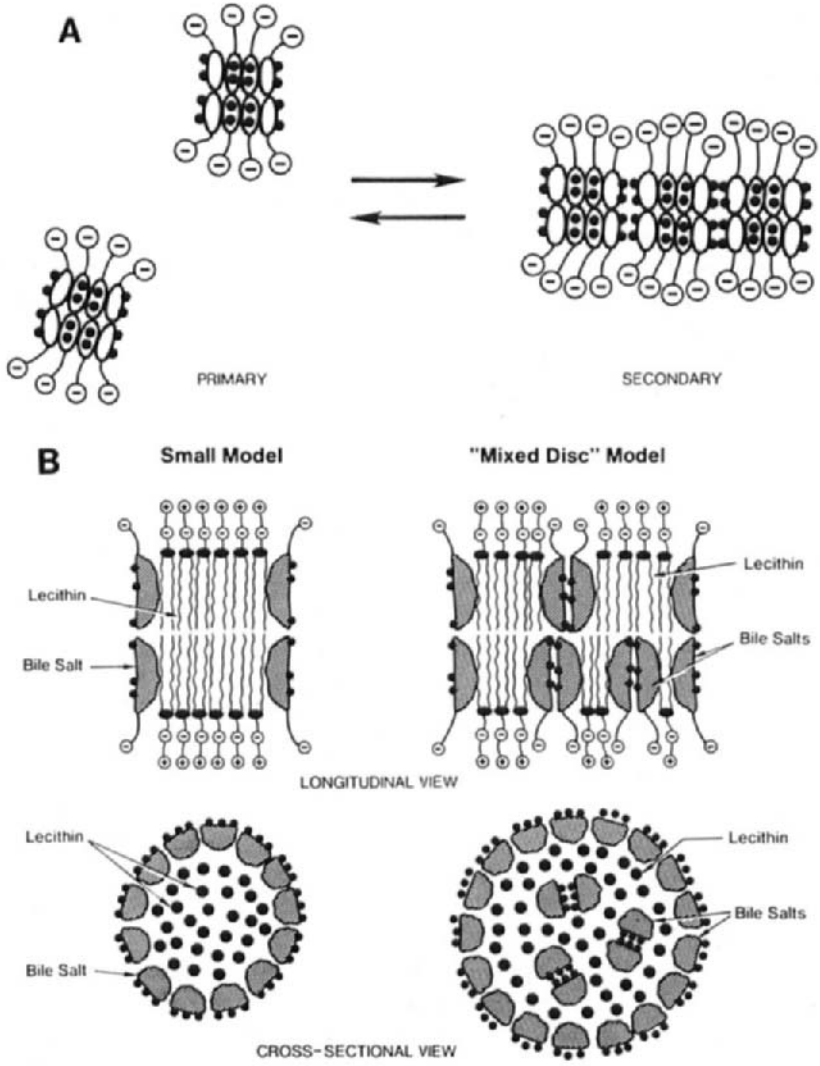

FIG. 2. Schematic diagrams of bile salt micelles (A) or mixed micelles (B), showing the molecular arrangement of bile salts and lecithin. The closed circles represent nonionic polar groups, and the open circles represent ionic polar groups of the molecules. [Redrawn from ref. (M20) with permission from *Biochemistry* **19**. Copyright (1980) American Chemical Society.]

in biological systems bile acids are mixed with other polar lipids. These lipids can be divided into two groups.

1. *Insoluble nonswelling amphiphiles* (H20). Lipids in this group have sufficient polarity to orient themselves at an air–water or air–oil interface, but do not interact with water sufficiently to dissolve in aqueous solutions. Examples of interest are cholesterol, triglyceride, the fat-soluble vitamins, and un-ionized fatty acids.

2. *Insoluble swelling amphiphiles* (H20). These lipids will interact with water in such a way that layers of water molecules can interpose themselves between layers of lipid, thus expanding or swelling the lipid structure. While still not truly soluble in water, these hydrated lipids can form a phase that is physically liquid, but has crystalline characteristics by X-ray analysis. Under certain conditions, liquid crystals can be seen under the polarizing microscope. Examples of lipids in this group are phospholipids, monoglycerides, and some ionized long-chain fatty acids.

The relevance of discussing these two classes of lipids becomes obvious when it is appreciated that bile acids alone can solubilize lipids with only low efficiency. However, when mixed with a swelling amphiphile, such as phosphatidylcholine, the capacity of bile acids for solubilizing a nonswelling amphiphile is greatly increased. For example, cholesterol in bile is solubilized by bile acid–phosphatidylcholine mixed micelles much more efficiently than by bile acid micelles alone. As is discussed later, mixed micelles are also important in fat solubilization and absorption in the intestine.

Schematic models for the expanded structure of bile acid–phosphatidylcholine mixed micelles are shown in Fig. 2B. The original model was proposed by Small in 1967 (S36). In this model the mixed micelle consisted of a phospholipid bilayer disk surrounded on its perimeter by bile acid molecules, which were oriented with their hydrophilic surfaces in contact with aqueous solvent and their hydrophobic surfaces interacting with the hydrocarbon chains of the phospholipid molecules. This model has recently been revised, based on further studies of mixed micelles using quasi-elastic light scattering spectroscopy (M20). In a new model for the molecular structure of bile acid–phospholipid mixed micelles, Mazer *et al.* (M20) propose a "mixed disk," in which bile acids are found not only on the perimeter of phospholipid bilayers, but also incorporated within their interior in high concentrations (Fig. 2B). The size of these mixed micelles was estimated to be as high as 200 to 400 Å in radius in some solutions, and disk-shaped particles in this size range were observed by transmission electron microscopy (M20). Micellar aggregates similar in size and structure to those found in model bile solutions have been demonstrated in dog bile (M22).

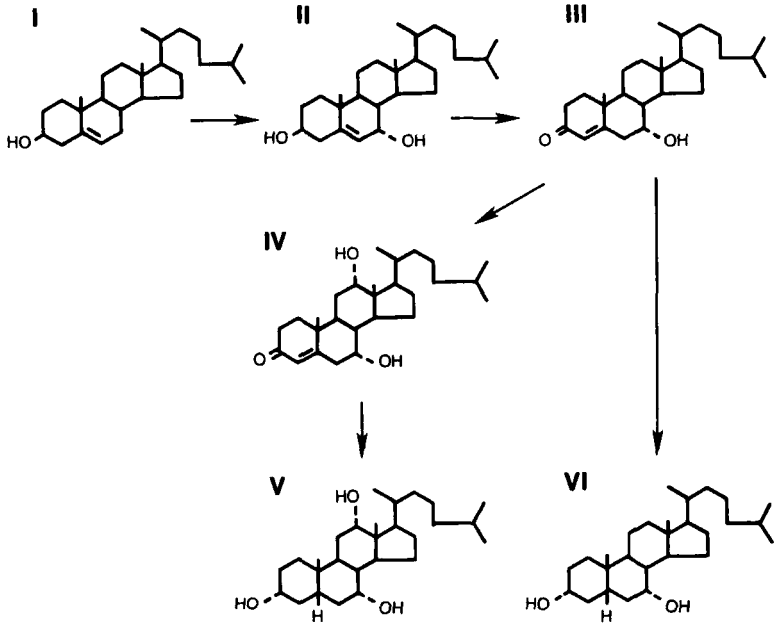

FIG. 3. Metabolic steps in the conversion of cholesterol (I) to the intermediate in cholic acid synthesis, 5β-cholestane-3α,7α,12α-triol (V), or the corresponding intermediate in chenodeoxycholic acid synthesis, 5β-cholestane-3α,7α-diol (VI).

3. The Biosynthesis of Bile Acids and Its Regulation

3.1. BIOSYNTHETIC PATHWAYS

The bile acids cholic acid and chenodeoxycholic acid are synthesized from cholesterol in the liver (D1, S3). Several structural modifications are necessary to convert cholesterol, with its 27 carbon atoms, C-5,6 double bond and 3β-hydroxyl group, to a 24-carbon atom, saturated, 3,7 and 12α-hydroxylated bile acid. The major reactions in this transformation are shown in Figs. 3 and 4. The reactions are catalyzed by mitochondrial, microsomal, soluble, and possibly peroxisomal enzymes.

During bile acid biosynthesis, modifications to the cyclopentanophenanthrene (steroid) nucleus are thought to precede the oxidation and cleavage of the cholesterol side chain. The first and rate-controlling step in bile acid synthesis is the 7α-hydroxylation of cholesterol (I) to form 7α-hydroxy-cholesterol (II) (Fig. 3). This step is catalyzed by cholesterol 7α-monooxygenase (cholesterol 7α-hydroxylase) (EC 1.14.13.17), a microsomal enzyme (M37). Further metabolism of 7α-hydroxy-cholesterol involves oxidation of the 3β-hydroxyl group and isomerization of the double bond from C-5,6 to C-4,5,

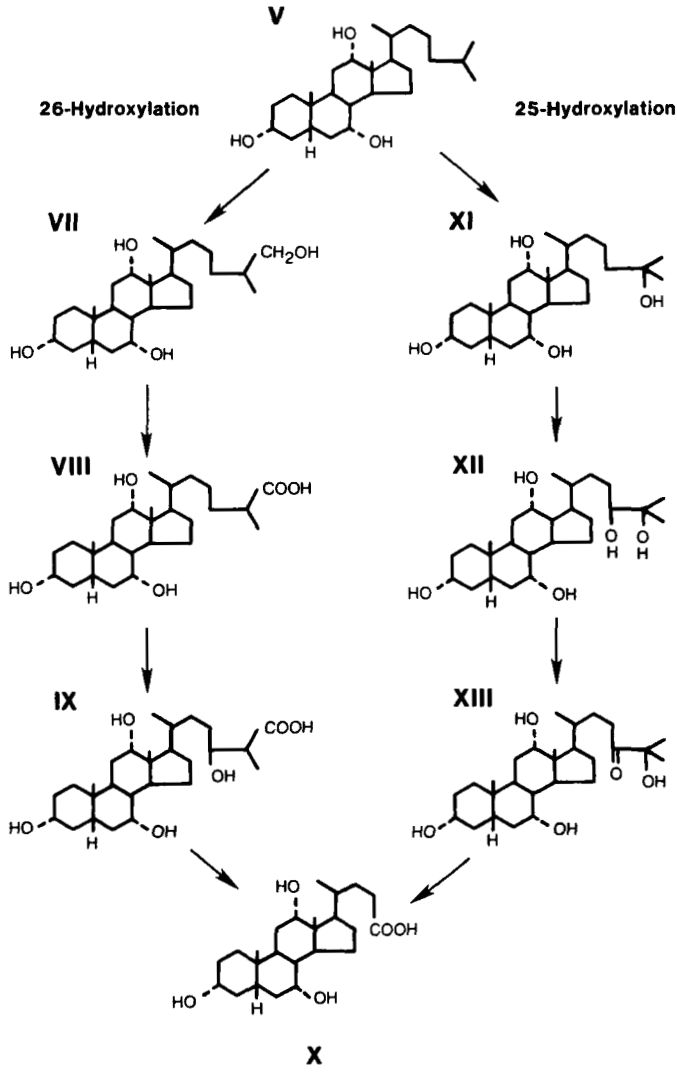

FIG. 4. The 26-hydroxylase and the 25-hydroxylase pathways for the oxidation of the hydrocarbon side chain of 5β-cholestane-3α,7α,12α-triol (V) to form cholic acid (X).

yielding 7α-hydroxy-4-cholesten-3-one (III). This compound is the last intermediate which is common to both cholic and chenodeoxycholic acid synthesis. For cholic acid formation, a further α-hydroxylation occurs to give 7α,12α-dihydroxy-4-cholesten-3-one (IV) which then undergoes reduction of the double bond and C-3 ketone to form 5β-cholestane-3α,7α,12α-triol (V). For

chenodeoxycholic acid formation, 7α-hydroxy-4-cholestene-3-one is similarly reduced to produce 5β-cholestane-3α,7α-diol (VI). An alternative pathway proposed for chenodeoxycholic acid formation involving an initial 26-hydroxylation of cholesterol, followed by oxidation of 26-hydroxy-cholesterol into 3β-hydroxy-5-cholen-24-oic acid, and conversion to lithocholic and finally chenodeoxycholic acid is thought to be of minor importance quantitatively (D1). Nevertheless, high serum concentrations of 3β-hydroxy-5-cholen-24-oic acid have been found in patients with liver diseases, especially primary biliary cirrhosis (S6).

Two pathways have been proposed for degradation of the cholestane side chain in the biosynthesis of bile acids. These differ in the site proposed for the first hydroxylation step in side-chain oxidation and are discussed below for the formation of cholic acid.

1. *26-Hydroxylation pathway.* The mitochondrial fraction of both human and rat liver contains a 26-hydroxylase enzyme (B23), which can convert 5β-cholestane-3α, 7α, 12α-triol (V) to 5β-cholestane-3α,7α,12α,26-tetrol (VII) (Fig. 4). This tetrol is oxidized to 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid (THCA, VIII) by liver cytosol (C5). Further hydroxylation at C-24 forms varanic acid (IX) and its side chain is then shortened with oxidation at C-24 to yield cholic acid (X) (Fig. 4).

2. *25-Hydroxylation pathway.* This alternative pathway has been demonstrated in both rat and human liver (S24). It involves the 25-hydroxylation of 5β-cholestane-3α,7α,12α-triol (V) to give 5β-cholestane-3α,7α,12α,25-tetrol (XI), followed by 24S-hydroxylation to yield 5β-cholestane-3α,7α,12α, 24S,25-pentol (XII, Fig. 4). The pentol is then oxidized to 3α,7α,12α,25-tetrahydroxy-5β-cholestan-24-one (XIII) (S26), which is degraded by cytosolic enzymes to cholic acid (X) and acetone (Fig. 4).

The exact contributions of these alternate pathways to total hepatic bile acid synthesis in normal subjects is not certain, although 26-hydroxylation is usually regarded as the major pathway. In addition, it should be pointed out that current views of hepatic cholic acid and chenodeoxycholic acid synthesis are based primarily on studies carried out in the rat. More recent studies, which have involved the administration of labeled bile acid intermediates to patients, have suggested that bile acid biosynthesis is more complex than previously thought and that multiple pathways exist to convert cholesterol to bile acids (V11).

3.2. Regulation of Bile Acid Synthesis

Bile acid synthesis plays an important role in maintaining cholesterol homeostasis, because cholesterol breakdown to bile acids and their subsequent excretion via the biliary system accounts for the majority of cholesterol

which is removed from the body (M37). It is well known that when bile acids are prevented from returning to the liver from the intestine, the rate of bile acid synthesis in the liver increases many-fold. This fact has led to the development of the concept that bile acids regulate their own synthesis by a negative feedback mechanism involving the rate-limiting enzyme of bile acid synthesis, cholesterol 7α-hydroxylase. The activity of this enzyme increases in parallel with bile acid synthesis after a number of experimental manipulations (M37). Also directly correlated with the activity of cholesterol 7α-hydroxylase in the liver is the activity of 3-hydroxy-3-methyl-glutaryl (HMG) CoA reductase (EC 1.1.1.34), the controlling enzyme of cholesterol synthesis. As newly synthesized cholesterol is regarded as the preferred substrate for bile acid synthesis, coordinate regulation of the activity of these two enzymes would seem an efficient way of maintaining cholesterol homeostasis in the liver (M37).

Cholesterol 7α-hydroxylase has been partially purified from rat and rabbit liver (H5). The enzyme is located in the smooth endoplasmic reticulum and is dependent on cytochrome *P*-450 and NADPH-cytochrome *P*-450 reductase for activity (H5). The particular cytochrome *P*-450 associated with microsomal cholesterol 7α-hydroxylase activity constitutes a small fraction of total liver cytochrome *P*-450 and, in the rabbit, it appears to be a subfraction of cytochrome *P*-450$_{LM_4}$ (B28). Measurement of the activity of this enzyme by isotope incorporation is complicated by dilution of added cholesterol by endogenous microsomal cholesterol. A method has now been developed to remove cholesterol from microsomes, so that the mass of 7α-hydroxycholesterol formed during enzyme assay can be accurately calculated (S25). Using this assay, cholic acid feeding was shown to suppress the activity of cholesterol 7α-hydroxylase in rat liver, whereas cholesterol feeding did not (S25).

A short-term regulation mechanism for cholesterol 7α-hydroxylase activity has been investigated recently in rat liver. The enzyme appears to exist in two forms, which are interconverted by cytosolic factors (K12). These factors may correspond to a protein kinase and a phosphatase, which have been proposed to regulate cholesterol 7α-hydroxylase activity by a phosphorylation (active form)–dephosphorylation (inactive form) mechanism (S9). Another enzyme utilizing cholesterol as substrate, acyl-CoA:cholesterol *O*-acyltransferase (EC 2.3.1.26), may also be regulated in this way, while the biosynthetic enzyme, HMG-CoA reductase, is inhibited in the phosphorylated form (S10). Thus, short-term regulation of the concentration of unesterified cholesterol in the liver may be achieved by coordinate control of these three key enzymes in cholesterol metabolism by reversible phosphorylation (S10).

An alternative mechanism for regulating bile acid synthesis is via cholesterol availability. Although cholesterol which has been newly synthesized in

the liver is believed to form the preferred substrate pool for bile acid synthesis (S15), radioactive cholesterol in serum lipoproteins can be converted to bile acids when administered *in vivo* (H1, M24) and therefore contributes to total bile acid synthesis. High-density lipoprotein (HDL) cholesterol is a better precursor for bile acids than low-density lipoprotein (LDL) cholesterol, both in man (H1) and the rat (M24). Recent studies with cultured rat hepatocytes found that bile acid synthesis and secretion was increased when the hepatocyte cholesterol pool was increased, either by adding mevalonic acid or lipoprotein fractions to the culture medium, or by feeding a cholesterol-rich diet (D4). Surprisingly, bile acids did not have a direct inhibitory effect on bile acid synthesis in this experimental system (D3). This finding conflicts with *in vivo* studies which have suggested that bile salts regulate their own synthesis by negative feedback control, but it is possible that a factor derived from the intestinal tract may be required for the feedback inhibition effect.

Cholic acid differs from chenodeoxycholic acid in having an extra hydroxyl group at C-12. The enzyme responsible for producing this difference, 7α-hydroxy-4-cholesten-3-one 12α-hydroxylase, thus acts at a key branch point in the biosynthesis of bile acids and might be expected to be regulated in order to control the relative amounts of cholic acid and chenodeoxycholic acid produced. Like other hydroxylation steps in bile acid biosynthesis, 12α-hydroxylation requires a specific form of cytochrome *P*-450, which is present in the cytochrome *P*-450$_{LM_4}$ fraction of rabbit liver microsomes (H6). The activity of 12α-hydroxylase has been postulated to be decreased in patients with liver cirrhosis to explain the low proportion of cholic acid relative to chenodeoxycholic acid in the bile of these patients (V9). Conversely, the activity of this enzyme may be high in patients with cerebrotendinous xanthomatosis, as the bile of these individuals contains mostly cholic acid (S2). More recent studies have shown that patients with cirrhosis are able to efficiently convert 7α-hydroxy-cholesterol into cholic acid (G8, P8), suggesting that 12α-hydroxylase activity is near normal. Other evidence from *in vivo* studies in man with labeled precursors suggests that 12α-hydroxylase activity is not important in the regulation of the ratio between cholic acid and chenodeoxycholic acid in human bile (B21). The possibility that different pools of cholesterol are utilized for the biosynthesis of cholic acid and chenodeoxycholic acid is now being investigated.

3.3. Inborn Errors of Bile Acid Synthesis

3.3.1. *Cerebrotendinous Xanthomatosis (CTX)*

This rare, lipid storage disease, which was first described in 1937, is characterized clinically by the presence of xanthomas or lipid deposits of cholesterol and cholestanol (5α-cholestan-3β-ol) in the brain and tendons.

Other clinical signs consist of progressive neurologic dysfunction, cataracts, and premature atherosclerosis (S1). The disease is inherited as an autosomal recessive trait, but is usually only detected in adults when cholesterol and cholestanol have accumulated over many years (S2). Biochemical features of the disease include striking elevations in tissue levels of cholesterol and cholestanol and the presence of unusual bile acids, termed bile alcohols, in bile. These bile alcohols are mainly 5β-cholestane-3α,7α,12α,24S, 25-pentol, 5β-cholestane-3α,7α,12α,23ϵ,25-pentol and 5β-cholestane-3α,7α,12α,25-tetrol (S2). As chenodeoxycholic acid is deficient in the bile of patients with CTX, it was postulated that early bile salt precursors are diverted into the cholic acid pathway and 12α-hydroxy bile alcohols with an intact side chain accumulate because of impaired cleavage of the cholesterol side chain and decreased bile acid production (S2). HMG-CoA reductase and cholesterol 7α-hydroxylase activity are elevated in subjects with CTX (N4, N5), so that sufficient 7α-hydroxycholesterol should be available for bile acid synthesis.

Although it was thought originally that side-chain oxidation of 5β-cholestane-3α,7α,12α-triol proceeded via a 26-hydroxylation pathway (see Fig. 4), the discovery of bile alcohols with hydroxyl groups at C-25 led to the demonstration of a 25-hydroxylation pathway for cholic acid biosynthesis in man (S24). Both normal subjects and CTX patients are able to convert 5β-cholestane-3α,7α,12α,25-tetrol into cholic acid (S5), via a 24S-hydroxylase reaction producing 5β-cholestane-3α,7α,12α,24S,25-pentol. This reaction is only 20% as active in the liver of CTX patients as in controls (S3), and a 24S-hydroxylase deficiency has been proposed as the defect in bile acid metabolism in CTX (S4, S24). However, there may be more than one defect, as convincing evidence of reduced mitochondrial 26-hydroxylase has also been published (B22, O3). If bile acid formation via 26-hydroxylation is blocked, perhaps the 25-hydroxylation pathway becomes prominent, with subsequent production of abnormal precursor metabolites and bile alcohols. Further studies are required to fully resolve the exact major and minor pathways of formation of chenodeoxycholic and cholic acids in CTX patients.

To diagnose CTX, advantage has been taken of the elevated cholestanol levels in plasma. If the ratio of cholestanol to cholesterol is measured by gas–liquid chromatography, a value of over 1% is obtained in patients with CTX compared with less than 0.5% for normal subjects (S2). More recently, capillary gas–liquid chromatography has been used to detect bile alcohol conjugates in the urine of CTX patients and this technique has been useful to assess the efficacy of treatment with orally administered chenodeoxycholic acid (W12).

3.3.2. *The Zellweger Syndrome*

The Zellweger or cerebro–hepato–renal syndrome is a severe neurological condition, which was first described in 1964 (B31). Clinical diagnostic features appear from birth and include hypotonia, growth and mental retar-

dation with abnormal skull shape and facial appearance, impaired liver function, and renal cortical cysts (B31). The incidence of the syndrome has been estimated as 1 per 100,000 births and it is transmitted as an autosomal recessive trait (D2).

A variety of biochemical defects have been reported in patients with the Zellweger syndrome. These include a defect in the catabolism of pipecolic acid (D2) and increased plasma, biliary, and urinary levels of intermediates in bile acid synthesis (H4, M15, M30). More recently, the accumulation of very-long-chain fatty acids, such as hexacosanoic acid, has been noted (M32) and elevated plasma phytanic acid with decreased fibroblast phytanic acid oxidase activity reported (P13).

The particular intermediates in bile acid synthesis which have been found in the Zellweger syndrome are 3α,7α, 12α-trihydroxy-5β-cholestan-26-oic acid (THCA); 3α,7α-dihydroxy-5β-cholestan-26-oic acid (DHCA); and varanic acid (H4, M15, M30). As discussed in Section 3.1, these metabolites are precursors of cholic and chenodeoxycholic acids that have undergone incomplete side-chain oxidation. The finding of these intermediates was taken originally as evidence that mitochondria are responsible for side-chain oxidation, since patients with the Zellweger syndrome have severe mitochondrial abnormalities (H4). However, it is of particular interest that in 1973, Goldfischer *et al.* reported that the liver and kidney cells of patients with this disease do not contain peroxisomes (G7). Rat liver peroxisomes are able to convert THCA to cholic acid (K9). Thus, peroxisomes may play an important role in bile acid synthesis. The Zellweger syndrome could be an example of a newly formulated group of diseases with disordered peroxisomal metabolism (G6), leading to the accumulation and excretion of the observed variety of metabolites.

Elevated plasma levels of THCA have also been found very recently in three young children with another inherited disease, infantile Refsum's disease (P14). This condition has in common many of the clinical and biochemical features of the Zellweger syndrome, so that the metabolic defect in these two diseases may be similar (P13).

3.3.3. *Intrahepatic Cholestasis*

In 1972, large amounts of THCA were found in the bile of two unrelated young children with intrahepatic bile duct abnormalities (E5), suggesting a block in the biosynthesis of cholic acid. Three years later, this bile acid intermediate was also found in the bile, serum, and urine of a brother and sister with a similar paucity of intrahepatic bile ducts and cholestatic liver disease, which proved fatal (H3). DHCA or varanic acid could not be detected in these patients, so that the metabolic defect in this condition appeared to be specific for the enzyme involved in the conversion of THCA to varanic acid. Hanson *et al.* speculated that THCA might have caused the

intrahepatic bile duct abnormalities and cholestasis seen in these patients (H3), but this seems unlikely as these features have not been reported in the Zellweger syndrome, in which THCA is known to accumulate.

4. The Enterohepatic Circulation of Bile Acids

4.1. The Enterohepatic Cycle

In healthy people, virtually all bile acids are confined to an enterohepatic circuit within the body (H18). This circuit involves the liver, gallbladder and biliary tract, the intestinal tract, and the plasma compartment and is shown diagrammatically in Fig. 5. The amount and concentration of bile acids in each of these anatomical sites fluctuates during the day according to the frequency of food ingestion. In the fasting state, in man or other animals with a gallbladder, most of the bile acid pool is located in the gallbladder. This organ acts as a storage site for bile and bile acids, and is able to concentrate bile secreted from the liver by absorbing water and electrolytes (W13). As the sphincter of Oddi, which is located at the lower end of the common bile duct, is contracted while fasting, most bile acids secreted from the liver are diverted into the gallbladder during this period. However, small amounts of bile acids probably do enter the duodenum during fasting to maintain an enterohepatic cycle (H18).

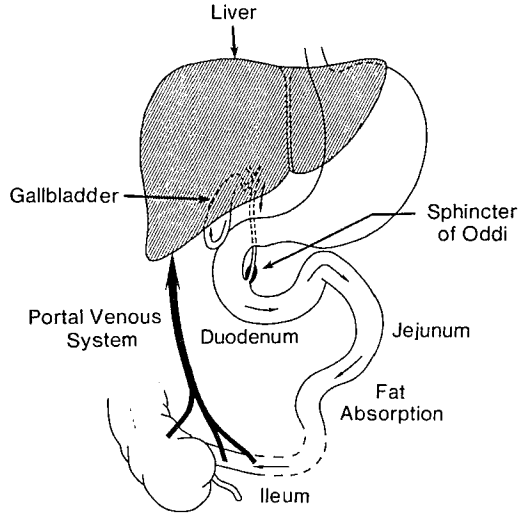

FIG 5. Diagrammatic representation of the enterohepatic circulation of bile acids.

The enterohepatic circulation of bile acids is activated following ingestion of a meal. Food reaching the intestinal tract, particularly amino acids, causes the release of cholecystokinin, which acts on the muscle of the gallbladder and sphincter of Oddi via the plasma (L9). Gallbladder contraction and sphincter of Oddi relaxation result in the passage of concentrated bile into the small intestine, where bile acids perform one of their physiological functions in aiding lipid absorption (see Section 5.3). Some glycine-conjugated dihydroxylated bile acids may be absorbed by passive diffusion in the proximal small intestine (A11). However, most bile acids are actively absorbed with 95% efficiency in the terminal ileum as they pass down the small intestinal lumen (H18). The ileal absorption process is competitive for the different bile acids (L3) and is sodium dependent (P10, W11). Bile acids which pass through the ileum move into the large intestine. Some further reabsorption may occur, while the unabsorbed bile acids are excreted in the feces.

Reabsorbed bile acids are transported back to the liver in the portal blood bound to albumin, where they are taken up by parenchymal cells for excretion into bile. The uptake process has been studied in isolated rat hepatocytes (S17), the perfused rat liver (R1), and cultured rat hepatocytes (V5), and a bile acid receptor protein has been partially characterized in liver cell membrane preparations (A1). Taken together, these studies suggest that uptake is via a coupled membrane carrier mechanism, whereby bile salt anions are cotransported with sodium cations across the liver cell sinusoidal membrane. Although the majority of bile acids are extracted from portal blood by the liver, a small fraction (less than 1% of the total bile salt pool)

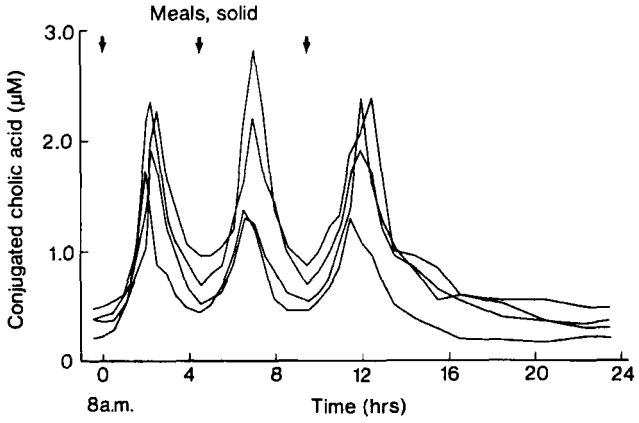

FIG. 6. Postprandial fluctuations in the level of conjugated cholic acid in serum, as determined by radioimmunoassay, in four healthy subjects over a 24-hour period. (Redrawn from H18 with permission.)

spills over into the peripheral blood. The level of serum bile acids thus reflects the instantaneous balance between intestinal input and hepatic uptake. From a low basal level, there is a 2- to 6-fold increase in the level of serum bile acids after a meal (Fig. 6). Since the fractional clearance by the liver for bile salts is constant, the postprandial increase in serum bile acids reflects a constant spillover of an increased amount of bile acids returning to the gallbladder via the liver. Because the hepatic fractional clearance for each bile acid is different, the pattern of bile acids in serum is not exactly the same as the pattern in bile (W6).

Bile acids, which have been taken up by the liver, are transported across the hepatocyte and secreted into the bile canaliculus. Newly synthesized bile acids, in a small amount just sufficient to balance the fraction lost by fecal excretion, join recycled bile acids for biliary secretion. Intracellular bile acid transport may be mediated by carrier proteins (B24, S42). The detailed mechanism of biliary secretion of bile acids and other organic anions into the bile canaliculus is not yet clear (B24). Possible mechanisms include vectorial vesicular transport, facilitated diffusion, or an energy-requiring carrier-mediated transport process (B24).

4.2. Metabolic Transformations during the Cycle

4.2.1. *7α-Dehydroxylation*

The two bile acids, cholic acid and chenodeoxycholic acid, which are synthesized from cholesterol in the liver, are termed primary bile acids. Each day, around one-third to one-quarter of the primary bile acid pool is lost or converted to secondary bile acids by anaerobic bacteria in the intestine. This is achieved by 7α-dehydroxylation, a process which converts cholic acid to deoxycholic acid (3α,12α-dihydroxy-5β-cholan-24-oic acid) and chenodeoxycholic acid into lithocholic acid (3α-hydroxy-5β-cholan-24-oic acid).

Once they have been formed, the fate of these two secondary bile acids is different. Up to one-half of the deoxycholic acid is reabsorbed and passes to the liver via the portal blood to be conjugated with glycine or taurine. Deoxycholyl conjugates are then secreted into bile to take part in recycling through the enterohepatic circulation in a manner similar to primary bile acids (H18). Unabsorbed deoxycholic acid is excreted in the feces. Lithocholic acid is relatively insoluble and only poorly absorbed in the intestine. Most of the small fraction which passes to the liver for conjugation with glycine or taurine is also sulfated at the C-3 position. Lithocholylglycine is sulfated to a greater extent than lithocholyltaurine (A5). After secretion via the bile, these sulfated conjugates are poorly absorbed from the small intes-

tine and pass into the colon, where they may be desulfated and deconjugated to a limited extent for passive reabsorption, or excreted in the feces.

As a consequence of the 7α-dehydroxylation process, the bile acid composition of bile in healthy subjects usually comprises around 30 to 40% conjugated cholic acid, 30 to 40% conjugated chenodeoxycholic acid, 10 to 30% conjugated deoxycholic acid, and less than 5% conjugated lithocholic acid, of which the majority is sulfated (H18).

4.2.2. *Deconjugation*

Around one-quarter of the bile acid conjugates reaching the ileum have the amino acid moiety removed by the action of bacterial enzymes. A deconjugating enzyme, choloylglycine hydrolase (EC 3.5.1.24) has been isolated from clostridial bacteria (N1) and is used in methods for the analysis of bile acids. The majority of deconjugated bile acids are reabsorbed and return to the liver, where they are efficiently reconjugated with glycine or taurine (V7).

Deconjugation of bile acids may become excessive when bacterial overgrowth occurs in the small intestine. This condition is referred to as the "stagnant loop" or "blind loop" syndrome and is characterized by diarrhea, often accompanied by steatorrhea because of a decrease in the effective concentration of conjugated bile salts and a reduction in the fat-absorptive capacity of the small intestine.

4.2.3. *Other Transformations*

The liver, and also bacteria in the small and large intestine, can cause other structural modifications to bile acids as they undergo their enterohepatic cycle. The formation of sulfate esters, already mentioned with respect to lithocholate in Section 4.2.1, is carried out primarily in the liver in man by a sulfotransferase (L11). Other bile acids can also be sulfoconjugated to a small extent, mainly at the 3α-hydroxyl position. Bacteria, which have been isolated anaerobically from human feces, are known to possess bile acid sulfatase activity, which removes the 3α-sulfate group of chenodeoxycholic and cholic acids (H24). The action of this bacterial enzyme probably explains why only trace amounts of sulfated bile acids, which are poorly absorbed in the intestine, are detected in the feces (I2). Another type of bile acid conjugate, which has been identified in the urine of healthy subjects and patients with hepatobiliary disease, is the glucuronide (A7, S41). Both the liver and extrahepatic tissues, such as the kidney and small intestinal mucosa, are capable of glucuronidation of bile acids in man (M14).

Ursodeoxycholic acid (3α,7β-dihydroxy-5β-cholan-24-oic acid) is present in human bile in small quantities and is of interest because of its therapeutic use as a gallstone-dissolving agent (D8). This bile acid is thought to be

formed in the liver from 3α-hydroxy-7-keto-5β-cholan-24-oic acid (7-ketolithocholic acid), which in turn may be formed in the intestine by bacterial dehydrogenation of chenodeoxycholic acid and its conjugates. Studies have shown that ursodeoxycholic acid can also be formed from chenodeoxycholic acid by bacterial metabolism in the human colon, with 7-ketolithocholic acid as an intermediate (F7). A complex variety of bile acids can be formed in the colon, as a result of bacterial action producing reduction, oxidation, and steric inversion of the hydroxyl groups of primary bile acids (D10).

4.3. Bile Acid Kinetics

Attempts to describe the enterohepatic cycle of bile acids in quantitative terms began in 1957 with the description by Lindstedt of an isotope dilution technique to measure the turnover and pool size of individual bile acids during the steady state (L10). This technique has become a standard procedure in bile acid research and involves the administration of a bile acid, labeled with ^{14}C or ^{3}H, and the subsequent collection of a bile sample each day by duodenal intubation over a period of 5 to 7 days. From the decay curve of the specific activity of the bile acid, the fractional turnover rate and pool size of the bile acid can be calculated (L10). The product of the pool size and daily fractional turnover rate equals the daily synthesis rate.

Using this technique, pool sizes of cholic acid and chenodeoxycholic acid have been estimated to be similar and around 1.0 to 1.5 g each in healthy subjects, with the total bile acid pool amounting to 2 to 4 g (H18, L10, V10). Cholic acid turnover is more rapid than for chenodeoxycholic acid, and the rate of hepatic synthesis of cholic acid (300 to 400 mg/day) is therefore approximately double that for chenodeoxycholic acid (150 to 200 mg/day) (H18, V10). In the steady state, total bile acid synthesis by the liver should equal bile acid loss in the feces, which is around 400 mg/day. Some studies have found that estimates of bile acid synthesis by the isotope dilution technique give values that are higher than those obtained by direct chemical measurement of fecal bile salts (S45), but good agreement has recently been claimed between the two methods (D10). The Lindstedt technique for measuring bile acid turnover and pool size has been modified so that only one bile sample need be collected after intravenous administration of the labeled bile acid. These modified methods measure either pool size alone (D9) or pool size and turnover if both ^{14}C and ^{3}H bile acid are administered at an interval of 24 hours apart (V6).

From studies using intestinal perfusion techniques with a recovery marker, it has been estimated that healthy subjects secrete about 4 to 6 g of bile acid in response to a liquid test meal (H18). This suggests that the enterohepatic cycle operates twice with each meal, so that for a three-meal day plus

a low basal secretion rate, around 20 g of bile acid enter the intestine. It can also be calculated that each bile acid molecule on the average cycles through the intestine 15 to 20 times before being excreted in the feces.

Most patients with cholesterol gallstones who are not obese have a reduced bile acid pool size within the enterohepatic circulation (V8). A small pool size could lead to decreased bile acid secretion and oversaturation of bile with cholesterol. There are three possible reasons for a decreased pool size of bile acids—increased loss, decreased synthesis, or increased cycling frequency. Evidence is available to suggest that decreased cholic acid synthesis (V8) and a reduced level of hepatic cholesterol 7α-hydroxylase activity (N5) occur in gallstone patients. On the other hand, bile acid secretion studies have suggested that there is an inverse relationship between pool size and recycling frequency, so that a small pool size recycles more frequently and the total hepatic secretion of bile acids remains constant (N6). The reason why bile acids could recycle more frequently in gallstone patients is not certain, although it has been proposed that gallbladder emptying is enhanced in response to food (N6, M19). Several studies suggest that gallbladder motor function may predispose to cholesterol gallstone formation by altering the dynamics of the enterohepatic circulation, but agreement has not been reached as to whether gallbladder emptying is increased, decreased, or unchanged in gallstone disease (L4). One explanation for these discrepancies is related to the difficulty in studying gallbladder emptying in the early stages of gallstone disease, before large gallstones have formed and caused secondary changes to gallbladder function.

5. Physiological Functions of Bile Acids

5.1. Bile Secretion

Nearly all bile acids are choleretic agents; that is, they increase bile flow when infused intravenously into various animal species. In all vertebrtae species examined, there is a close relationship between bile flow and the hepatic excretion rate of bile acids (B24). Acute interruption of the enterohepatic circulation of bile acids in man by diversion of bile flow causes the rate of bile secretion to decrease by about 50% (T10). Thus, the excretion of bile acids from the liver is the major determinant of bile water and solute excretion, predominantly because of the osmotic activity of bile acids in bile. Some interesting studies in dogs have been performed with the bile salt taurodehydrocholate (taurine conjugate of 3,7,12-triketo-5β-cholan-24-oic acid), which, for stereochemical reasons, cannot form micelles and should therefore have greater osmotic activity than other bile acids. At the same

bile acid secretion rate, it has been shown that the bile flow rate associated with taurodehydrocholate is greater than that associated with taurocholate, but less than that associated with free cholate (O4). These results indicate that micelle formation is not essential for bile acid secretion across the liver cell canalicular membrane and that nonosmotic factors must also play a role in determining the choleretic properties of individual bile acids.

Until recently, it was considered that hepatic bile flow was still possible even if no bile acids were excreted. This portion of bile flow was termed bile acid independent and was estimated to account for around one-third of canalicular bile flow in man (B33, P15). More recent evidence suggests that the relationship between bile flow and bile acid excretion is curvilinear, so that bile flow decreases progressively more rapidly as bile acid excretion rates decrease to very low values (B2, B24). Bile-salt-independent bile flow may therefore be much lower than previously thought and represent only a very small fraction of total bile flow (B24).

Lithocholic acid and its conjugates are bile acids which are not choleretic, but have the opposite effect in causing intrahepatic cholestasis in experimental animals (F5, J1, O2), and presumably man. The cholestatic effect of these bile acids is abolished if cholic acid is administered simultaneously, probably because of the ability of cholic acid to solubilize lithocholic acid in micelles (K2, L5). To explain the pathogenesis of lithocholate-induced cholestasis, it has been suggested that lithocholate binds to the bile canalicular membrane, increases its cholesterol content and reduces its permeability to water and ions (K1, K2).

5.2. Solubilization of Biliary Cholesterol

Cholesterol is nearly insoluble in water, but in gallbladder bile the concentration of cholesterol can be over 20 mmol/liter (A2). This dramatic increase in cholesterol solubility is brought about by the action of bile acids, which in combination with phosphatidylcholine (lecithin) dissolve cholesterol in mixed micelles (see Section 2.2). The importance of mixed micelles, containing both bile acids and lecithin, in biliary cholesterol solubilization was established by systematic phase equilibria studies involving various aqueous mixtures of these three lipids (S35). From these studies of artificial bile solutions, cholesterol solubility limits were established by Admirand and Small (A2), so that the maximum quantity of cholesterol that could be dissolved in mixed micelles of varying bile acid/lecithin molar ratio could be determined from a triangular coordinate diagram (Fig. 7). This diagram shows the physical state of all possible combinations of bile acids, lecithin, and cholesterol in aqueous solutions containing a total of 10% solids by weight. The composition of any bile containing these three lipids was repre-

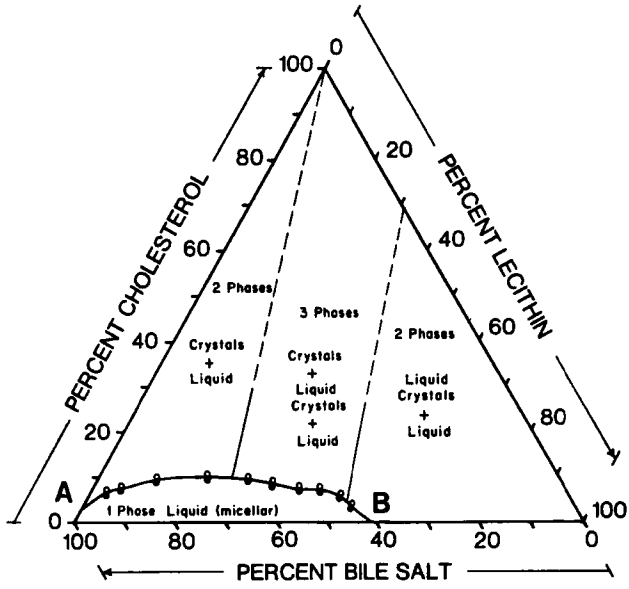

FIG. 7. Phase diagram showing the physical state of all possible combinations of cholesterol, bile salts, and lecithin (expressed as mole percent) in aqueous solutions. The line AB represents the maximum amount of cholesterol, according to Admirand and Small (A2), which can be dissolved by any mixture of bile salts and lecithin. [From ref. (A2). Reproduced from *The Journal of Clinical Investigation*, 1968, **47**, by copyright permission of The American Society for Clinical Investigation.]

sented by a single point within the triangular coordinates. Cholesterol was completely in micellar solution only in biles with a composition corresponding to a point below the line of maximum cholesterol solubility. Biles with a lipid composition above this line (line AB on Fig. 7) contained cholesterol in crystalline form as well as in mixed micelles.

These studies of cholesterol solubility in model bile solutions led to the discovery of a physicochemical basis for cholesterol gallstone formation in man. After measuring the concentrations of biliary lipids, gallbladder bile from cholesterol gallstone patients was predicted to be supersaturated with cholesterol, while normal bile was not (A2). This finding stimulated an enormous amount of further work on factors affecting cholesterol solubility in human bile. While it is still agreed that the pathogenesis of cholesterol gallstones is related to the capacity of bile to maintain cholesterol in micellar solution, in the last 15 years several important developments have occurred, which require some modification be made to the conclusions of Admirand and Small (A2). These developments include the following points.

1. The precise limits of cholesterol solubility in model bile solutions appear lower than first determined (H13, H23).

2. The total lipid concentration in bile is an important factor affecting cholesterol solubility and must be taken into account when calculating biliary cholesterol saturation (C7).

3. Bile supersaturated with cholesterol is common in normal subjects, and there is considerable overlap between estimates of the cholesterol saturation of bile in patients with and without gallstones (C7, H23). Thus it is not possible to completely separate patients with cholesterol gallstones from controls simply by examination of biliary lipid composition.

4. Supersaturation of gallbladder bile with cholesterol is now regarded as a necessary prerequisite for cholesterol gallstone formation, but other factors such as the presence of nucleating or antinucleating agents in bile may influence whether cholesterol will crystallize from micellar solution (B36, H21, H22, W7). Of particular interest are studies which suggest that the gallbladder of patients with cholesterol gallstones may add a nucleating factor to bile, thus facilitating cholesterol crystal formation (B36, G9).

5.3. Lipid Digestion and Absorption

Although there can still be considerable hydrolysis and absorption of fat in the absence of bile (P12), there is no doubt that bile acids greatly enhance intestinal lipid absorption. Nearly all dietary fat is ingested in the form of triglycerides. Hydrolysis of fat begins in the stomach, but most triglyceride lipolysis occurs in the duodenum. Here, after bicarbonate secretion has raised the pH to 6 to 6.5, pancreatic lipase hydrolyzes triglyceride at the 1 and 3 positions, leaving β-monoglyceride, diglycerides, and free fatty acids.

For pancreatic lipase to be active, an additional protein, termed colipase, is required (B26). Pure pancreatic lipase is inhibited by bile salts in concentrations exceeding their critical micellar concentrations (B27). The function of colipase is to restore lipase activity in the presence of bile salts. Although colipase by itself has no lipolytic activity (B27), defective fat digestion and absorption occur if either lipase or colipase activity is low in the small intestine. Patients with steatorrhea due to either isolated lipase deficiency (F4) or isolated colipase deficiency (H16) have been reported. A lipase which requires bile acids for activity is human milk lipase (O1). This enzyme comprises 1% of the protein of human milk, but is inactive against milk fats until its activity is stimulated by bile acids in the small intestine.

The products of triglyceride hydrolysis have only limited solubility in the aqueous environment of the intestinal lumen, as do other lipids which are present, such as cholesterol and fat-soluble vitamins. For efficient lipid sol-

ubilization and absorption, the formation of mixed micelles containing bile salts, monoglyceride, fatty acids, and cholesterol, is essential. Micelles allow easier penetration of the unstirred water layer, which covers the surface of the intestine and forms a significant barrier to the diffusion of hydrophobic lipids to the microvillus membrane of the intestinal epithelial cells (T9). It follows that any disease which results in a reduced concentration of bile acids within the intestinal lumen will impair the micellar solubilization of lipids and may lead to steatorrhea. Conditions where this occurs are (1) biliary obstruction or cholestatic liver disease, which leads to decreased delivery of bile salts to the intestinal lumen; (2) ileal disease or resection, which may lead to an increased intestinal loss of bile salts, which is too large to be compensated by increased hepatic synthesis; and (3) stasis syndromes which are characterized by bacterial overgrowth in the small intestine with bile salt deconjugation (G5). Drugs which bind bile acids, such as cholestyramine resin, can also affect micelle formation and lipid solubilization and absorption in the small intestine.

6. The Biochemical Assay of Bile Acids

Interest in methods for the quantitative measurement of bile acids has grown rapidly in recent years. This is because it has been recognized that alterations in bile acid metabolism may be the cause or consequence of several diseases and that bile acid analysis may be useful in evaluating disturbed liver or intestinal function. Of the body fluids, gallbladder bile contains the highest concentration of bile acids (Table 1), because nearly all of the bile salt pool is confined to the enterohepatic circulation and is stored in the gallbladder between meals. Gallbladder bile can be collected directly from the gallbladder by needle puncture during abdominal surgery or it can be aspirated from the duodenum along with pancreatic secretions via an oral tube after contraction of the gallbladder and ejection of bile into the duodenum (W3). As already described in Section 4.1, bile acids are also present in the systemic circulation, although at concentrations which are very much less than found in bile (Table 1). The main excretory route for bile acids is via the feces, but small amounts are also detectable in the urine (Table 1).

For the quantitative estimation of bile acids in body fluids and tissues, consideration must be given as to whether (1) an extraction step is necessary to partially purify the bile acids prior to further analysis; (2) hydrolysis is required to remove glycine and taurine or other conjugate moieties; and (3) the method of analysis will be of the required sensitivity and provide infor-

TABLE 1

CONCENTRATION RANGES OF TOTAL BILE ACIDS PRESENT IN NORMAL HUMAN BODY FLUIDS

Specimen	Major bile acids present[a]	Concentration range	Comments	References
Gallbladder bile	Glycine and taurine conjugates of C, CDC, DC	150–350 mmol/liter	Concentration dependent on gallbladder function	C7
Hepatic bile	As for gallbladder bile	4–60 mmol/liter	Collected from common bile duct	C7
Duodenal bile	As for gallbladder bile	20–160 mmol/liter	Concentration dependent on intubation technique	W3
Fasting serum (peripheral venous)	As for gallbladder bile, with up to 50% unconjugated bile acids	1–6 μmol/liter	Concentration increased after a meal	A9, P9, S34, S37
Fasting serum (portal venous)	As for peripheral venous serum	9–50 μmol/liter	Collected at surgery	A3
Urine	Sulfate and glucuronide conjugates of CDC, DC, and LC	7–45 μmol/day		A6, A7
Feces	Unconjugated DC and LC	200–1500 μmol/day	Amount excreted dependent on diet	D6, P11, S27

[a] Abbreviations used for bile acids are C, cholic acid; CDC, chenodeoxycholic acid; DC, deoxycholic acid; LC, lithocholic acid.

mation on total or individual bile acid concentrations. These aspects are considered below.

6.1. Extraction Methods

There are several methods available for the extraction of bile salts from serum or plasma. The most convenient methods utilize some form of liquid–solid extraction. An early procedure involved the anion-exchange resin, Amberlyst A-26 (S8), but considerable time and effort was required to perform column chromatography and to concentrate the eluate from the column. The introduction in 1972 of the neutral resin, Amberlite XAD-2, improved the ease of extracting bile acids and their conjugates from serum samples (M6). Further improvement occurred in 1977 with the description of a batch extraction technique using the related neutral resin, Amberlite XAD-7 (B5). With this technique, serum is diluted in 0.1 *M* sodium hydroxide to release bile acids from albumin and mixed with resin for 1 hour. After washing the resin in dilute alkali, bile acids are eluted with methanol, which can be removed on a rotary evaporator (B5).

More recently, the use of cartridges packed with octadecylsilane-bonded silica has been proposed as faster, more efficient, and generally superior to extraction procedures using Amberlite resins (S19, S20), especially for sulfated bile acids. However, one commercially available cartridge of this type is not stable under the alkaline conditions used for serum bile acid extraction and can cause problems in later gas–liquid chromatographic analysis (W1). Nevertheless, these cartridges are simple to use and offer a rapid method for quantitative recovery of all the common bile acids and their conjugates in a small volume of organic solvent. For a more detailed analytical profile, serum bile acids can then be further fractionated into unconjugated, taurine and glycine-conjugated and sulfate-conjugated groups. This is achieved by anion-exchange chromatography on a small column of diethylamino-hydroxypropyl (DEAP) Sephadex LH-20, using stepwise elution with acetic acid in ethanol (S19). Profiles of bile acids in urine also have been determined using this lipophilic anion-exchange material (A6).

The extraction of bile acids from solid material, such as feces or tissues, requires a different approach since bile acids are likely to be firmly bound to bacteria or proteins. A variety of solvents have been used, such as alcoholic alkali (G14), toluene–acetic acid (E4), methanol–acetone (B35), and methanol–chloroform (E1), sometimes with refluxing or Soxhlet extraction (B17, E1). The state of ionization of the carboxyl group of bile salts, and thus the solvent pH, would be expected to affect the efficiency of extraction with organic solvents, with better extraction at acid pH. However, in practice an

alkaline pH can be more favorable, presumably due to inhibition of bile acid binding to proteins.

6.2. Hydrolysis of Bile Acid Conjugates

The fact that bile acids are generally conjugated with glycine or taurine as *N*-acyl conjugates at the C-24 carboxyl group and may also form sulfate esters or glucuronides via the hydroxyl groups, particularly at the C-3 position, means that a complex mixture of bile acid species can exist in biological fluids. This is especially true for the low amounts present in serum and urine, whereas in bile virtually all the bile acids are conjugated with glycine or taurine and levels of unconjugated or sulfated bile acids are very low (C4, M4). In urine, nearly all bile acids are conjugated with glycine or taurine and most are also sulfated (A6, M4). Bile acid glucuronides also occur in the urine of healthy subjects and account for 12 to 36% of the total urinary bile acid excretion (A7). Although the pattern of glucuronides in urine is complex, C-6 hydroxylated bile acids form a major fraction of the glucuronides, which are otherwise unconjugated (A7).

While it is recognized that a significant fraction of serum bile acids may be unconjugated and that sulfated bile acids are present in serum, few studies have been carried out to simultaneously isolate and quantitate all the different conjugate fractions in order to document their relative proportions in serum in health and disease. With the development of new extraction methods for fractionating bile acids into their conjugate classes (A6, S19), future studies of bile acid profiles should be more comprehensive. Present indications are that the serum of healthy subjects contains a small but variable proportion of sulfated bile acids (B9, C3, M4, S19). This proportion may rise dramatically in hepatobiliary disease (M4), along with the total serum bile acid concentration, and if not measured will cause underestimation of the serum bile acid concentration. In addition, unconjugated bile acids, which were once thought to be present in negligible amounts in the serum of normal subjects, may account for up to one-third of total nonsulfated bile acids (M5, S37). Serum unconjugated bile acid levels show a diurnal variation with increased levels after meals (S18). In cholestatic liver disease, the enterohepatic circulation of bile acids is greatly reduced and negligible amounts of unconjugated bile acids are found in the serum (B9, M35).

To deconjugate bile acids for further analysis, particularly by gas–liquid chromatography, different methods are required for the hydrolysis of the peptide bonds in glycine and taurine conjugates than for hydrolysis of the ester sulfate and glucuronide bonds. Glycine and taurine may be removed by either alkaline or enzymatic hydrolysis (R11). Alkaline hydrolysis is often

carried out in Teflon tubes using 2 *M* aqueous or ethanolic sodium hydroxide for 2 or 3 hours at 120°C (B14, G14, L6, R11). This procedure usually gives good recoveries of the primary and major secondary bile acids, but can cause losses of keto bile acids, especially if hydrolysis is carried out in alcoholic solution (L6). A variation on the method of chemical hydrolysis, which gives excellent recoveries in a much shorter hydrolysis time of 15 to 20 minutes, is to use 20% potassium hydroxide in ethylene glycol under reflux at a temperature of 210°C (E4, E6).

Enzymatic hydrolysis of glycine and taurine bile acid conjugates became possible after the discovery in 1967 of the enzyme choloylglycine hydrolase (EC 3.5.1.24), which was isolated from *Clostridium perfringens* (N1, N2). This enzyme can now be purchased from commercial sources. The substrate specificity of the enzyme is such that bile acids with free hydroxyl groups on the steroid skeleton are more easily hydrolyzed than keto bile acids and glycine conjugates are hydrolyzed faster than taurine conjugates (B14, N1). Provided that sufficient enzyme is used, rapid quantitative hydrolysis of all the common bile acids can be achieved at 37°C (B14, R11). Enzymatic hydrolysis has a number of advantages over chemical hydrolysis. The most important of these is the more gentle hydrolysis conditions, which minimize the breakdown of bile acids and artefact formation. Sulfated bile acid conjugates in particular may be unstable under the conditions used for chemical hydrolysis, but are hydrolyzed slowly without desulfation by cholylglycine hydrolase preparations (C3, P1). In addition, for a bile acid such as taurolithocholic acid, enzymatic hydrolysis is far superior to chemical hydrolysis, which requires prolonged alkali treatment for 24 to 48 hours (N1).

Chemical hydrolysis or solvolysis is the only method available at present to remove sulfate groups from bile acids. It is known that some colonic bacteria possess a sulfatase which can utilize sulfated bile acids as substrate (H24), but this enzyme has not yet been purified. The position of the sulfate moiety in monosulfated bile acids is nearly always at C-3 and this group is easily removed by acid hydrolysis in ethereal solution after removal of glycine or taurine (V1). This method, however, does not remove sulfate groups from C-7 or C-12 (P1). For complete removal, other methods have been described, including solvolysis in acidified methanol–acetone for 18 hours at 37°C (P6), acidified ethyl acetate–ethanol for 16 hours at 39°C (A6), or acidified 2,2-dimethoxypropane for 12 hours at room temperature (C3).

Bile acid glucuronides may be hydrolyzed by β-glucuronidase (EC 3.2.1.31) from bovine liver or the digestive juice of *Helix pomatia* (S41). The latter enzyme preparation also contains sulfatase activity (S41), but this is likely to be an aryl sulfatase which may not use bile acid sulfates as substrates.

6.3. Quantification of Total and Individual Bile Acids

6.3.1. *Enzymatic Estimation with 3α-Hydroxysteroid Dehydrogenase*

Early methods for the determination of total bile acids involved heating samples with concentrated sulfuric acid to form compounds which could be measured spectrophotometrically by their absorbance of ultraviolet light (S33). Other colorimetric reactions, which utilized strong acids and other reagents to produce blue or red colored products, were also used. These methods were lacking in specificity, but this problem was overcome in 1964 with the introduction of an enzymatic method for bile acid determination (I3). The enzyme 3α-hydroxysteroid dehydrogenase (EC 1.1.1.50), which is isolated from *Pseudomonas testosteroni*, catalyzes the conversion of all 3α-hydroxycholanic acids to 3-ketocholanic acids, with the concomitant reduction of NAD^+ to NADH. The NADH formed in the reaction is then determined spectrophotometrically at 340 nm. To ensure complete reaction, hydrazine is usually added to bind the 3-keto products (P2). The optimum conditions for enzymatic assay include a pH of 9.0 to 9.5 and reaction temperature in the range 20 to 40°C (T13). Reaction rates for individual bile acids may not be identical, but the assay is normally carried out as an endpoint determination. Alternatively, the addition of bovine serum albumin appears to overcome the problem of variable affinity of 3α-hydroxysteroid dehydrogenase for different bile acids if reaction rates are to be measured (S13).

The enzymatic assay method has gained wide acceptance for the determination of bile acids, since all the major bile acids, including both free and glycine- and taurine-conjugated forms, can be determined. With gallbladder or hepatic bile samples, or with samples of duodenal aspirate which contain bile, the concentration of bile acids is sufficiently high for samples simply to be diluted with methanol or isopropanol prior to assay. Samples may need to be centrifuged to remove precipitated protein. For serum bile acid estimation, the sensitivity of the enzymatic method must be increased in order to detect the small amounts of NADH produced in the reaction. This has been accomplished by direct fluorimetric estimation of NADH (M33, S16), either using a single beam spectrofluorimeter or a double beam instrument to cancel nonspecific fluorescence (S31). Double beam spectrofluorimetry also allows the direct analysis of serum without time-consuming preliminary extraction steps (S31).

More recently, the sensitivity of enzymatic assays has been further increased by a number of coupling methods (Table 2). These transfer hydrogen from NADH to acceptor compounds, such as a tetrazolium dye to produce diformazan (M11) or the dye resazurin to produce resorfin (M9). The enzyme

TABLE 2

COMPARISON OF THE SENSITIVITY OF DIFFERENT PHOTOMETRIC METHODS USING 3α-HYDROXYSTEROID DEHYDROGENASE TO MEASURE TOTAL BILE ACIDS

Method	End product	Measurement[a]	Detection limit	Upper limit of linear range[c] (amount/assay)	CV[b]	Reference
Spectrophotometric	NADH	A_{340}	5–10 nmol	—	—	I3, P2
Spectrofluorimetric	NADH	E_{460}	0.5–1 nmol	16 nmol	2.0	M33, S16, S31
Spectrophotometric (coupled)	Diformazan	A_{540}	1 nmol	50 nmol	4.3	M11
Spectrofluorimetric (coupled)	Resorfin	E_{580}	0.1 nmol	15 nmol	0.9	M9
Spectrophotometric (coupled and amplified)	Testosterone	A_{248}	0.3 pmol	15 pmol	7.5	N3
Luminometric	Light	Luminometer	1 pmol	20 nmol	8.2	S13

[a] *A*, absorbance; *E*, emission.

[b] In the assay of normal serum.

diaphorase (EC 1.8.1.4) is used to catalyze the transfer and to regenerate NAD^+ for further reaction, and the end products are measured spectrophotometrically or spectrofluorimetrically (Table 2). The fluorimetric estimation of resorfin has been incorporated into a continuous flow system suitable for the routine determination of serum bile acids in the clincial laboratory (M10). A further increase in the sensitivity of the enzymatic determination of bile acids has recently been achieved using bioluminescence. The principle of this method is to utilize NADH to generate light via coupled reactions catalyzed by a bacterial oxidoreductase and luciferase isolated from either *Photobacterium fischeri* (R5) or *Beneckea harveyi* (R6). This method has a number of outstanding advantages, which include sensitivity to the level of 1 pmol per assay so that 10 μl of normal serum is sufficient (R6, S13), and rapid and precise determination, as light output is immediate and reaches a plateau for measurement within one minute after reagents are mixed (R6). Alternatively, conditions can be chosen so that kinetic measurement of light intensity can be related to the concentration of bile acids (S44). A continuous-flow bioluminescence method utilizing nylon-immobilized enzymes has been described for measuring primary bile acids in serum, so that automation of the method for the clinical laboratory is possible (R5). Automation has also been achieved with more simple enzymatic methods (B30, S40).

An interesting variation on coupling procedures to increase the sensitivity of NADH measurement, which deserves further attention, has been described by Nicolas *et al.* (N3). In this method, each pmol of NADH produced by hydroxysteroid dehydrogenase-catalyzed reaction with serum bile acids is used to generate several thousand pmol of testosterone, which can then be easily measured spectrophotometrically at 248 nm (N3). Coupling enzymes involved are 3β,17β-hydroxysteroid dehydrogenase (EC 1.1.1.51) and 3-ketosteroid Δ^5,Δ^4-isomerase (EC 5.3.3.1) from *Pseudomonas testosteroni*, along with the substrate dehydroepiandrosterone. This system is unique in that amplification of the NADH present is accomplished by cycling of NADH between the reduction of the 17-keto group of dehydroepiandrosterone and the oxidation of the 3β-hydroxyl group of androsterone-3β,17β-diol to produce a stoichiometric amount of 17β-hydroxy-3-keto-Δ^5-androstene, which is converted to stable testosterone by the isomerase. During an overnight incubation, around 7000 cycles occur to produce the desired level of amplification (N3).

In addition to bile and serum bile acids, fecal bile acids can be estimated by the enzymatic method, provided that they are first extracted to remove interfering lipids. Methods for this purpose are continually being simplified and improved (B16, D6, V3). Enzymatic determination of fecal extracts slightly underestimates total bile acid excretion, since 3-keto bile acids and bile acids which are sulfated at the 3α position occur in feces and are not

measured by this technique. However, 3-keto bile acids can be reduced to 3α-hydroxy bile acids by sodium borohydride (B14), or directly assayed using the hydroxysteroid dehydrogenase reaction in the reverse direction and measuring the decrease in NADH by spectrophotometry (A12). To date, a bile acid 3α-sulfatase has not been purified to remove sulfate groups and permit to the enzymatic assay of bile acid sulfates, which occur in serum in hepatobiliary disease (C3, M4).

3α-Hydroxysteroid dehydrogenase is available from several commercial sources, but different enzyme preparations vary in purity and the degree of contamination with other dehydrogenases. Interference has been reported due to alcohol dehydrogenase (E3), 3β-hydroxysteroid dehydrogenase (T13), malate dehydrogenase (B8, W4), and aldehyde dehydrogenase (S13). A mutant form of *P. testosteroni* which does not produce 3β-hydroxysteroid dehydrogenase is of limited value because its 3α enzyme has different activities with different bile acids (B20). Endogenous NADH-generating enzymes present in serum must be inactivated before total serum bile acids can be measured accurately. Methods for accomplishing this include heating at 67°C for 30 min (M9), sodium pyruvate (M11), dialysis (M10), and alkali (H2). Alkali pretreatment appears to be the preferred method (H2).

As well as the enzyme 3α-hydroxysteroid dehydrogenase, a 7α-enzyme has been isolated from *E. coli* and a 12α enzyme from a strain of *Clostridium* (M1). These group-specific enzymes can be used to measure the amounts and ratios of the three main bile acids (cholic, chenodeoxycholic, and deoxycholic acids) in bile specimens. Bioluminescent assays suitable for serum bile acid analysis have been described using 7α-hydroxysteroid dehydrogenase (R6) and 12α-hydroxysteroid dehydrogenase (S12), as well as for the 3α enzyme (S13, S44).

6.3.2. *Radioimmunoassay*

Because the low levels of bile acids in serum have been difficult to measure by most photometric techniques, much effort has been devoted to developing radioimmunoassays for specific bile acids. Antibodies can be prepared by immunization of rabbits with the appropriate bile acid covalently coupled to bovine serum albumin by a mixed anhydride (M34) or carbodiimide (S30) technique. These antibodies are usually of low titer, but by preliminary treatment of rabbits with antitubercular vaccine and the use of microgram quantities of antigen, antisera of much higher titer can be obtained (R4). Surprisingly, the use of free cholic acid coupled to albumin as immunogen produces the highest titer of antibodies to conjugated cholic acid, suggesting that the amide linkage of bile acid to protein is an important antigenic determinant (B13). Incorporating a spacer between bile acid hapten and carrier protein decreases the antiserum titer (B13).

In the last decade, several research groups have developed radioimmunoassays to individual bile acids and the characteristics of these assays are shown in Table 3. Most methods have used the glyco-conjugated bile acid as immunogen and the resultant antibodies show equal affinity for the tauro-conjugate and usually a low cross-reactivity with the free bile acid. Assays are therefore bile acid specific but do not usually distinguish the glyco- and tauro-conjugates of that bile acid. Conjugated bile acids, for which radioimmunoassays have been developed, are cholic acid (B3, M2, M12, M23, M25, M26, M34, R8, S30, S38, V2), chenodeoxycholic acid (B3, M2, R8, S11), deoxycholic acid (M2, M13), sulfated lithocholic acid (C8), nonsulfated lithocholic acid (C9, R9), and ursodeoxycholic acid (H17, M3) (Table 3). Assays have also been reported for the glycine conjugates of cholic, chenodeoxycholic, deoxycholic and sulfolithocholic acids (D5), and tauro-β-muricholic acid (B29).

Most radioimmunoassay methods for serum bile acids do not use any form of extraction prior to assay. Two studies have compared assay results with and without serum extraction and have found no significant differences (B3, B12). Early radioimmunoassays used ^{3}H ligands, but the later synthesis and use of ^{125}I-labeled bile acid derivatives, such as bile acid–histamine (B12, S38), increased the sensitivity and precision of assays while decreasing assay time and cost. To separate bound and free ligand, polyethylene glycol and saturated ammonium sulfate have been used in preference to dextran-coated charcoal, possibly because the first radioimmunoassay described for a bile acid found that charcoal was unsatisfactory (S30). In another study, the apparent specificity of the antibody was altered by using charcoal as compared to polyethylene glycol (S38). Assay sensitivities and normal fasting serum concentrations of individual bile acids are shown in Table 3. Most radioimmunoassays are sufficiently sensitive to consistently detect the increase in serum bile acid concentrations which occurs after a meal.

Radioimmunoassay kits have become available for various serum bile acids, in particular glycocholate, glycochenodeoxycholate, conjugated primary bile acids, and sulfoglycolithocholate. Critical evaluation of these kits has indicated that they are acceptable with respect to accuracy, precision, stability, and analytical recovery (R7) with one exception (K3). The specificity of antisera can vary widely with the antibodies from different manufacturers showing different cross-reactivities for glyco-, tauro-, and free cholic acid (R7). Different kits may therefore give slightly different results in estimating bile acids. A comparison of one radioimmunoassay with an enzymatic–fluorimetric method for determining total bile acids in serum concluded that the ease of radioimmunoassay was outweighed by its greater cost and poorer analytical performance (S39). In addition, due to problems with cross-reactivity, radioimmunoassays are best directed towards measurement of one

TABLE 3

THE DEVELOPMENT AND CHARACTERISTICS OF RADIOIMMUNOASSAYS USED TO MEASURE BILE ACIDS IN SERUM

Year of publication	Bile acids assayed[a]	Serum extraction	Isotope	Separation method[b]	Assay sensitivity (pmol/assay)	Normal fasting serum conc. range (μmol/liter)	Reference
Cholic acid conjugates							
1973	CG,[c] CT	No	^{3}H	PEG	5	0.2–1.0	S30
1974	CG,[c] CT	Ethanol	^{3}H	AS	10	0.6–1.8	M34
1976	C,[c] CG, CT	XAD-2	^{3}H	PEG	5	0.2–1.3	M12
1976	CG,[c] CT	No	^{3}H	SP	5	0.8–2.0	V2
1976	CG[c]	No	^{3}H	AS	10	0.2–0.3	D5
1977	CG,[c] CT	No	^{14}C	AS	5	0.1–0.7	R8
1979	CG,[c] CT, C	Ethanol	^{125}I	PEG	2	0.1–0.8	M2
1979	CG,[c] CT	No	^{3}H	AS	5	0.5–1.3	B3
1979	C,[c] CT, CG	No	^{125}I	DC	10	0.4–1.9	M26
1981	CG,[c] CT	No	^{125}I	PEG	0.5	0.0–1.3	M25
Chenodeoxycholic acid conjugates							
1976	CDCG[c]	No	^{3}H	AS	10	0.1–0.3	D5
1977	CDCG,[c] CDCT	No	^{3}H	AS	5	0.4–2.5	R8
1977	CDCG,[c] CDCT	No	^{3}H	AS	2	0.3–3.8	S11

1979	CDCG,[c] CDCT, CDC	Ethanol	^{125}I	PEG	0.5	0.0–0.9	M2
1979	CDCG,[c] CDCT	No	^{3}H	AS	2	0.6–2.0	B3
1979	CDC,[c] CDCG, CDCT	No	^{125}I	AS	1	0.0–2.2	B12
Deoxycholic acid conjugates							
1976	DCG[c]	No	^{3}H	AS	10	0.0–0.1	D5
1977	DC,[c] DCG, DCT	XAD-2	^{3}H	PEG	8	0.2–0.9	M13
1979	DCG,[c] DCT, DC	Ethanol	^{125}I	PEG	2	0.1–0.6	M2
Lithocholic acid conjugates							
1977	LCG,[c] LCT, LC	No	^{3}H	PEG	20	0.0–0.6	C9
1978	LCG,[c] LCT, LC	No	^{3}H	AS	1	0.0–0.3	R9
Sulfolithocholic acid conjugates							
1976	SLCG[c]	No	^{3}H	AS	10	0.0–0.1	D5
1977	SLCG,[c] SLCT, SLC	No	^{3}H	PEG	10	0.5–4.0	C8
Ursodeoxycholic acid conjugates							
1978	UDC,[c] UDCG, UDCT	No	^{3}H	PEG	10	0.1–0.4	M3
1983	UDCG,[c] UDCT, UDC	No	^{125}I	AS	1	0.1–0.7	H17

[a] The abbreviations used are CG, cholylglycine; CT, cholyltaurine; C, cholic acid; CDCG, chenodeoxycholylglycine; CDCT, chenodeoxycholyltaurine; CDC, chenodeoxycholic acid; DCG, deoxycholylglycine; DCT, deoxycholyltaurine; DC, deoxycholic acid; LCG, lithocholylglycine; LCT, lithocholyltaurine; LC, lithocholic acid; SLCG, sulfolithocholylglycine; SLCT, sulfolithocholyltaurine; SLC, sulfolithocholic acid; UDCG, ursodeoxycholylglycine, UDCT, ursodeoxycholyltaurine; UDC, ursodeoxycholic acid.

[b] PEG, polyethylene glycol; AS, ammonium sulfate; SP, solid phase; DC, dextran-coated charcoal.

[c] Bile acid used as hapten.

bile acid, rather than towards attempting to measure total serum bile acid concentrations.

The low concentrations of bile acids in urine have also been measured by radioimmunoassay. In one study, total cholic and chenodeoxycholic acid conjugates were measured after extraction and solvolysis to remove sulfate groups, giving a mean urinary excretion of 0.6 μmol/24 hours for cholic acid and 1.2 μmol/24 hours for chenodeoxycholic acid in normal subjects (S7). These estimates can be compared with values of 2.1 μmol/24 hours for conjugated cholic acid and 8.4 μmol/24 hours for sulfoglycolithocholic acid obtained for the urinary excretion of bile acids using commercially available radioimmunoassays (W10).

Using the same principle as radioimmunoassay, but employing an enzyme–antigen conjugate instead of radioactive isotope tracer, enzyme-linked immunoassays have been developed for chenodeoxycholic acid (B4) and ursodeoxycholic acid (O7). The sensitivity and specificity of these assays are similar to those of radioimmunoassay, but the use of a radioactive isotope is avoided and enzyme activity is measured spectrophotometrically. Another interesting variation on the use of antiserum to bile acids has been reported with the synthesis of a glycochenodeoxycholyl–fluoresceinthiocarbamyl ethylenediamine conjugate as a fluorescent label. The antibody was coupled to magnetizable particles to allow a simple separation of bound and free ligand in a fluoroimmunoassay (S29).

6.3.3. *Gas–Liquid Chromatography (GLC) and Mass Spectrometry*

Gas–liquid chromatography is a well established and widely used method for the analysis of individual bile acids. In addition to its sensitivity, this technique has the advantage of simultaneous separation of complex bile acid mixtures and their quantification, usually by flame ionization detection. When combined with mass spectrometry, unequivocal identification of unknown bile acids can be achieved. Thus, this method is regarded as the reference method against which other methods are compared. However, GLC is laborious and time consuming and needs considerable expertise on the part of technical staff for good results.

Unlike enzymatic or radioimmunoassay methods, GLC requires lengthy sample preparation before bile acid concentrations can be determined. In the case of serum, bile acids must be extracted (see Section 6.1) and hydrolysis carried out to remove glycine and taurine, and also sulfate groups, if they are likely to be present (see Section 6.2). The free bile acids are then converted to volatile derivatives.

The carboxyl group of the bile acid molecule is methylated carefully with diazomethane (K10, R12, W6) or sometimes with 2,2-dimethoxypropane

(S23) or acidified dry methanol (C2). Hydroxyl groups on the steroid nucleus are also derivatized, most commonly as trifluoroacetates (K10, R12, W6) or silyl ether derivatives (K8), but occasionally as acetates (R10) or formates (A7). Trifluoroacetate derivatives have the advantage that electron capture can be used to increase the sensitivity of detection to the pmol level (D4, K6), but they may not be as stable as other derivatives (A6). Other recent variations on derivative formation include methylation of the carboxyl group and all hydroxyl groups, called permethylation (B7), or the formation of heptafluorobutyrates with the carboxyl and hydroxyl groups (M36). The use of heptafluorobutyric acid anhydride has the advantage that deconjugation and derivatization is carried out in the one step.

Internal standards which have been used for GLC of bile acids are numerous and include 7-ketolithocholic acid (K10, R12), hyodeoxycholic acid (K8), 7-ketodeoxycholic acid (L1, W6), 5β-cholanic acid (C2), coprostanol (S19), and 23-nor-deoxycholic acid (R10). Standards are added either to the sample to be analyzed (R12) or prior to derivative formation (S19) and bile acid quantitation carried out from gas–liquid chromatograms by determining the ratio of the peak area of the bile acid to the peak area of the internal standard. This ratio is then compared with that obtained with bile acid standards, which are used to define the linear range of detector response and to determine the detector response for each bile acid.

Once suitable derivatives have been prepared, mixtures of bile acids can be separated on a number of commercially available liquid stationary phases. With packed columns, commonly used phases for methyl trifluoroacetates are SP-2401 (J2, W6), OV-210 (R12) and QF-1 (C2, K10), while for methyl acetate and trimethylsilyl derivatives, PPE-20 (G1) and Poly S-179 (S46) are preferred. Packed columns are slowly being replaced by capillary columns, due to their greatly increased resolution. This development represents an important step towards the complete metabolic profiling of complex mixtures of bile acids with widely different polarity, as occur in feces. However, the choice of open-tubular glass capillary columns for bile acid analysis is restricted to a limited number of phases. Setchell and Matsui (S19) have reported the separation of serum bile acids as the methyl trimethylsilyl ether derivates on a 25-meter glass capillary column wall-coated with silicone OV-1, using temperature programming from 220 to 275°C. The sensitivity of their method, which used a solid injection device, was comparable to radioimmunoassay (S19). Other liquid phases which have been used successfully to determine bile acids by capillary GLC are polyethylene glycol 20,000 (K8), polyethylene glycol-HT (T3), and SE-54 (D4).

The sensitivity and specificity for bile acid determination can be further increased if mass spectrometry is combined with GLC. By the use of a multiple ion detector unit for selected ion monitoring (mass fragmentogra-

phy), individual bile acid concentrations can be measured in serum (A10, S19) and also in small volumes of skin interstitial fluid (B9). Bile acids labeled with deuterium are used as internal standards to correct for losses in preliminary extraction, deconjugation, and derivatization steps (A10). For a comprehensive analysis, computerized mass spectrometry (mass chromatography) has been used in a few studies (A6, M29). Greater detail on the use of mass spectrometry to analyze bile acids is given in a recent review by Street *et al.* (S43).

6.3.4. *High-Performance Liquid Chromatography (HPLC)*

Because HPLC offers the possibility of rapid separation, quantification, and recovery of both free bile acids and bile acid conjugates, much interest is currently centered on this technique. A significant advantage of HPLC compared to GLC is that potentially destructive hydrolysis steps can be avoided and bile acid conjugates can be analyzed as they occur in bile or serum. The method is relatively fast as the major bile acids in human bile have been separated in less than 1 hour by reverse-phase HPLC (B25, M8).

The first report on the use of HPLC to separate bile acids appeared in 1976 (S22). Since that time, a great number of methods have been published describing the use of various solvent systems and straight-phase or reverse-phase columns, which are effective in separating mixtures of bile acids and their conjugates. The features of some of the methods which were described in 1983 are summarized in Table 4. Most recently developed methods use reverse-phase columns with acidic alcoholic eluents. Radial compression cartridges have been found to be quicker and more reproducible than stainless steel columns (R2, R13). HPLC analysis of bile acids can be simplified if a preliminary separation into groups of free, glyco-, and tauro-conjugates is first carried out by ion-exchange chromatography of bile (G12) or serum (G13). Three separate analyses are then performed to separate bile acids of interest (cholic, chenodeoxycholic, ursodeoxycholic, deoxycholic, and lithocholic acids) or their glycine or taurine conjugates. Alternatively, HPLC methods have now been described which can resolve these 15 bile acids in one run over a period of up to 2 hours (H8, R2, T1).

Once separated, the bile acids can be quantified by a number of detection systems. Unfortunately, a major disadvantage of HPLC at present is that direct detection methods, such as changes in refractive index or absorption of ultraviolet light in the 200 nm region, are insensitive for bile acids, which lack unsaturation or aromaticity in their molecular structures. Therefore, bile acids have been derivatized before chromatography by reaction via the carboxyl group with ultraviolet-absorbing labeling reagents, such as *p*-bromophenacyl bromide (M27) or *o,p*-nitrobenzyl-N,N′-diisopropylisourea (S21). To further increase sensitivity for the detection of serum bile acids,

TABLE 4

FEATURES OF RECENT HPLC METHODS FOR THE SEPARATION AND QUANTIFICATION OF BILE ACIDS AND THEIR CONJUGATES

Column packing	Mobile phase	Bile acids separated[a]	Run time (minutes)	Detection system	Application	Detection limit	References
Ultrasphere IP (C-18)	Acetonitrile/water / tetrabutyl ammonium phosphate	Glycine and taurine conjugates of C, CDC, DC	15	Absorbance at 214 nm	Bile	26–40 pmol	W9
Cosmosil 5 (C-18)	Potassium phosphate / methanol, pH 6.0	Anthroyl derivates of C, UDC, CDC, DC, or their glycine or taurine conjugates	20	Fluorescence at 470 nm	Serum	20 fmol	G13
Radial Pak A	Acetonitrile / methanol/water	Acetylpyrene derivatives of C, UDC, CDC, DC, LC, and their glycine conjugates	65	Fluorescence at 440 nm	Bile or serum	5–10 pmol	K4
Bile Pak	Acetonitrile / postassium phosphate / ammonium carbonate / NAD (gradient)	C, UDC, CDC, DC, LC, and their glycine and taurine conjugates	120	Immobilized 3α-HSD[b]; fluorescence at 470 nm to detect NADH	Bile or serum	0.2–2 pmol	T1
W-260	Acetonitrile / triammonium phosphate	C, UDC, CDC, DC, LC, and their glycine and taurine conjugates	130	Immobilized 3α-HSD; fluorescence detection of NADH	Serum	1–4 pmol	H8

[a] The abbreviations used are C, cholic acid; UDC, ursodeoxycholic acid; CDC, chenodeoxycholic acid; DC, deoxycholic acid; and LC, lithocholic acid.
[b] HSD, hydroxysteroid dehydrogenase.

fluorescent derivatives have also been prepared, using bromoacetylpyrene (K4) or anthroyl nitrile (G13). Reaction with the latter reagent occurs through the C-3 hydroxyl group rather than the carboxyl group of the side chain and does not require hydrolysis of glycine and taurine conjugates (G13). This approach looks promising since quantitative formation of anthroyl derivatives occurred after heating at 60°C for 20 min and the fluorescence detection limit was 20 fmol, which permitted the analysis of free, glyco-, and tauro-conjugated bile acids in normal serum (G13). Another way of detecting bile acids which have been eluted from HPLC columns has involved the post-column reaction with immobilized 3α-hydroxysteroid dehydrogenase to produce NADH for fluorimetric (O6) or electrochemical (K5) analysis. This method has enabled the measurement of bile acids in umbilical venous blood (O6).

6.3.5. *Thin-Layer Chromatography (TLC)*

Thin-layer chromatography is a relatively simple technique which has been used for many years to separate mixtures of bile acids. Advantages of TLC are that it is reasonably rapid, inexpensive, and offers a means of preliminary purification of bile acids. Methods for bile acid analysis by TLC have been reviewed previously (E2). In general, most methods use silica gel G as chromatographic support and different solvent systems are required to separate individual free bile acids (or their methyl esters) and individual glycine and taurine conjugated bile acids. A solvent system is available which conveniently separates bile acids into free, glycine-, and taurine-conjugate groups on the one TLC plate (G10). Alternatively, two-dimenstional TLC with different solvent systems in each dimension can resolve mono-, di-, and trihydroxy bile acids and their conjugates on the one thin-layer chromatogram (I1). While mono-, di-, and trihydroxy bile acids can be resolved, it is not easy to separate either the glycine or the taurine conjugates of the dihydroxy bile acids, chenodeoxycholic acid and deoxycholic acid. Success has been claimed with particular solvent mixtures (B11, G11) or reverse-phase TLC (T12). The use of high-performance TLC improves resolution and allows more rapid separation of bile acids, either in normal-phase (R3, S28) or in reverse-phase mode (L7). The TLC characteristics of bile acid sulfates have been determined in a number of solvent systems (P7).

Detection and quantitation of bile acids can be carried out directly on a thin-layer chromatogram with a suitable spray reagent followed by densitometry. By using an acidified solution of phosphomolybdate and ceric ammonium sulfate and heating chromatograms at 110°C for 2 to 5 min to develop blue spots, as little as 50 pmol of bile acid from duodenal bile was detected (R3). Other reagents used to produce colored or fluorescent bile acid spots include sulfuric acid (L7, T12, V4), acidified anisaldehyde (O5),

and iodine vapor (P4). Sometimes, areas of interest on the chromatogram are scraped into tubes and bile acids extracted with solvent prior to quantification. Using a coupled enzymatic method and spectrofluorimetry, Beher *et al.* (B15) reported a sensitivity level of 250 pmol per bile acid spot by this approach. An interesting new TLC technique, which has been applied to bile acid separation, involves the use of Chromarods, which consist of an inner quartz rod with an outer sintered silica gel layer (B18). Quantitation is carried out by scanning the rods in a flame ionisation detector and takes only 1 minute (B18).

7. Measurement of Bile Acids in Clinical Tests

Although research has shown that bile acids play an important role in liver and intestinal function, and that the metabolism or enterohepatic circulation of bile acids is altered in certain diseases, tests involving bile acids are uncommon in the clinical chemistry laboratory. This is probably because bile acid tests are not absolutely necessary for the diagnosis of any important or well-known disease and they are generally regarded as too specialized for routine use (H12). Thus, at present bile acid tests tend to be confined to gastroenterology units with research interests in bile acid metabolism. However, there is currently much interest in evaluating serum bile acid levels as a liver function test (H19) and bile acid tests have been developed to assess various aspects of intestinal function, as described below.

7.1. Serum Bile Acids and Liver Function

In normal, healthy subjects, the fasting level of serum bile acids is low and is less than 5 μmol/liter. This level is greatly increased in various hepatobiliary diseases (A9, B6, F2, F3, P9, S34, T11). For example, some liver diseases and their reported range of fasting serum bile acid concentrations (in brackets) are liver cirrhosis (5–100 μmol/liter), viral hepatitis (78–405 μmol/liter), and extrahepatic biliary obstruction (5–230 μmol/liter) (P9). An elevated serum bile acid concentration is highly specific for liver disease, but there is no specificity as to the type of liver disease. Determination of the profile of individual bile acids and calculations such as the cholic to chenodeoxycholic acid ratio have been proposed as useful in the differential diagnosis of liver disease (P9). In practice, however, there is too much overlap between diseases, so that the pattern of serum bile acids does not normally provide useful diagnostic information.

Serum bile acid concentrations as a test of liver function can be measured either in the fasting state or after a meal when there is a 2- to 6-fold increase

in total concentration (H18) (see Fig. 6). Early studies claimed that the use of postprandial, rather than fasting, serum bile acid levels improved test sensitivity (B6, F1, K7). Present indications are that there is no major advantage in using postprandial serum samples (M7), especially now that radioimmunoassays and equally sensitive enzymatic assay methods are available to measure bile acids in fasting serum.

To assess the value of serum bile acid measurements relative to the commonly used tests of liver function, prospective studies are now being carried out to compare the sensitivity, specificity, and predictive values of the various tests. However, the patient population under study needs to be carefully defined, as disease prevalence has a marked effect on the predictive value of any test. Serum bile acid concentrations could be used for screening for liver disease, for the diagnosis of suspected liver disease, or for estimating the severity of disease and prognosis of patients with known liver disease. In a study of nearly 600 subjects (97 healthy subjects, 138 patients with nonliver disease, and 344 patients with hepatobiliary disease), serum bile acids and aspartate transaminase showed similar sensitivity (78 versus 74%), specificity (93 versus 92%), and predictive value of a positive test (94 versus 93%) to detect liver disease (F2). When these data were stratified with respect to the severity of the disease, bile acids were not as good as aspartate transaminase and γ-glutamyl transferase in the detection of mild liver parenchymal cell disease, but were superior to all conventional liver tests in patients with severe, chronic liver disease, such as cirrhosis. Fasting serum bile acid levels have been found to be very sensitive in detecting all forms of cirrhosis (F2, F3, M7, S34) and are also equal to or better than any of the conventional tests to detect chronic hepatitis (F2, S34), possibly because they measure a combination of liver injury, excretory function and portal–systemic shunting of blood (H19). For the detection of acute viral hepatitis, the sensitivity of serum bile acids is about equal to aminotransferases (H19, T11), while for alcoholic liver disease and other mild liver disease, γ-glutamyl transferase and aminotransferases are more sensitive (G2, H19, M28). These findings are summarized in Table 5, which compares the sensitivities of various liver function tests, according to the type and severity of hepatobiliary disease.

It is still too early to tell whether serum bile acid determinations will be added to the currently available combination of liver function tests or replace individual tests, such as the measurement of bilirubin (H19). In correctly diagnosing patients with histologically defined liver disease, serum bile acids appear to slightly improve the results from conventional liver tests, if used in combination with these tests (F3). Perhaps when sensitive analytical methods such as bioluminescence, which can be applied to serum bile acid analysis, become available and established in diagnostic laboratories, serious consideration will be given to routine measurement of serum bile acid levels to

TABLE 5
COMPARISON OF THE SENSITIVITY OF FASTING SERUM BILE ACID LEVELS WITH THAT OF ROUTINE LIVER FUNCTION TESTS IN THE DIAGNOSIS OF HISTOLOGICALLY CLASSIFIED LIVER DISEASE[a]

Liver disease	Number of patients	Liver function test[b]						References
		SBA	ALT	AST	AP	GGT	BILI	
Severe parenchymal[c]	300	91	66	87	69	83	69	A9, B6, F2, S34
Severe cholestasis[d]	88	98	81	91	93	86	82	B6, F2, S34
Mild parenchymal[e]	68	50	65	75	32	71	19	F2, S34
Mild cholestasis[f]	61	56	26	30	56	54	30	B6, F2

[a] Sensitivities were calculated from the combined data of the studies cited and are expressed as the percentage of patients with values above the normal reference range.

[b] Abbreviations used are SBA, fasting serum bile acids; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AP, alkaline phosphatase; GGT, γ-glutamyl transferase; BILI, bilirubin.

[c] Includes alcoholic and cryptogenic cirrhosis, acute viral and active chronic hepatitis.

[d] Includes primary biliary cirrhosis, bile duct obstruction.

[e] Includes alcoholic fatty liver, chronic persistent hepatitis.

[f] Includes drug-induced cholestasis.

detect liver disease. Apart from detecting the more common liver diseases, there are certain circumstances where serum bile acid estimations appear to be of value. In intrahepatic cholestasis of pregnancy, serum bile acid levels, in particular cholic acid, are markedly elevated and can be used to monitor the disease (H14, H15, L2). Elevated levels sometimes precede the symptom of itching (H15, L2), although direct measurement of bile acids in skin interstitial fluid has suggested that contrary to popular belief, they do not cause pruritus (B9). Serum bile acid concentrations are normal in Gilbert's syndrome, a benign condition characterized by unconjugated hyperbilirubinemia (B34, D7). Bile acid determinations may therefore help in differentiating this syndrome from structural liver disease and avoid liver biopsy in patients with this syndrome.

Two other areas which are being explored and involve serum bile acid analysis deserve mention. While many studies have concentrated on their diagnostic value, serum bile acids may have potential for predicting the prognosis of chronic liver disease. Monroe *et al.* have found that the degree of serum bile acid elevation correlated with histological severity of liver damage in chronic hepatitis patients (M31) and serial bile acid determina-

tions may be useful in these patients to monitor the disease for early signs of relapse (J3, K11). There is also current interest in bile acid clearance tests as a dynamic measure of liver function. In this respect, it has been speculated that serum bile acids could become the "creatinine of the liver" (H19). The principle of the test is to administer an oral dose of a bile acid, such as ursodeoxycholic acid, which is not normally present in large amounts in the enterohepatic circulation, and measure its subsequent appearance and disappearance in blood (G4, T4). The magnitude of the rise and the time for serum bile acid levels to return to baseline will depend on hepatic function. Unfortunately, the requirements of multiple serum samples and a sensitive and specific assay for ursodeoxycholic acid, make this test unattractive for routine use and the clinical value of oral loading test has yet to be proven.

7.2. Tests of Intestinal Function

There are two tests involving bile acids which have aroused the interest of gastroenterologists, who wish to assess intestinal function in individual patients. The first is a bile acid breath test to detect abnormal exposure of the bile acid pool to bacteria, such as occurs in conditions of bacterial overgrowth of the small intestine or in bile acid malabsorption. The test is simple and is based on the principle that when bacteria deconjugate bile acids, the amino acid is released and is metabolized to form CO_2 (T5). A patient undergoing the test swallows a meal or drink containing glycocholate with the glycine labeled with ^{14}C. At various time intervals later, by breathing into a tube immersed in a measured amount of base and a pH indicator, $^{14}CO_2$ produced by bacterial deconjugation of [^{14}C]glycocholate and metabolism of [^{14}C]glycine is trapped for scintillation counting. The specific activity of expired $^{14}CO_2$ is then determined. Patients with excessive bacterial growth in the small intestine produce abnormally large amounts of $^{14}CO_2$ (T5).

The clinical value of the bile acid breath test remains limited, due to reports of both false positive and false negative results (H12). It has been suggested that simultaneous measurement of fecal ^{14}C-labeled bile acid excretion allows a more definite diagnosis in patients with bile acid malabsorption (T5). In this test, however, the bile acid should be labeled on the steroid nucleus to avoid loss of label due to bacterial degradation. After oral administration, a portion of a 24-hour fecal collection is oxidized to release $^{14}CO_2$ for radioactive counting. Bile acid malabsorption, as occurs in disease of the ileum and idiopathic diarrhea, can then be assessed. This method of counting fecal radioactivity is inconvenient and unpopular, and efforts have been made to produce a γ-emitting bile acid analog (B32). One such compound, 23-[^{75}Se]selena-25-homocholic acid, has been used in preliminary studies

with abdominal gamma counting of patients with gastrointestinal disorders (T6). The role of bile acid and other chemical tests in the diagnosis of intestinal malabsorption has recently been reviewed (T7).

8. Gallstone Dissolution with Bile Acids

Until recently, the only available active treatment for gallbladder stone disease was cholecystectomy; that is, surgical removal of the gallbladder and stones. This situation has changed over the last decade with the demonstration that two bile acids can be effective as cholesterol gallstone dissolution agents, when administered orally in doses ranging from 500 to 1000 mg per day for periods up to 2 years. These two bile acids, chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholan-24-oic acid) and ursodeoxycholic acid (3α,7β-dihydroxy-5β-cholan-24-oic acid), are now used as pharmacological agents for the treatment of gallstone disease, and surgery can be avoided (D8).

The original rationale for administering bile acids to dissolve gallstones was to increase the bile acid pool size within the enterohepatic circulation, since the pool is reduced in gallstone patients (V8). An expected consequence was that bile acid secretion from the liver would increase, leading to reduced cholesterol saturation of gallbladder bile. Surprisingly, early studies found that there was a specificity in the action of bile acids, in that chenodeoxycholic acid and ursodeoxycholic acid were effective for gallstone dissolution, but cholic acid was not (D8, T8). It is now known that both chenodeoxycholic acid and ursodeoxycholic acid convert supersaturated bile to the unsaturated state by decreasing the hepatic secretion of cholesterol into bile. Exactly how this is achieved is not certain (A8), although both these bile acids suppress the activity of hydroxymethylglutaryl-CoA reductase, the rate-limiting enzyme of hepatic cholesterol synthesis (M16).

Recent investigations into the mechanism of action of these bile acids indicate that ursodeoxycholic acid has certain advantages over chenodeoxycholic acid in the context of the overall homeostasis of cholesterol metabolism (F6). In contrast to chenodeoxycholic acid, ursodeoxycholic acid does not suppress bile acid synthesis (H7), possibly because the α-orientation of the 7-hydroxyl group of chenodeoxycholic acid is required to inhibit cholesterol 7α-hydroxylase activity. Thus, cholesterol breakdown into bile acids is not reduced by ursodeoxycholic acid. Other favorable factors are that ursodeoxycholic acid has a reduced capacity to solubilize cholesterol into micellar solution compared to chenodeoxycholic acid and intestinal cholesterol absorption is decreased by this bile acid (F6, H7). However, in gallbladder bile the relative limitation of ursodeoxycholic acid for micellar solubilization of cholesterol is compensated for by an ability to transport

cholesterol in a liquid–crystalline form (P5), so that gallstone dissolution rates are similar for patients treated with chenodexocyholic acid or ursodeoxycholic acid.

Careful patient selection for gallstone dissolution therapy is important to exclude patients with a minimal chance of successful treatment. Primary selection criteria are the presence of small stones, less than 15 mm in diameter, in a functioning gallbladder. The stones must be radiolucent on X ray, which implies that they are composed predominantly of cholesterol. The efficacy of bile acid treatment to dissolve the gallstones of patients selected in this way has been evaluated in large clinical trials in Australia, Japan, the United Kingdom, and the United States. To date, by far the most extensive and well-controlled study is the American National Cooperative Gallstone Study (NCGS), which was carried out over 8 years at a substantial cost of over $11 million. Nine hundred and sixteen patients with radiolucent gallbladder stones were treated with chenodeoxycholic acid or placebo for 2 years at 10 different medical centers. Patients were randomly allocated to receive 750 or 375 mg/day of chenodeoxycholic acid, or a placebo with a similar taste (S14). The incidence of complete gallstone dissolution, which was assessed radiographically, was 14, 5, and 1% in each group, respectively. In retrospect, it was appreciated that higher doses of bile acid are required for optimum gallstone dissolution. In a study of 125 gallstone patients, who were treated with chenodeoxycholic acid at a dose of 13 to 15 mg/kg/day (equivalent to 1000 mg/day in a patient of normal weight), Maton *et al.* in the United Kingdom reported an incidence of complete gallstone dissolution of 33% (M17). Other clinical trials with both chenodeoxycholic acid (T2) and ursodeoxycholic acid (B1) have achieved similar results.

There are several factors which contribute to this relatively low success rate of bile acid therapy to dissolve gallstones. First, around 15% of patients who were recruited for clinical trials have withdrawn before completing at least 6 months treatment. As dissolution is a slow process, 6 months is considered the minimum treatment time before a successful outcome is likely to be achieved. On the other hand, if at least partial dissolution is not observed after 12 months of treatment, then dissolution is unlikely to occur. Second, some treatment failures are undoubtedly due to the fact that not all gallstones are high in cholesterol content. In Western countries, approximately 10% of gallbladder stones contain virtually no cholesterol (W2), and are termed pigment stones. A gallbladder stone which is high in cholesterol content and contains low amounts of calcium salts will appear radiolucent by radiographic examination (oral cholecystography). However, between 14 and 20% of radiolucent stones are low in cholesterol content (B19, W2) and will not respond to dissolution therapy with bile acids. Unfortunately, there is no completely reliable method for distinguishing patients with cholesterol stones from those

with noncholesterol or pigment stones. Some small radiolucent stones float in the gallbladder and these are invariably composed of cholesterol and are very amenable to dissolution therapy (S14). Aside from pigment stones, some radiolucent stones of high cholesterol content may not dissolve because they contain calcium salts. These salts, mainly calcium carbonate, can form a thin, dissolution-resistant layer, either on the surface of the stone (W5) or in the interior, resulting in partial dissolution until the layer is reached. Thus, partial stone dissolution may not necessarily lead to complete dissolution and cannot be regarded as a successful outcome of treatment. Gallstone calcification during treatment with cessation of the dissolution process has been reported in several patients taking ursodeoxycholic acid (B10). Last, other factors which have been found to decrease the likelihood of successful gallstone dissolution are a large volume of gallstone, obesity, and inexplicably a serum cholesterol level of less than 5.9 mmol/liter (S14). If patients are carefully selected for factors favorable to the outcome of dissolution treatment, a success rate of 60 to 70% can be achieved (M17).

The biochemical monitoring of gallstone patients treated with bile acids involves liver function tests prior to treatment and at 6-monthly intervals. Specialized treatment centers consider it valuable to obtain pre- and post-treatment samples of fasting gallbladder bile to ensure that bile has become unsaturated with cholesterol during treatment. The bile is collected by duodenal intubation, after stimulation of gallbladder contraction by intravenous administration of cholecystokinin, and is analyzed for cholesterol, bile acids, and phospholipid before calculating the degree of cholesterol saturation as a saturation index (C7). Patient compliance in taking bile acid capsules or tablets can also be assessed by determining the bile acid profile of bile and checking that the administered bile acid has become the major species in the bile acid pool. To avoid bile collection for monitoring patient compliance, a method has been developed whereby the bile acid profile in fasting serum is used to predict the bile acid composition of bile (W6).

When chenodeoxycholic acid therapy was first introduced, there was some anxiety that this bile acid, or its bacterial metabolite lithcholic acid, might cause liver damage in man. This possible complication has not eventuated. Lithocholic acid is toxic to the liver in many animal species but in man, it is converted to sulfolithocholate and excreted (A5). Nevertheless, up to one-third of patients undergoing chenodeoxycholic acid treatment do show transient rises in serum levels of aspartate aminotransferase activity. The mechanism of this hypertransaminasemia is obscure, although it could possibly be related to lithocholate formation (D8). In any case, hepatotoxicity very rarely occurs at a clinically significant level (S14).

Another side effect of chenodeoxycholic acid treatment is diarrhea. This is dose dependent and is due to spillover of excessive amounts of bile acids into

the colon, as the capacity of the small intestine to absorb bile acids is exceeded. Ursodeoxycholic acid does not generate the side effects caused by chenodeoxycholic acid. For this and other reasons, ursodeoxycholic acid is likely to become the bile acid of choice for dissolving gallstones, although it is more expensive to manufacture and is not yet available for prescription in some countries. It was predicted that prolonged bile acid administration might lead to hypercholesterolemia by suppressing the catabolism and biliary excretion of cholesterol. Some studies have reported a slight increase in serum cholesterol levels, mainly in the low-density lipoprotein fraction, during treatment with chenodeoxycholic acid (A4, S14) but this increase might not be clinically important. Ursodeoxycholic acid does not seem to affect serum cholesterol levels. On the other hand, chenodeoxycholic acid, and possibly ursodeoxycholic acid, lower serum triglyceride levels and can be used in the treatment of hypertriglyceridemia (C1).

Although the cholesterol gallstones of selected patients can be dissolved by bile acid treatment, the place of gallstone dissolution therapy in the management of patients with gallstone disease is limited. It has been estimated that less than 10% of the gallstone patients presenting to a teaching hospital in Australia could be successfully treated by this form of therapy (W2). Cholecystectomy is a safe and effective treatment for most gallstone patients. A major drawback with dissolution therapy is that gallstone recurrence is frequent 1 to 2 years after successful treatment and the recurrence rate may be as high as 50% (R14). Whether lower maintenance doses of bile acid or dietary measures can prevent gallstone recurrence is under investigation. Nevertheless, although surgery will remain the main form of treatment for gallstone disease, dissolution therapy is a particularly valuable, alternative treatment for those patients who represent a poor surgical risk because of other diseases.

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CLINICAL CHEMISTRY OF FOLIC ACID

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1. Introduction

Folic acid was first recognized in 1930 by Lucy Wills as a factor present in the autolyzed yeast preparation "marmite," which was able to cure a macrocytic anemic occurring among Hindu women in Bombay, particularly during pregnancy (W1, W2, W3). This early work by Wills stimulated interest in what is now recognized as nutritional megaloblastic anemia. The active agent in the "marmite" was subsequently identified as folic acid. This was the name first given to a hematopoietically active material isolated from spinach and other "foliage" by Mitchell *et al.* in 1941 (M1). The material was later identified as pteroylglutamic acid. In 1945, Angier and his colleagues (A1) successfully synthesized folic acid in the form of pteroylmonoglutamate with a therapeutic activity equal to the natural material.

The hematological abnormalities resulting from folate deficiency are indistinguishable from those associated with vitamin B_{12} deficiency. However, while vitamin B_{12} deficiency may also result in neurological changes, it was thought that this was not a characteristic of folate deprivation. This view is almost certainly incorrect because during the past 15 years there have been increasing numbers of reports of neurological and neuropsychiatric disease associated with a deficiency of this vitamin. Folate deficiency is one of the most common vitamin deficiency states seen in clinical practice and is particularly prevalent among people in the lower socioeconomic groups. Like vitamin B_{12} deficiency, a suboptimal intake of folate is associated with a generalized disorder of cellular metabolism.

Rapidly dividing cells have a high requirement for folate and antagonists have been developed which act as spurious substrates for the associated enzyme dihydrofolate reductase. Methotrexate is one such agent, and it has proved to be a very effective drug in the treatment of certain malignancies and in the suppression of lymphocyte activity following autologous bone marrow transplantation. Other folate antagonists specifically affect bacterial

or protozoal folate reductase, and these agents are used in the treatment of a wide range of infections.

1.1. Nomenclature

In the past the vitamin has been known as vitamin B_c, folic acid, vitamin M, and pteroylglutamic acid. The term folic acid was originally used by Mitchell (M1) because he found the material in leafy vegetables (foliage). The International Union of Pure and Applied Chemistry (I1) has ruled that pteroylglutamic acid should be used to describe the pure substance and that folic acid may be used as the generic term. In fact of course, the term "folic acid" has now been used for so long and is so widely understood that it would be difficult to change it.

2. Chemistry and Biochemistry

2.1. Chemistry

Work on the isolation and identification of folic acid started following the description by Snell and Peterson (S1) of an unidentified growth factor for *Lactobacillus casei*. Shortly after this, Stokstad at the Lederle Laboratories found that various by-product fractions of liver extraction were potent sources of the so-called *L. casei* factor (S2). Work was then started on the production of this *L. casei* factor by a fermentation process. The "active material" was subsequently identified as pteroyltriglutamate. This material proved to be only 70% as active as liver folic acid for *L. casei* and only 5% as active for *S. faecalis* as liver folic acid. Removal of two molecules of glutamic acid from the fermentation product boosted its activity to that of the liver folic acid. It is clear now that *S. faecalis* does not respond to the polyglutamate forms of folate. At this time liver folic acid was identified as consisting of a pteridine plus *p*-aminobenzoylglutamic acid and there was evidence suggesting a methylene bridge between the two components. An understanding of the structure and synthesis of the vitamin was finally achieved by chemists at the Lederle Laboratories and at the Calco Chemical Division of American Cyanimide in 1945 (A1). We now know that folic acid or pteroylglutamic acid is a naturally occurring pteridine derivative comprising three basic parts, a pteridine, *p*-aminobenzoic acid, and glutamic acid (Fig. 1). Pteroic acid is the parent compound and this is able to support the growth of *S. faecalis*. When pteroic acid is conjugated with one molecule of L-glutamic acid, the resultant compound is pteroylglutamic acid. While the synthetic

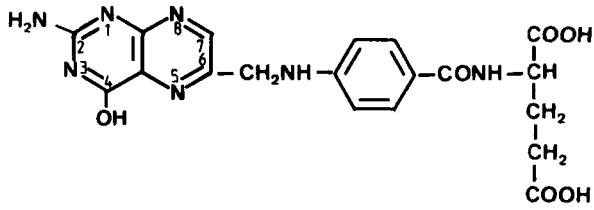

FIG. 1. Pteroylglutamic acid.

form of the vitamin is available as a monoglutamate, this form does not occur in nature, where folates are always found as polyglutamates with up to 11 glutamic acid residues (Fig. 2). The link between the second and subsequent glutamic acid molecules is through the γ-carboxyl group to the amino group. The parent compound pteroylglutamic acid when crystallized from an aqueous solution produces yellow spear-shaped crystals. It has a molecular weight of 441.4 (C1). On heating to 140°C two molecules of water are lost. The free acid is poorly soluble in water and organic solvents but as the disodium salt it is freely soluble.

2.2. BIOCHEMISTRY

The active coenzyme forms of folic acid are polyglutamates having between four and six glutamic acid residues, with the majority being in the form of a pentaglutamate (H1). The glutamic acid chain is required to bind the coenzyme to the apoprotein (W4). These polyglutamate forms are present within the cells while the monoglutamate 5-methyltetrahydrofolate is found in the blood plasma and other extracellular fluids and probably represents the transport form of the vitamin. Certain bacteria used in the study of folic acid such as *L. casei* are only able to respond to short-chain polyglutamates while *S. faecalis* responds only to the monoglutamate, and the vitamin

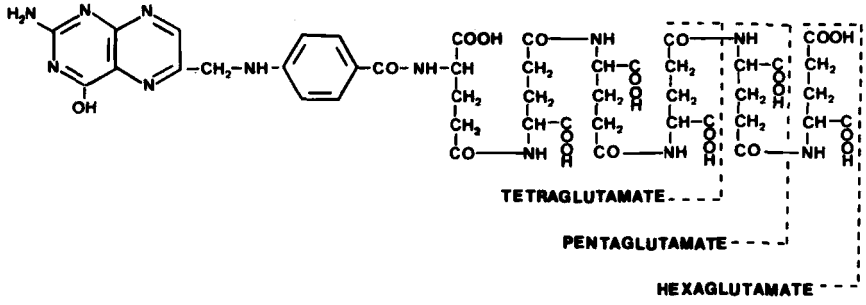

FIG. 2. Folate polyglutamates.

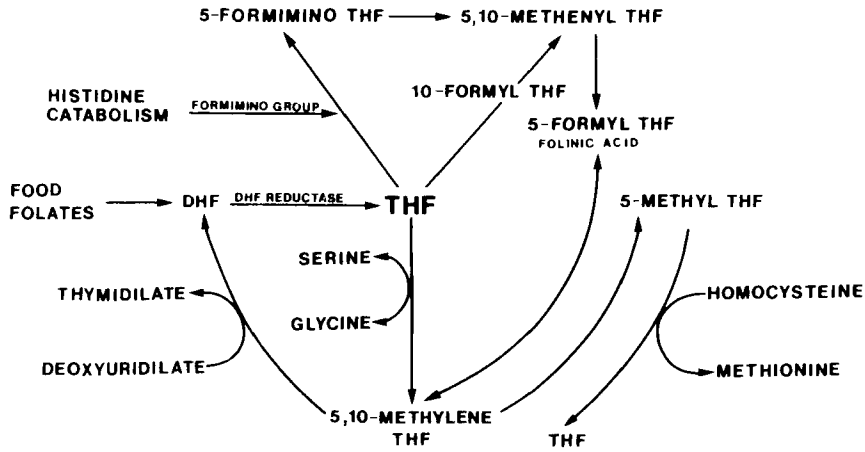

FIG. 3. Abbreviated diagram of folate metabolism. DHF, dihydrofolate; THF, tetrahydrofolate.

must therefore first be treated with a conjugase to reduce the number of glutamic acid molecules to one.

Folic acid can undergo a reaction at almost any ring position. The addition of hydrogen atoms in the 7,8- and 5,6- positions give rise to the dihydro- and tetrahydroforms required in man and animals (Fig. 3). The enzyme dihydrofolate reductase (EC 1.5.1.3.) catalyzes the NADPH-dependent reduction of 7,8-dihydrofolate to 5,6,7,8-tetrahydrofolate, which is the coenzyme form and has a central role as a carrier of one-carbon units in intermediary metabolism (Fig. 3). The one-carbon unit can exist as a methenyl, methylene, or methyl group depending on the state of oxidation. In these forms the group may be transferred to an acceptor. For example, the methyl group of 5-methyltetrahydrofolate can be passed to homocysteine for the formation of methionine. This reaction is vitamin B_{12} dependent. Another example is the participation of 5,10-methylenetetrahydrofolate in the conversion of deoxyuridilate to deoxythymidilate (Fig. 4). Tetrahydrofolate is also a cofactor in phenylalanine hydroxylation (W5).

Folic acid also has an important role in histidine catabolism (Fig. 4), where the formimino group of the end-stage product formiminoglutamic acid is transferred to tetrahydrofolate, giving formiminotetrahydrofolate.

2.3. FOLATE ANTAGONISTS

The development of antagonists to folic acid or, perhaps more correctly, the enzymes which play a vital role in the folate pathway is without doubt one of the success stories of medical science. In man, animals, protozoa, and

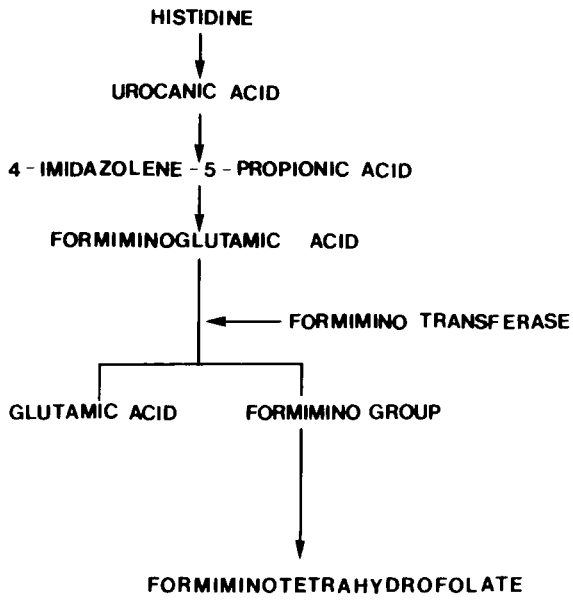

FIG. 4. Catabolism of histidine.

some bacteria tetrahydrofolic acid is the active coenzyme form of the vitamin. The enzyme dihydrofolate reductase catalyzes the reduction of dihydrofolate to tetrahydrofolate. It acts by binding to the pyrimidine portion of the pteridine ring, which is 2-amino-4-hydroxy compound. The substitution of this by a 2,4-amino compound which tightly binds the enzyme is the basis for the success of the folate antagonists. The reductases in man, protozoa, and bacteria all have small differences (B1). A number of spurious substrates have been developed for these reductases, and they vary in their affinity for a particular species form of the enzyme. For example, trimethoprim has a high affinity for bacterial dihydrofolate reductase but is much less active against the human reductase. Methotrexate, on the other hand, is active against all types of dihydrofolate reductase.

2.3.1. *Trimethoprim*

Many bacteria, including many common pathogens, synthesize their own folate and to do this they require *p*-amniobenzoic acid together with other basic building blocks. Once formed the folate is reduced by dihydrofolate reductase. In 1968 a new antibacterial compound was introduced which combined a sulfonamide (sulfamethoxazole) with trimethoprim (2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyrimidine). This drug combination had a powerful synergic bactericidal action. The sulfonamide blocked the utilization of *p*-aminobenzoic acid and therefore blocked the synthesis of folate, while

the trimethoprim acted as a spurious substrate for the bacterial dihydrofolate reductase, preventing the reduction of any folate that might be formed. The combination of these two agents was found to be highly effective and the development of resistant organisms was and remains unusual. The use of a drug combination able to block a metabolic pathway at two points is probably unique. At the time of its introduction, the concentration of trimethoprim used was thought to be harmless to man and in fact Burchall and Hitchings (B1) claimed that bacterial dihydrofolate reductase was more sensitive to trimethoprim by a factor of 50,000 than the mammalian enzyme. Davis and Jackson (D1) examined the tetrahydrofolate levels in 24 patients receiving trimethoprim/sulfamethoxazole for a total of 104 treatment weeks. They found no change in the serum concentration of tetrahydrofolates but pointed out that the drug may have an adverse effect on folate metabolism in certain "high-risk" patients. From time to time reports of patients developing a folate responsive megaloblastic anemic have appeared (C2, C3), and it now seems that the margin of safety in some patients is less than was previously supposed. This was supported by the work of Sive *et al.* (S4), who showed that the deoxyuridine suppression test, a very sensitive method for measuring folate activity, gave slightly abnormal results in all patients tested, even though they were only taking a standard therapeutic dose of the drug combination. However, considering the millions of treatment courses of this drug combination that have been prescribed over the last 15 years, the number of patients suffering complications is very small indeed and many of these have been associated with a sensitivity to the sulfamethoxazole component. Some patients have developed a megaloblastic anemia during treatment with this drug combination, but the folate status of some may already have been compromised before treatment was commenced (R1).

2.3.2. *Pyrimethamine*

Pyrimethamine [2,4-diamino-5-(*p*-chlorophenyl)-6-ethylpyrimidine] is most active against protozoal dihydrofolate reductase. However, there is only a small margin between the dose required to kill the organisms and that which will affect the host. It is most commonly used in the treatment of toxoplasmosis but more recently has been used at a lower and safer dose of 25 mg weekly for malarial prophylaxis. This dose is unlikely to produce any adverse hematological complications in the short term. Larger doses are required to treat toxoplasmosis and where it exceeds 25 mg daily a megaloblastic anemia will develop in many of the patients so treated (N1).

2.3.3. *Methotrexate*

Methotrexate (4-amino-10-methylpteroylglutamic acid) was one of the first folate antagonists to be used clinically and it has now been in use for more than 30 years. It is useful for the treatment of a number of malignancies and

has been shown to have a curative role in choriocarcinoma (L1). The drug binds tightly to dihydrofolate reductase and may persist in this bound state for long periods of time. Williams (W6) found that the potent inhibition of dihydrofolate reductase by methotrexate occurred as a consequence of a two-stage reaction involving the rapid formation of an enzyme–NADPH–methotrexate complex followed by a slow reversible isomerization reaction. The treatment goal is to obtain a high intracellular concentration of the drug which will effectively block the intracellular dihydrofolate reductase. To achieve this it is necessary to have a very high extracellular concentration of the drug and this would prove very toxic to the patient. To overcome this a procedure known as folinic acid rescue has been used in recent years. Following a large dose of methotrexate several hours are allowed to elapse and then a large dose of folinic acid (5-formyltetrahydrofolate) is given. This is a reduced form of folate which bypasses the reductase-mediated step and by dilution removes some of the intracellular methotrexate. Cells may become resistant to the effect of methotrexate by either an excessive production of dihydrofolate reductase or by membrane changes which impair transport of the drug. The use of high-dose methotrexate may cause mucosal sloughing and compromise renal function.

2.3.4. *Anticonvulsant Drugs*

The association between anticonvulsant drug therapy and a reduced concentration of serum folate and, less frequently, erythrocyte folate is well recognized. Mannheimer (M2) first reported an association between long-term anticonvulsant therapy and the development of a megaloblastic anemia due to folate deficiency. In this case phenytoin was the drug used. Many other anticonvulsant drugs have since been found to interfere with folate metabolism including phenobarbital (F1), phensuximide (D2), and primidone (G1). However, although the development of a megaloblastic anemia is a known problem associated with the use of anticonvulsant drugs, such an occurrence is not common. Davis and Woodliff (D3) measured the serum folate levels in 149 patients in a single institution who were being treated with anticonvulsant drugs, 38 received phenobarbital alone, 15 diphenylhydantoin (Dilantin) alone, 3 primidone (Mysoline) alone, and the remainder various combinations of two or three anticonvulsant agents. Eighty-seven patients were found to have a serum folate concentration below the lower limit of the reference range (2.5 μg/liter). A reduced folate level was more frequent in females (72%) than in males (49%); this may possibly have been due to the additional burden of menstrual blood loss because in women over 50 years of age only 60% had a reduced level of folate. No association was found between a particular anticonvulsant drug or combination of drugs and a reduced serum level of folate (Fig. 5). It appears that most of these drugs

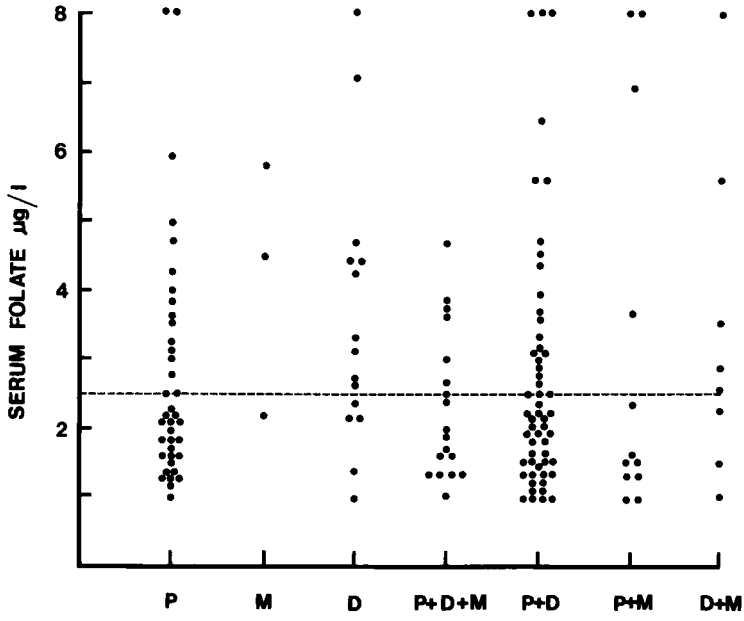

FIG. 5. Folate levels in patients receiving anticonvulsant drugs. P, phenobarbitone; M, Mysoline; D, Dilantin. [From Davis and Woodliff (D3), © 1969, The Medical Journal of Australia, reprinted with permission.]

are able to interfere with normal folate metabolism although their mode of action may be different.

Davis *et al.* found that folate was not the only B group vitamin which was reduced in patients receiving treatment with anticonvulsant drugs (D4). In a study of 68 patients suffering from severe epileptic seizures they found that 18 patients had a low folate, 10 a low serum pyridoxal, and in 15 both the folate and pyridoxal were reduced. Only two patients in this series had a reduced erythrocyte folate, and this is in accord with the infrequency with which a macrocytic anemia is seen in these patients. All the patients in this series had a normal hemoglobin concentration and a normal mean corpuscular volume. However, in a study of 75 epileptic children Maxwell (M3) found both the serum and erythrocyte folate levels to be reduced in 60% and similar observations have been made by other workers (M4, N2).

It has been suggested that anticonvulsants inhibit the absorption of folate by the inhibition of intestinal conjugases (S5) but results from various studies have been conflicting. Some workers have reported impaired absorption of folate when given with diphenylhydantoin (B2, E1) while others have failed to demonstrate this effect (R2, S6). There is some evidence that anticonvul-

sant drugs stimulate hepatic enzyme induction of pathways that require folate (D5) and this in turn could lead to a deficiency of folate. The deoxyuridine suppression test has also been found to give conflicting results in patients receiving these drugs (W7, T1). Diphenylhydantoin has been shown to impair lymphocyte division (B2) and may also be associated with a lymphadenopathy indistinguishable histologically from Hodgkin's disease or non-Hodgkin's lymphoma (K1). In most cases the lymphadenopathy was found to regress when the drug was stopped but in some patients a true Hodgkin's disease or non-Hodgkin's lymphoma developed. The role played by the hydantoin derivatives in the development of these malignancies is not clear.

The treatment of folate deficiency in patients receiving anticonvulsant drugs is not without difficulties. Chanarin (C3) described an epileptic patient who had developed a megaloblastic anemia due to folate deficiency. Treatment with folic acid apparently precipitated convulsions. Up to this time the patient had been free of seizures while on phenobarbital for 4 years. There have since been a number of reports of treatment with folic acid precipitating seizures in patients with epilepsy (D6, R3, W8).

Folic acid has been found to have active convulsant properties in animals (B4, H2) and it has been postulated that the antifolate affect of anticonvulsants may be related to their therapeutic effect. There has been some clinical and experimental support for this suggestion (H3, H4), but it has been denied by others (R4).

Phenytoin has also been reported to cause peripheral neuropathy when used in high doses. Shorvon (S7) found that at a serum phenytoin concentration of 30 mg/liter there was a reduction in sural nerve conduction velocity in seven of nine patients and in median nerve sensory velocity in four of nine patients. It was not clear if the peripheral neuropathy was due to a toxic effect of the drug or, as seems more likely, to a folate deficiency associated with the anticonvulsant drug (H5).

2.4. Absorption and Transport

There remains a good deal of uncertainty regarding the mode of folate absorption. In nature the majority of folates are found as polyglutamates of varying chain length with various substitutions on the pteridine ring. The principal forms representing some 90% of dietary folates are present as 5-methylpteroylpolyglutamates while most of the remainder is in the 10-formyl form (S8, B5); these are deconjugated by an intestinal conjugase, and the resultant monoglutamates are taken up by the enterocytes. The exact site of the conjugase activity is not known but it is possible that it takes place

within the enterocyte. Reisenauer (R5) has reported the presence of two separate enzymes or conjugases in the human jejunum, one intracellular with a pH optimum of 4.5 and the other localized to the brush border with a pH optimum of 7.5. Some of the polyglutamates would also be deconjugated by the conjugases that occur naturally in such vegetables as broccoli, Brussels sprouts, cabbage, cauliflower, lettuce, and spinach (L2). There is experimental evidence that the vitamin is converted to 5-methyltetrahydrofolate within the enterocytes and released into the portal circulation (P1). This conversion is probably a rate-limited phenomenom because, if pteroylmonoglutamic acid is administered it is absorbed largely unchanged, although if small doses are used some of the vitamin will be methylated.

Earlier work by Swenseid (S9) showed that absorption, based on the urinary excretion of the vitamin, was similar for both the synthetic pteroylmonoglutamate and a pure form of a heptaglutamate. On the other hand, when a yeast form of pteroylpolyglutamate was used, the urinary excretion of the vitamin was much less. It was later confirmed that yeast contained a natural inhibitor to polyglutamate hydrolysis (P2, S10). The pH dependence of the activity of the conjugase was confirmed by the observation that orange juice reduced the availability of polyglutamate but not monoglutamate forms of the vitamin (T2). It was subsequently shown that orange juice, when adjusted to a pH 7, did not affect absorption of the vitamin (N3). Cooper (C4) observed that some oxidation of folate may occur during normal food preparation and, although there have been numerous studies on the folate content of prepared foods, there has been only limited work on determining the forms of folate present in food as eaten. It seems likely that results from such an undertaking would be subject to a wide variation and would therefore be of limited value.

It was suggested by Hillman (H6) that an enterohepatic cycle has an important role in folate homeostasis. This was based on the observation that alcohol appeared to reduce the level of folate supplied to serum and tissue by interfering with folate clearance into bile. Studies in rats of this aspect of the folate cycle confirm that the enterohepatic pathway has a major role in folate homeostasis. It has been shown that when small amounts of radio-labeled methylpteroylmonoglutamate and pteroylmonoglutamate are placed in the rat jejunum they are both rapidly absorbed and transported to the liver. They are then cleared into the bile predominantly in the methyl form. The original methyl form appears in the bile unchanged 10 min after administration and the pteroylmonoglutamate appears in the bile after conversion to the methyl form in a similar time period. This confirms that only a very short period of time is required for methylation. Bile folate is reabsorbed for

distribution to liver and other tissue. With bile drainage, the serum folate level falls by 30–40% of normal within 6 hours (S11).

2.5. Folate-Binding Proteins

It had earlier been thought that folate was not bound to a specific serum protein and that if it was bound at all it was loosely bound to albumin. This was supported by the fact that the vitamin could be dialyzed and megaloblastic anemia had been reported in uremic patients undergoing long-term hemodialysis (H7). It was later shown that albumin did bind folate and that it had two binding sites per molecule. Binding was found to be maximal at pH 6.0 (S12). A second serum binder for folate has been described (W9, R6). Although this binder has a high affinity for folate, its capacity is low; moreover it binds oxidized folate well but 5-methyltetrahydrofolate poorly. Folate attached to this protein is not removed by charcoal, indicating that the bond is firm. Vitamin bound to this protein is not given up to cells and possibly represents a storage form.

This second folic acid binding protein is present also in human milk, leucocyte lysates from patients with chronic myeloid leukemia, and cow's milk. Binding is pH dependent, and is not inhibited by alcohol or anticonvulsant drugs. Waxman (W10) has reported that in serum protein-bound [^{3}H]polyglutamates appear as two peaks on Sephadex G-200 gel filtration. One peak has a molecular weight greater than 200,000 while the second and smaller peak appears to have a molecular weight of 50,000. Studies by other workers have in the main only been able to confirm the presence of a binder having the characteristics of a glycoprotein, giving a single peak and having a molecular weight of 40,000 (F2, C5). The level of this high-affinity binder in normal serum has been reported to range from 45 to 76 ng/liter (W10, G2). Pregnant women and patients with folic acid deficiency, cirrhosis of the liver, and uremia appear to have increased serum levels of the high-affinity binder. Eichner (E2) examined the serum unsaturated folate-binding capacity in 489 hospital patients: 20% had a mild to moderate elevation while 6% had a marked elevation. These raised levels correlated with metastatic cancer, active granulomatous disease, and particularly with liver disease. They found that the unsaturated folate-binding capacity was poorly correlated with the folate status of the patient.

Cerebrospinal fluid contains a folate binder which appears identical to that present in serum except that it is unsaturated. Similar binders have been found in bile and urine but have not been well characterized (C6).

Human milk folate-binding protein appears to have two components, one with a molecular weight in excess of 200,000 and another with a molecular weight of 26,500. Like the binder in serum at a physiological pH, it has a

greater affinity for oxidized folate. However, at a pH of 9.3 it binds both oxidized and reduced folate equally (G3), and this property has been used to advantage in designing isotopic methods for assaying folate.

Cow's milk has a folate binder which is a low-molecular-weight protein (35,000) and is associated with β-lactoglobulin (W11). It appears to bind pteroylglutamic acid in preference to 5-methyltetrahydrofolate and shows three peaks on isoelectric focusing (H8).

The function of folate binders in folate metabolism remains unclear, except in the case of milk where they have recently been shown to enhance the absorption of folate in breast-fed infants and possibly protect the folate against utilization by intestinal bacteria (C7). The binder may also have a function in sequestering folate from the mother's circulation. It has not been shown to have a role in polyglutamate synthesis nor does it appear to play a part in enabling cells to take up folate; on the contrary, Waxman and Schreiber (W12) have shown that the binder prevents the uptake of folate by cells in tissue culture.

3. Methods of Measurement

Microbiological assay techniques were designed early in the history of folate. Their use was restricted to the assay of materials which had a relatively high concentration of the vitamin. They were not found suitable for clinical use because they failed to differentiate between deficient and nondeficient patients. This was partly due to the poor sensitivity of the methods but also because the labile nature of the folate present in blood was not appreciated at that time. They did provide some useful information when used to measure the urinary output of folate following an oral loading dose of the vitamin but this was a rather crude method of trying to assess folate status. Because of the lack of sensitivity associated with these early microbiological systems, tests were developed based on the catabolism of histidine and measured the concentration of formiminoglutamic acid excreted following an oral loading dose of histidine.

With the introduction in 1960 (B6) of an improved and sensitive microbiological assay for folate the measurement of formiminoglutamic acid was no longer used as a diagnostic test, although it continues to be used as a research tool. The microbiological assay has in turn now been replaced in many laboratories by isotope dilution techniques.

3.1. Measurement of Formiminoglutamic Acid

The pathway in the catabolism of L-histidine is to urocanic acid, 4-imidazolene-5-propionic acid, and formiminoglutamic acid. The formimino

group is then split from the glutamic acid with the release of ammonia (Fig. 4). In the presence of adequate folate the formimino group is transferred to tetrahydrofolate to give formiminotetrahydrofolate which is immediately converted to methenyltetrahydrofolate. When there is insufficient folate available to permit this transfer the formiminoglutamic acid is excreted in the urine and may be accompanied by a lesser amount of urocanic acid.

Although a number of earlier tests had been published for the measurement of formiminoglutamic acid, that described by Tabor and Wyngarden in 1958 (T3) was the first to receive any widespread use in clinical medicine. Urine to be assayed was incubated with a mixture of transferase, cyclodeaminase, and tetrahydrofolic acid. The mixture was then acidified, and the concentration of 5,10-methenyltetrahydrofolate calculated from the absorbance measured at 350 nm. The test was simple and reliable. Very little formiminoglutamic acid is present in the urine of patients with an adequate folate intake and who have normal enzymes in that part of the metabolic pathway. To enhance the excretion of formiminoglutamic acid it was usual to give patients a 15-g oral loading dose of L-histidine monohydrochloride before collecting urine over a set period of time. Care is required in giving this dose of histidine to patients with severe liver dysfunction as it may precipitate hepatic coma. With histidine loading healthy individuals excrete less than 17 mg of formiminoglutamic acid over the following 5 hours (D7).

Because of the apparent usefulness of this test a number of alternative methods for measuring formiminoglutamic acid were devised. These were based on high-voltage electrophoresis (K2), conventional electrophoresis (L3), and thin-layer chromatography (R7). If formiminoglutamic acid is heated, the glutamic acid is freed from the formimino group and can then be assayed microbiologically using *L. arabinosus* as the test organism (S13). Unfortunately, urine normally contains a varying amount of glutamic acid which interferes with the assay. Davis and Onesti (D8) devised a two-stage method in which the glutamic acid initially present in the urine was first removed.

3.2. The Microbiological Assay of Folic Acid

Three organisms have been found useful for measuring folate in blood and serum, they are *Lactobacillus casei* (American Type Culture Collection (ATCC) 7469), *Streptococcus faecalis* (ATCC 8043), and *Pediococcus cerevisiae* (ATCC 8081). Assays using *L. casei* as the test organism have been more extensively used because they give higher results and respond to the major form of folate present in blood, i.e., 5-methyltetrahydrofolate. *L. casei* responds to triglutamate and other short-chain forms of pteroylglutamic acid but shows only a limited response to the longer-chain folates (T4). *S. faecalis*

uses monoglutamates other than the methyl form and has been used mainly to determine the concentration of folate in biological material other than the methyl form. In the past it has frequently been used to assay the folate in urine following an oral loading dose of the vitamin. *P. cerevisiae* can only utilize tetrahydrofolates other than the methyl form and is seldom used except for experimental purposes.

The method described by Baker in 1959 (B6) for the microbiological measurement of folate in serum provided a means of measuring folate which he claimed could clearly identify patients who were folate deficient. However, a number of workers found that in their hands the test gave a degree of overlap which they claimed made it of little value (C8). It had been recognized earlier that ascorbic acid was necessary to "protect" the folate (T5) but it was Herbert (H9) who found that the concentration of ascorbate was critical if the method was to be used as a clinically reliable test for folate depletion. Studies by Davis and Kelly (D9) showed that the test gave reliable results if serum samples were diluted with nine volumes of a buffer containing 1.5 g/liter of ascorbic acid. They also observed that serum did not deteriorate if it was frozen without added ascorbate. The ascorbate appeared to prevent oxidation of the serum 5-methyltetrahydrofolate during the heat sterilization. Although many workers have added ascorbate to the standards as well as the assay tubes, the pteroylglutamic acid used for the standards does not require "protection" and it has been found that ascorbate increases growth in the standard tubes, which will in turn result in lower estimates of folate (T6). Up to this time the test was based on conventional microbiological practice which required the careful sterilization of all materials used in the assay. In 1966 Herbert published an aseptic addition technique which greatly simplified the test (H10). It was based on the presterilization of the flasks containing the assay medium and ascorbic acid and then adding the serum to be assayed aseptically. The flasks were then inoculated with the test organism and incubated in the usual manner.

In 1970 Davis (D10) described an automated microbiological assay for the measurement of folate activity. The method used a choloramphenicol resistant mutant of *L. casei* and this eliminated the need for sterilization or aseptic addition. Using an automatic turntable, a series of diluters and a recording colorimeter it was possible to set up tests at a speed of 160 per hour and following incubation to measure growth at the same rate. The method has since been upgraded and is now controlled by a small desk top computer which automatically examines the turbidity readings of the test samples and integrates them on the standard curve. It then prints the results in micrograms per liter. The method has been found to be more reproducible than manual methods and is particularly useful for population surveys where very large numbers of assays are required.

TABLE 1
SERUM FOLATE CONCENTRATIONS IN NORMAL SUBJECTS

Authors	Reference	Range (μg/liter)	Mean	Number of subjects
Toennies *et al.* (1956)	T5	2.6–10.5	5.5	—
Waters and Mollin (1961)	W13	5.9–21.0	9.9	100
Grossowicz *et al.* (1962)	G4	3.2–15.0	8.3	43
Davis and Kelly (1962)	D8	2.7–18.5	5.9	100
Spray (1964)	S14	2.1–28.0	7.8	94
Spector and Hutter (1966)	S15	3.0–11.5	7.4	—
Cooperman *et al.* (1960)	C8	4.0–45.6	15.9	38
Herbert (1966)	H9	7.0–15.9	—	2161

Tennant (T7) has also described an automated method using a Technicon continuous flow system and a short incubation time of 4 hours. Growth response of the organism to folate was estimated by measuring the rate of reduction of 2,3,5-triphenyl tetrazolium chloride.

As the clinical usefulness of the microbiological assay for folate became more widely recognized so the number of variations and modifications to the method increased. As a consequence there was a wide range in the reported values found in normal subjects and a considerable variation in the level below which a patient was considered folate deficient. Some of these are shown in Table 1. Some workers have even suggested that folate levels in serum be graded as low, intermediate, or normal. In general it appears that serum folate levels below 3.0 μg/liter reflect a deficient state.

Attempts have been made to identify some of the variables in the microbiological assay procedure. Anderson and Cowan (A2) found that the growth properties of the assay medium were destroyed by direct sunlight and impaired by bright reflected sunlight and this reduced the sensitivity of the assay. Cowan and her colleagues (C9) described an inhibitor present in serum from patients with tropical sprue, idiopathic steatorrhea, from some pregnant women, and from some patients with liver disease. It was not found in healthy subjects and was not present in deproteinized serum. Streeter and O'Neill (S16) studied the effect of incubation time on the assay, and found that reproducible results could not be obtained unless growth of the organism was complete and this depended on the physiologic condition of the inoculum and the number of viable cells present, the type of media used, and the conditions of incubation. Most laboratories now use an incubation time of 16 to 18 hours, or alternatively wait until the absorbance of the top standard has reached a predetermined level. It is most important with all of these assays that several samples with known high and low values are included with each batch of test samples.

The measurement of erythrocyte folate has the advantage of being a less labile index of folate status than the measurement of serum; it also appears to be laid down early in the life of the cell so the erythrocyte folate reflects past folate status. It is good practice to measure both erythrocyte and serum levels of the vitamin when investigating a patient's folate status. Hoffbrand (H11) described a method for measuring erythrocyte folate in 1966. Whole blood was hemolyzed by diluting with 1.0% ascorbic acid. The sample was then further diluted with an ascorbate-containing buffer, the proteins were precipitated, and the supernatant assayed in a manner similar to that used for serum. If the serum folate concentration and the hematocrit are known, the erythrocyte folate concentration can be calculated. It was found that higher results were obtained if serum was present in the final reaction tube. The reason for this is not clear. Only a very small quantity of serum is required and it does not seem to be involved in the deconjugation of polyglutamate forms of the vitamin present in erythrocytes. Incubating the hemolyzates with chicken pancreas conjugase produced a mean fall in red cell folate activity of 4.1% and from this it could be concluded that any folate polyglutamates present in erythrocytes were of the short-chain variety and could therefore be utilized by the test organism. The erythrocyte folate concentration in 40 healthy subjects ranged from 166 to 640 μg/liter with a mean of 316 μg/liter. Forty patients with a megaloblastic anemia due to folic acid deficiency had levels ranging from 8 to 143 μg/liter (mean 79 μg/liter). Spray (S17) examined the erythrocyte folate concentration in 81 control subjects and obtained a range between 80 and 470 μg/liter. This was lower than that obtained by Hoffbrand (H11) and appears to be too low. Among healthy subjects very few are found with a level below 150 μg/liter.

With the introduction of automated and semiautomated microbiological methods, the measurement of erythrocyte folate was simplified and only required predilution with 1% aqueous ascorbate after which the procedure was the same as for serum (M5).

3.3. Measurement of Folate by Saturation Analysis

Waxman, Schreiber, and Herbert first described a radioisotopic assay for the measurement of serum folate in 1971 (W14) which they claimed gave almost identical results to those obtained by with the *L. casei* microbiological assay and could separate low, borderline, and normal folate levels. Serum was mixed with [^{3}H]methyltetrahydrofolic acid in a phosphate buffer and a folate binder was added which, in this case, was Carnation brand instant powdered milk. Following incubation, free vitamin was separated from that which was bound using hemoglobin-coated charcoal. The radioactivity of the supernatant was determined, and from this the folate concentration in the serum sample could be calculated using appropriate standards and blanks.

The folate-binding protein in milk is a β-lactoglobulin and as this was available commercially its use in place of the whole milk resulted in a much simplified assay (W15). Following the introduction of this assay numerous modifications of the methodology were reported (A3, D11, M6, S18), although there were few major changes. Because of the apparent success of the assay and its modifications a large number of kits was placed on the market, mostly for the measurement of folate in serum but some claimed to be satisfactory for the measurement of erythrocyte folate. Although the kits were popular and used by a large number of laboratories in preference to microbiological assays, they were expensive, and a careful evaluation of the costs by Hill and Dawson (H12) showed that a radioisotopic assay cost more than five times that of a microbiological assay. Kubasik and his colleagues (K3) eavluated six commercial radioassay kits in which either tritium or iodine isotopes were used to measure serum folate. They found serious discrepancies among the manufacturers' kit with regard to reagent stability, within-run precision, minimum detection limits, results of analysis of patient samples, and the analytical recovery of both added pteroylglutamic acid and 5-methyltetrahydrofolic acid. Baril and Carmel (B7) evaluated kits using ^{125}I-labeled pteroylglutamic acid and [^{75}Se]selenofolate. They also compared kits using [^{3}H]pteroylglutamic acid with the *L. casei* assay. They found significant discrepancies between the microbiological and isotopic results and felt that, because a number of patients were on antibiotics and as a result had a low microbiological assay, the isotopic assay may be preferred. Four of their samples gave a low result with the isotopic method because the blood had previously been contaminated with isotopes. It was concluded that none of the assay methodologies was consistently reliable. Workers skilled in the use of microbiological assays are usually able to identify serum samples containing antibiotics by appropriate dilution techniques.

Investigation into the discrepant results found with some radioassay kits when compared with the *L. casei* assay led to the recognition that the uptake of 5-methyltetrahydrofolate by the β-lactoglobulin binder was pH dependent. When assays were run at a pH of 7.4, binding of the methyl form was weak and it was surprising that, in view of the earlier experiments on folate-binding proteins, this was not carefully examined before the early kits were made available. When the pH was raised to 9.3, the binder appeared to have a strong affinity for the 5-methyl form of the vitamin (L4) and under these conditions the assay was shown to give satisfactory results. Trials in the United States (W17) and Great Britain (J1) concluded that results from the measurement of serum correlated well with both the *L. casei* assay and the clinical picture. The measurement of erythrocyte folate, however, was less satisfactory, although Johnson and Rose (J2) were able to improve the results by modifying the technique.

More recently, kits have appeared which measure both the serum vitamin B_{12} and folate concentrations concurrently. The procedures have been greatly simplified, although it must be remembered that the optimum pH for the measurement of folate is 9.2 or 9.3 and this is not satisfactory for the measurement of vitamin B_{12}.

3.4. The Deoxyuridine Suppression Test

Thymidine is required by replicating cells and may be obtained via a synthetic pathway or a salvage pathway which recycles preformed thymidine. The synthetic pathway depends on the methylation of deoxyuridine, the methyl donor being 5,10-methylenetetrahydrofolate. This reaction is vitamin B_{12} dependent, and a deficiency will result in an accumulation of 5,10-methylenetetrahydrofolate due to an inability to transfer the methyl group. In the presence of deoxyuridine and adequate folate, thymidine will be synthesized. However, when the available folate is reduced, thymidine will be obtained via the salvage pathway. Killmann (K4) designed a test based on this reaction which made it possible to determine the role played by vitamin B_{12} or folate in the pathogenesis of disordered hemopoiesis. The method underwent a number of modifications, but the technique described by Wickramasinghe (W16) appears to be reliable and is relatively simple to perform.

Bone marrow is placed in a balanced salt solution and a single-cell suspension is prepared. The nucleated cell count is adjusted to $5–10 \times 10^6$/ml and deoxyuridine added. Following incubation, tritiated thymidine is added and the mixture incubated for a further hour. An aliquot is placed on a filter paper disk and dried. The activity of the disk is determined using a beta counter. The amount of labeled thymidine incorporated into the DNA of the nucleated cells is calculated, and the results are expressed as the percentage uptake when compared with the activity of a sample which has not been preincubated with deoxyuridine. This is called the deoxyuridine depressed value. The normal range is 1.4 to 1.8% (W16).

A depressed uptake of deoxyuridine brought about by a deficiency of folate can be reversed by the addition of as little as 50 μg/ml of the vitamin.

3.5. Folate Absorption Tests

Absorption of the vitamin may be measured by giving an oral dose of tritiated folic acid 24 hours after giving a 15 mg intramuscular injection of "cold" folic acid. This parenteral dose serves to saturate the tissues. The patient then empties his bladder and is given another 15 mg intramuscular injection of folic acid. This is the flushing dose, which serves to flush all the

absorbed tritiated folate into the urine. Urine is collected for 24 hours and the radioactivity measured; the amount of folate excreted can then be calculated (F3). A simpler alternative is to saturate the patient as described above and then, following an oral dose of the vitamin, blood samples are collected at regular intervals over a period of 3 hours and the peak concentration of folate compared with that obtained in healthy controls (P3). This was initially rather a crude test, because at that time *S. faecalis* was being used as the assay organism and it would only respond to a relatively large concentration of pteroylmonoglutamate. With the introduction of the more sensitive *L. casei* assay it became possible to use very small test doses of the vitamin (200 μg), and this provided more physiological test conditions (H13).

There may be a case for using folate with a long-chain glutamic acid residue such as that contained in yeast for absorption studies rather than a pteroylmonoglutamate. This should provide more useful information on the patient's ability to absorb food folates. However, in practice this is seldom done.

4. Folate—Nutritional Requirements

The most satisfactory way of determining the daily requirement of a particular nutrient is to devise a diet which is complete except for the nutrient being studied and to feed it to healthy volunteers together with graded supplements of the nutrient in question. The supplement which just fails to produce clinical change when fed over a period of some weeks represents the minimum daily requirement. This is easily done with animals, provided a suitable diet can be devised, but there are problems when using humans for this type of study because it may be necessary to take the diet for many weeks or months before clinical signs of a deficiency occur.

In 1962, a physician (H14) placed himself on a diet which, when assayed, was found to provide only 5 μg of folate daily. Up to that time no diet had been devised that contained so little folate, and it was made possible only by boiling each 100 g portion of all those constituents that contained more than a trace of the vitamin for 10 minutes in two liters of water. The water was then discarded and the procedure repeated twice (H15). On this diet early megaloblastic change was noted after 137 days. The serum level of the vitamin fell after 14 days followed by the appearance of hypersegmented neutrophils. Formiminoglutamic acid then appeared in the urine and shortly before the megaloblastic change occurred the red cell folate fell. This experiment provided important information on body stores and the sequence of events in folate deficiency. While the diet was not completely free of folate, its content had been carefully measured and was very small. It was calcu-

lated from this study that the daily folate requirement was approximately 50 μg (H16).

In a follow-up experiment three volunteers were placed on this same very-low-folate diet and supplemented with 25, 50, and 100 μg of folate daily (H17). After 1 month there was a fall in the serum folate level from 10.3 to 4.2 μg/liter in the volunteer receiving the 25 μg supplement, while in the two subjects receiving the 50 and 100 μg supplements the serum folate level remained essentially the same. These results served to confirm that the minimum daily requirement was approximately 50 μg.

The World Health Organization has recommended that the daily dietary intake of folate should be 40–50 μg in infants up to 6 months of age, 120 μg between the age of 7 and 12 months, 200 μg from 1 to 12 years, and 400 μg over 13 years. They also recommend that pregnant women should receive at least 800 μg; this is most conveniently given in tablet form as a daily supplement and can be reduced to 600 μg during lactation (W18). In 1979, a British Department of Health and Social Security report (D12) recommended a daily intake of 200 μg of available folate for adults (i.e., 270 μg as total folate, if conjugated forms make up 50% of the total and are 50% utilized). They recommended 500 μg daily for pregnant women and 400 μg during lactation. In 1981, however, these recommendations were withdrawn on the basis that "The requirements of healthy people (for folic acid) are not yet established firmly enough for a recommended daily amount to be set" (D13). This shows that there remains uncertainty about the daily folate requirement, at least in Great Britain, and that further research is needed to resolve this problem. The form in which the vitamin is present in food needs to be taken into consideration when calculating dietary intake. Patients with infections, particularly of the urinary tract (M7) or any condition associated with an increased cell turnover, will have an increased daily requirement for the vitamin.

4.1. The Presence and Availability of Folate in Food

Although it appears that there are folate-synthesizing organisms in the human gut, the folate that they produce does not appear to contribute significantly to the host's folate nutrition. As a consequence man is wholly dependent on food as a source of the vitamin.

During the decade 1950–60 much work was done on determining the folate content of various foods, and there was sometimes a considerable variation in the results obtained. It is not the purpose here to present an extensive account of this earlier work. More recent methods of measurement and the protection of some labile forms of the vitamin with ascorbate have resulted in higher assay results for many foodstuffs. In some instances the

folate concentration assigned to a particular food will, if it is to be meaningful, depend on the method of preparation or cooking. For example, when cooking cabbage, even though only a small amount of water may be used, nearly 50% of the folate will move out into the water. Thus, with respect to folate, the cooking water is just as nutritious as the cabbage. Most foods will lose a considerable part of their folate during cooking, and it has been suggested that there is a greater loss of free folate than of the conjugated vitamin (T8). Exposure of green vegetables to sunlight, as may occur when they are on display in stores, may result in some loss of folate, depending on the number of days of such exposure.

The best organism to use for measuring folate in food seems to be *L. casei* and, although it is sensitive to the short-chain polyglutamates, to obtain the highest result food should first be treated with a conjugase before being assayed. Many foods have been shown to contain a conjugase, and it follows that this may well have reduced some of the long-chain polyglutamates before the food even arrives in the assay laboratory. Many foods also contain a large percentage of 5-methyltetrahydroglutamic acid, and it is therefore a sound policy to protect the food folate with a reducing agent such as ascorbic acid.

Two important papers on the folate content of food are those by Hoppner and his colleagues in Canada (H18) and Perloff and Butrum in the United States, (P4) whose article lists 299 food items. Books such as McCance and Widdowson's *The Composition of Foods* (P5) provide detailed information on the vitamin content of a great variety of foods. The vegetable with perhaps the highest concentration of folate is spinach with 204 μg/100 g. This is more than is found in beef or pork liver but somewhat less than the 275 μg/100 g present in lamb's liver. Dong and Oace (D14) measured the folate in orange, grapefruit, lemonade, and grape juices. The juices were frozen concentrates obtained from a local grocery store and reconstituted according to the manufacturer's instructions. Total folate was 526 μg/liter in the orange, 211 μg/liter in the grapefruit, 23 μg/liter in the lemon, and 47 μg/liter in the grape juice. Approximately half of the total folate was present as a polyglutamate in all four juices. In the citrus juices over 95% of the folate was present as methylfolate compared with only 26% in the grape juice.

Manufacturing processes can result in a substantial loss of the vitamin; for example, during the milling of whole wheat to white flour, 75% of the folate is lost (B8). Bread was found to contain more folate than the flour from which it was made and this was due to the effect of the added yeast. Folate deficiency has been found to be relatively uncommon in Iran and this prompted Russell (R8) to measure the folate content of various Iranian breads since bread is the staple food in that country. They found village wholemeal bread to have a free folate concentration of 340 μg/kg and leavened breads made

TABLE 2
FOLATE CONTENT OF SELECTED FOODS

	Total folate range (μg/100 g)
Bread (white)	15–30
Whole wheat flour	32–54
Beans white, dry	129–290
Asparagus	64–109
Potatoes	7–36
Cabbage	22–30
Broccoli	4–56
Pumpkin	5–19
Brussels sprouts	14–49
Spinach	91–240
Oranges	4–46
Orange juice	2–53
Tomatoes	1–39
Grapefruit juice	2–21
Bananas	22–30
Liver cooked (various)	145–240
Peanuts	44–106

from flours of high extraction rates, which are widely consumed in the cities, as high as 710 μg/kg. The total folate would be much higher than this. White bread made from refined flour contained only 130 μg/kg and oatmeal bread only 90 μg/kg. Hoppner (H19) measured the folate concentration in a wide range of prepared frozen dinners. They found that potatoes provided 19% and other vegetables 49% of the total folate present in the meal. They also found a difference among potatoes, depending on the method of cooking. French fried potatoes contained generally higher levels of folate activity than those prepared by other methods. The dinners showed a wide range of folate activity and those at the lower end of the range did not contain sufficient folate if they were to be used as the sole source of the vitamin. Reheating the dinners resulted in an average loss of 22% of the free folate but did not result in any significant difference in the level of conjugated folate. The folate content of some of the more common foods are shown in Table 2.

Many investigators have found that cooking results in a substantial loss of folate. Banerjee and Chatterjea (B9) observed that cooking fish in an open pan for 10 minutes at 110–120°C resulted in a loss of up to 96% of folate activity, and there have been many reports of folate losses of up to 90% following the cooking of vegetables by steaming.

In the past some manufactured infant milk foods have been found to be low in folate (N4) but fortunately most babies are given additional food

supplements before they have a chance to develop signs of a frank deficiency state. Most manufactured infant foods are now supplemented with folate.

Goat's milk is sometimes fed to infants, particularly those who are sensitive to cow's milk. Goat's milk contains very little folate. Nicol and Davis (N4) found goat's milk to contain only 7 compared with 52 μg/liter in cow's milk. Heating the milk reduced the folate in the goat's milk to 1 μg/liter and halved that in the cow's milk.

Maize is poor in folate and where this is the staple food of a community clinical signs of folate deficiency may appear when there is an increased demand for the vitamin, as occurs in pregnancy. Colman and his colleagues (C10, C11) have done considerable work on the fortification of maize meal with folate and they concluded that it was a practical and worthwhile proposition. The problem in fortifying any foodstuff is that the quantity of the vitamin actually consumed should be as close as possible to that which would be obtained from a well-balanced diet. Once the intake rises much above this there is the risk that it may mask vitamin B_{12} deficiency by preventing megaloblastic hemopoiesis. It has no effect on the neurological changes associated with vitamin B_{12} deficiency and the patient may not seek medical advice until the neurological deficit has reached a serious and sometimes irreversible state. With the exception of some infant foods and milk formulae there is little evidence so far of any widespread supplementation of foods with folate.

5. Conditions Associated with a Deficiency of Folic Acid

A deficiency of folic acid results in the development of a megaloblastic anemia and more rarely neuropathy. The deficiency may result from a reduced intake, an increased demand (e.g., pregnancy), abnormal metabolism, or as a result of various drug therapies.

5.1. Pregnancy

As has already been stated there is an increased requirement for the vitamin during pregnancy. This means that large numbers of women in the lower socioeconomic groups in many if not most countries will become folate deficient during pregnancy unless they are given additional folate to supplement that present in their diets. A similar problem exists with iron, and several pharmaceutical companies market a capsule which contains both iron and folic acid.

During pregnancy the serum folate level progressively falls. Davis (D15) measured the serum folate in blood samples collected from 100 consecutive

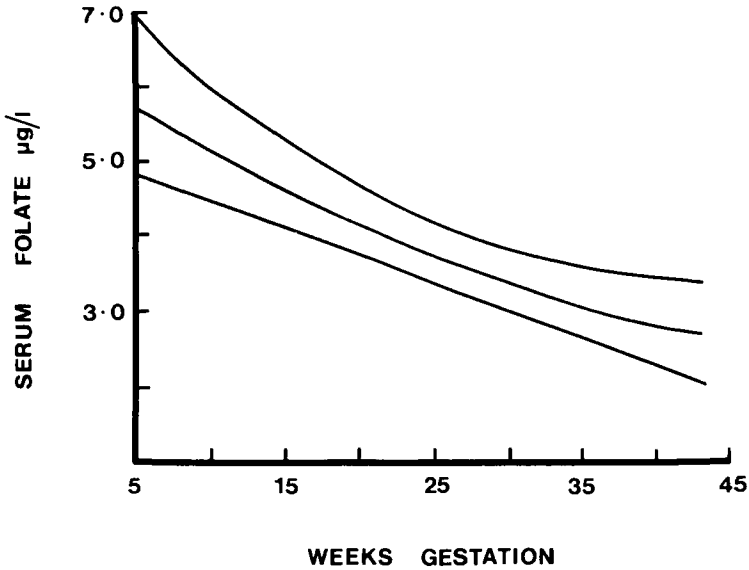

FIG. 6. Progressive fall of serum folate with advancing pregnancy. The outer curved lines represent the 95% confidence limits. [From Davis *et al.* (D14), © 1971, The Medical Journal of Australia, reprinted with permission.]

women on their first attendance at one obstetrician's clinic. No other selection criteria were used. The folate level fell progressively as pregnancy advanced and the level tended to drop below the lower limit of the reference range in some patients close to term (Fig. 6). No other abnormality was found in these patients. Ek and Magnus (E3), in a study of 43 pregnant women, also found that the serum folate concentration fell as pregnancy advanced. However, they found no similar change in the erythrocyte folate concentration. This natural fall in the serum folate concentration can make it difficult to interpret results of folate assays in pregnancy, particularly when the patient is close to term. Hall and her colleagues (H20) thought that the fall in serum folate that occurs in normal pregnancy was principally due to plasma volume expansion. The total hemoglobin concentration was also found to fall in a similar manner. Low urinary estrogen excretion has also been associated with a low serum folate level in pregnancy (M8), and low urinary estrogen is known to be associated with reduced fetal weight. Women with a megaloblastic anemia of pregnancy tend to have smaller babies than those without this condition. If the observed folate deficiency were important in pregnancy, it would be in relation to the estrogenic response of the uterus. Ovarian hormones are able to modify the effects of a deficiency of thiamin or pyridoxal but they cannot do so with folate. Thus, once a deficien-

cy has occurred, repair of any resulting damage may not be possible. Therefore, if folate plays any role in the prevention of certain cases of unsuccessful pregnancy, it would have to be given before conception in order that the greatest response of the uterus to estrogen may be obtained when pregnancy does occur.

The view is now held by some that neural tube defects may be the result of a preconceptual folate deficiency (H21). Unfortunately, in at least one of the clinical trials a multivitamin preparation containing vitamin A and folic acid was used, and, although the trial appeared to be very successful, doubts have been expressed about the safety of vitamin A administration (E4). Smithells (S19) in a multicenter study reported that of 178 infants of multivitamin-supplemented high-risk mothers only one had a neural tube defect, while in the control group comprising 260 mothers 13 gave birth to infants with nueral tube defects. The answer may come from the several trials that are now under way particularly that mounted by the Medical Research Council in Great Britain. If it is confirmed that vitamin supplementation reduces the incidence of neural tube defects the difficulty remains of offering supplementation to women, the majority of whom may not need it. Moreover, to be effective, the vitamin supplement must be taken before conception, therefore the pregnancy must be planned. If, as seems likely, folic acid is the vitamin able to reduce the incidence of neural tube defects, then widespread supplementation would need to be considered.

A number of studies have shown an association between anticonvulsant drugs and congenital malformations. Since a reduced level of serum folate is not uncommon in patients taking antiepileptic drugs folate deficiency may be responsible for the malformations. Elshove and Van Eck (E5) found the frequency of malformations in infants born to mothers on antiepileptic treatment eight times greater than in a control group and others have found the frequency to be two to three times greater (L5, S20). In a study of congenital malformations associated with the taking of anticonvulsant drugs, Biale (B10) found that of 56 births in their series 9 infants were born with malformations. Four were born dead or died shortly after birth. Among the abnormalities found were congenital heart disease, cleft lip with or without cleft palate, neural tube defects, and skeletal abnormalities. These same authors reported on two women suffering from *grand mal* epilepsy, both of whom had their blood folate measured in the seventh month of their first pregnancy. A subnormal result was recorded in both and, in the seventh month, one delivered spontaneously a male infant with anencephalus, cleft lip, and palate. The infant lived for 2 hours. The second woman delivered at term a male infant with hypospadias (B11). Although it is necessary to adjust the level of anticonvulsant drug used when administering folate to patients with epilepsy because the vitamin has been reported to worsen seizure activity (S21),

there does appear to be a need for a clinical trial of folate supplementation in epileptic women who are anticipating pregnancy.

Folate deficiency has been shown to be responsible for birth defects in rats. Armstrong and Monie (A4) have shown that malformations of the eyes occurred in folate-deficient fetuses and these included anophthalmia or microphthalmia, reduction or absence of the optic nerve fibers, duplication of the optic cup, and a number of other defects. However, in the rat these defects were associated with a deficiency of maternal folate during pregnancy particularly between the seventh and ninth days.

Apart from the effect on the fetus, folate deficiency may have serious consequences for the mother. Although the mother is more likely to be affected by a deficiency developing during pregnancy, the fetus appears more likely to be affected by the mother's folate status at conception and possibly during the first few weeks of pregnancy. The most common presentation in the mother is a megaloblastic anemia which cannot be differentiated on morphological grounds from the megaloblastic anemia resulting from vitamin B_{12} deficiency. The anemia may be accompanied by a profound leucopenia and thrombocytopenia. In the stained blood film the granulocytes show a shift to the right with large hypersegmented forms. Patients usually respond to treatment with folic acid given orally but occasionally require transfusion with packed erythrocytes. Chanarin *et al.* (C12) studied the folate concentrations in a large number of pregnant women. They found 13% of 105 women had megaloblastic hemopoiesis; none was anemic and only three had minor changes in their peripheral blood films. Megaloblastic change was three times as frequent in the winter months compared with other times of the year. Women who were found to have megaloblastic bone marrows in late pregnancy also had a significantly lower red cell folate level at the fifteenth week of pregnancy as compared with patients who remained normoblastic, suggesting a nutritional deficiency of folate as the underlying cause. An unusual presentation of puerperal psychosis resulting from folate deficiency was reported by Thornton (T9). The patient had an unremarkable pregnancy and delivery, but 12 weeks postpartum she was observed by her family to become progressively withdrawn, and emotionally labile. This progressed to visual hallucinations associated with agitation and panic, frequent episodes of confusion, and disorientation. The patient was confined in a private institution and later in a government psychiatric institution. She was treated with electroconvulsive therapy, chlorpromazine, trifluoperazine, and benztropine. After several suicide attempts she was seen in consultation and a blood count and vitamin assay done. Her hemoglobin was 108 g/liter with a mean corpuscular volume of 101 fl. The serum folate was 0.8 μg/liter on one occasion and two further samples were reported as undetectable. All other drugs were stopped and the patient was treated with folic acid and

made an uneventful recovery. She was followed up for 2½ years without evidence of psychiatric disturbance.

The fetus is able to accumulate folate from the mother regardless of the mother's folate status. Soon after delivery, the infant's serum and red cell folate levels are almost always higher than the mothers (H22) and this remains true even when the mother is depleted to the point of having a severe megaloblastic anemia (G5). Newborn infants whose mothers had developed a severe megaloblastic anemia due to folate deficiency were found to have normal hemoglobin values (P6). These studies have provided some evidence that folate is transferred against a concentration gradient from mother to fetus, and this indicates active placental transport (S22). Although the placenta seems able to supply the fetus with adequate folate regardless of the nutritional state of the mother, there is little information about how folate is provided during organogenesis or before the placenta and its circulation have developed.

5.2. Folate Nutrition in Infancy

Folate deficiency in breast-fed infants is a very rare occurrence, and most bottle-reared infants receive adequate folate when fed one of the proprietary infant milk formulae or diluted cow's milk provided that it has not been overheated. Almost all infant milk formulae now contain added folate. Folate deficiency is not often seen in infants, but one of the most frequent causes is that which has sometimes been called "goat's milk anemia" (B12). This usually develops in infants who are fed entirely on goat's milk which is naturally low in folate. Rohm (R9) described an 18-month-old girl who was treated for tonsillitis by a general practitioner. After recovering from the tonsillitis she remained weak and took only bottle feeds and no solid food. Her hemoglobin was found to be 49 g/liter and a stained blood film showed macroovalocytes; she was treated with ferrous lactate. It was later found that the child had been fed on the infant milk food "Lactogen" until 6 weeks of age, after which she was fed only goat's milk and took no solid foods. At this time she was drinking 1.6 liters of goat's milk daily. On admission to hospital she was treated with oral folic acid and intramuscular iron and responded with a reticulocytosis of 34%. She was taught to eat solid food and discharged. Within 3 weeks all of her hematological parameters had returned to normal.

5.3. Effect of Oral Contraceptive Steroids

The effect of oral contraceptive agents on serum folate levels has been the subject of numerous studies (S23, S24, P7). Davis and Smith (D16) as part of

a population survey examined the serum and red cell folate concentrations in 1067 women, 166 of whom were taking oral contraceptives. They found that both the mean serum and erythrocyte levels were significantly lower than that found in the women not taking these agents. They interpreted this as indicating that the folate pool was reduced in women taking oral contraceptives. However, only eight women (4.8%) in the contraceptive group had a serum folate concentration below the lower limit of the normal range compared with 65 (7.2%) in the control series. These results were probably due to the inclusion of a greater number of women of relatively low socioeconomic status in the controls. There was no difference in the mean hemoglobin levels of the two groups. Several studies have, however, failed to find any depression of serum folate levels of women taking oral contraceptives (A5, P8).

Although it appears that the serum folate concentration may be related to the length of time that the oral contraceptive has been taken it has also been shown that in short-term users (4 months) there is a significantly lower serum folate during the first week of the menstrual cycle in these women despite an adequate folate intake (P9).

Folic acid supplements have been reported to result in improvement in cervical dysplasia in women using oral contraceptives (B13). In a study of 47 young women with mild or moderate dysplasia of the uterine cervix, 22 received 10 mg of oral folate daily while 25 received a placebo (10 mg ascorbic acid). After three months each participant was biopsied and the biopsy slides and smears reviewed and scored without knowledge of the patient's treatment. Mean biopsy scores from folate-supplemented subjects were found to be significantly better than from those receiving the placebo. Interestingly, the erythrocyte folate in this group of women was significantly lower than that found in women taking oral contraceptives without cervical dysplasia, who in turn had a lower concentration than women not taking these agents.

Although there does not appear to be any difference in the hemoglobin concentrations in women taking oral contraceptives, some studies have shown that there is a small but significant increase in the mean corpuscular volume as compared with women not taking oral contraceptives (C13, F4). Occasionally a megaloblastic anemia has been associated with the taking of these agents, usually when they have been taken over a period of several years. Although most of the affected women are said to have consumed an adequate diet, dietary factors cannot be entirely eliminated. Streiff (S25) described several patients who were taking oral contraceptives and developed a severe megaloblastic anemia. Ryser and his colleagues (R10) found a patient presenting with a low hemoglobin and an MCV of 132 fl. A bone marrow biopsy showed gross megaloblastic hyperplasia. She had been taking

oral contraceptives for the past 3 years. A jejunal biopsy was normal and there was no other evidence of malabsorption. The patient appeared to have an adequate diet except for a dislike of meat and she was not taking any other drug. Treatment with 200 μg of folate intramuscularly resulted in a characteristic reticulocyte response. The anemia in other patients has been found to respond to withdrawal of the oral contraceptive (W19). In a number of these women evidence of intestinal malabsorption has accounted for their folate deficiency (W19, T10). Green (G6) described a megaloblastic anemia in a 39-year-old vegetarian woman who had been taking an oral progestogen agent for 8 years. Although she ate moderate amounts of raw vegetables her dietary folate was calculated to be 70 μg daily. Following treatment with folate her hemoglobin returned to normal.

It is probable from the evidence available so far that oral contraceptives marginally depress the body folate pool. In many, if not most women, this is probably of little consequence; however, this folate-depleted state is important should pregnancy take place shortly after ceasing the oral contraceptive agent because the vitamin has an important role in the early stage of fetal development.

5.4. Folate and Alcohol

Alcoholics may suffer from a deficiency of a number of B-group vitamins and supplementation may not have the expected beneficial effect while the individual continues to take alcohol. The reason for this is that enzymes necessary to convert the vitamin into an active coenzyme form may be depressed by alcohol. This was demonstrated in the classic experiment by Sullivan and Herbert (S26). Their patient, an alcoholic woman, was admitted to hospital with a megaloblastic anemia due to folate deficiency. While in the hospital she continued to receive alcohol in the form of one pint of whiskey daily. When given folic acid under these conditions she showed no response. Withholding the alcohol resulted in a brisk reticulocytosis. This was repeated several times with similar results. It seemed from this that either the alcohol was inhibiting a particular reaction in the folate metabolic pathway or it was in some other way blocking the elaboration of a particular coenzyme form of folate. Bone marrow biopsy of acute alcoholics has shown vacuolization of the early red cells (M9) and the presence of ringed sideroblasts (H23). These changes are not generally appreciated because bone marrow biopsies are not often performed in the acute stages of alcoholism.

A high alcohol consumption is not infrequently associated with liver disease, megaloblastic anemia which can also be sideroblastic, dietary deficiency, and the Wernicke–Korsakoff syndrome. Results from the numerous studies which have been conducted on alcoholics have varied depending on

the severity of alcohol abuse in the group studied. These have ranged from the so-called "skid-row" alcoholics (S27), which represent one end of the spectrum, to studies of moderate or heavy social drinkers who are almost at the other end of the spectrum (W20). With the introduction of automatic equipment for routinely counting and sizing blood cells it has become apparent that one of the most common blood changes in alcoholics is a macrocytosis. This may or may not be associated with anemia. Thrombocytopenia is also very common (L6), but recovers within a few days following withdrawal of alcohol and is more likely to be a reaction to the toxic effect of the alcohol rather than to a deficiency of folate.

Davis and Leake (D17) studied the urinary excretion of formiminoglutamic acid following histidine loading before and after folic acid therapy in 17 alcoholic patients. Five patients excreted formiminoglutamic acid before and after treatment with folic acid, which suggested that they had a problem associated with histidine catabolism and appeared unable to transfer the formimino group to 5-methyltetrahydrofolate. The interesting point in this study was that the tests were done several days after the withdrawal of alcohol and probably reflected significant liver damage.

Halsted *et al.* (H24) studied the absorption of folate in recently intoxicated alcoholics. Using small doses of folic acid (15 μg/kg) they found that the urinary excretion of the vitamin was seldom out of the normal range. However, the plasma levels showed a striking deviation from the normal and failed to peak in the same manner as the controls. They concluded that folate absorption was impaired by a period of prolonged alcoholism and that this could be corrected by abstinence. The effect of acute alcohol ingestion on folate kinetics has shown that it can reduce the serum folate level (i.e., the 5-methyltetrahydrofolate level) in a matter of hours. Alcohol was thought to exert a blocking effect on the mobilization and transport of 5-methyltetrahydrofolic acid from tissue stores to plasma (L7). Studies on rats have shown that alcohol ingestion appears to create a situation whereby folate is trapped in the liver, resulting in an increase of both the monoglutamate and pentaglutamate stores at the expense of folate released into bile. This reduction in the concentration of folate in bile results in a drop in the level of folate in the intestine available for reabsorption, and this is responsible for the immediate fall in serum concentrations. Alcohol thus interferes with the folate enterohepatic cycle and this is reflected in a reduction in the serum level of the vitamin (H6). However, McGuffin and his colleagues (M10) could not confirm that there was any block to the release of folate from liver and observed that, in rats receiving alcohol, both the serum and liver folate fall at about the same rate. They concluded that alcohol suppressed the level of 5-methyltetrahydrofolate but could not offer an explanation of how this came about. Studies so far indicate that alcohol interferes with the transfer of non-

methyl folate to bile without affecting the biliary excretion of 5-methyltetrahydrofolate. This greatly reduces the folate available for reabsorption and results in a rapid fall in serum folate (A6). In addition to the direct effect of alcohol on folate metabolism, alcoholics, by the very nature of their problem, often consume a diet poor in many essential nutrients including folate. Lindenbaum and Roman (L8) found that the combination of a poor diet and a high consumption of alcohol was the most common cause of megaloblastic anemia due to folate deficiency in the United States. They pointed out that in New York City this accounted for 89.6% of patients seen with a megaloblastic anemia. Dietary deficiency of folate alone was found to be a relatively uncommon cause of megaloblastic anemia accounting for only 2%. Alcoholic patients with a megaloblastic anemia who had been previously treated were placed on a low-folate diet and given ethanol equivalent to 1 pint of whiskey daily. They developed a low serum folate level in 4 days and a megaloblastic bone marrow within 2 to 3 weeks (H25). Alcohol appeared to greatly accelerate the development of a folate deficiency in these patients.

5.5. Nutritional Folate Deficiency

A nutritional deficiency of folic acid, with the exception of that found in pregnancy, is not common and is usually found in people in lower socioeconomic groups, those who have unusual dietary habits, and those who have an increased demand associated with an increased cell turnover, as found in infections or malignant disease, which cannot be met by their dietary intake. A marked deficiency is characterized by a megaloblastic anemia with leucopenia and thrombocytopenia. The blood and bone marrow appearances are identical to those found in vitamin B_{12} deficiency. This is to be expected because a deficiency of vitamin B_{12} causes a block in the folate cycle, which results in the suppression of DNA synthesis. It is this depression of DNA synthesis in the face of normal RNA production which results in asynchronous development of the red cells. While the cytoplasm matures normally the nucleus remains large and primitive. These megaloblasts are fragile cells and many survive in the circulation for only a relatively short time. Neutrophil dysfunction in the form of reduced phagocytic and to a lesser degree bacteriocidal action has been reported in patients with protein-calorie malnutrition and folic acid deprivation (Y1).

5.6. Folate and Neuropathy

Neuropathy is not usually thought to be a feature of folate deficiency although it was suggested in 1969 by Brain and Walton (B14) that the serum folate should be measured in cases of unexplained polyneuropathy, myelopa-

thy, and dementia. At that time the role of folate deficiency in these disorders was not appreciated. Reynolds and his colleagues (R11) compared the neurological status of 24 patients with severe folate deficiency with a control group having a normal serum folate. They found a significant increase in organic brain syndrome and pyramidal tract damage together with a higher prevalence of neuropathy in the folate-deficient patients. These findings were independent of the presence of alcoholism or the degree of anemia, although they did find that neurological complications were more common among patients who were both folate deficient and alcoholic. Fehling (F5) described a young man presenting with paresthesia in the forearm and dorsum of the hand. Examination revealed diminished vibration sensitivity in the thumbs and big toes, weak knee reflexes, and diminished cutaneous sensation on the dorsum of the hand. After 3 weeks atrophy of the extensor muscles of the left forearm was noted and this was followed by neurogenic changes in several muscles of both forearms, hands, and legs. His hemoglobin concentration was normal but his serum and erythrocyte folate concentrations were very low. Treatment with thiamin, riboflavin, niacin, pyridoxine, and pantothenic acid had little effect. This was followed by treatment with 2.5 mg of folic acid daily which resulted in a slow improvement which was, except for some minor residual damage, complete after 12 months. Gastroenterological studies showed that the patient was unable to absorb folate tetraglutamate and absorption of monoglutamate was impaired. Neuropsychiatric changes, depressed ankle jerks, diminution of vibration sense, and stocking-type tactile hypoesthesia appear to be the most common neurological signs associated with a deficiency of folate. Several families with the restless-legs syndrome and mild neurological disturbances who were also folate deficient have been reported by Botez (B15). All responded to treatment with folic acid. Elderly patients with neuropathy due to folate deficiency have shown a dramatic improvement following treatment with the vitamin. Manzoor and Runcie (M11) described 10 patients between the ages of 52 and 93 years all of whom were folate deficient and suffering from a psychotic or confused mental state, nine were bedridden, and the tenth was confined to a chair. Following treatment with folic acid seven became ambulant and three were confined to a chair. Normal mental function was restored in six and improved in three. A significant improvement in mental function has also been reported in younger folate-deficient patients following treatment with the vitamin (B16).

Although the neuropathy and mental changes associated with folate deficiency are usually not as pronounced as those observed in vitamin B_{12} deficiency, this is not always the case. Enk and his colleagues (E6) described a woman who presented with what they described as massive neurological manifestations, which included symmetrical weakness and atrophy of the

muscles in all limbs, diminished sense of touch, pain, and vibration sense below the knees, loss of all tendon reflexes except biceps, and an atactic gait. She had difficulty in walking because of the numbness and tingling in her feet. Sixteen years previously she had a partial gastrectomy performed for peptic ulceration. Laboratory investigation showed a normal serum vitamin B_{12} level but her absorption of the vitamin was impaired. Because of the abnormal absorption test a diagnosis of subacute degeneration of the spinal cord was made and she was treated with vitamin B_{12}. After 20 months she was reexamined and her condition was found to have deteriorated and she now showed evidence of a moderate degree of dementia with severe impairment of memory. The patient was not anemic nor was her MCV raised; however, her serum and erythrocyte folate which at this stage was measured for the first time was low. She was also found to be excreting formiminoglutamic acid. Folate absorption was normal. Treatment was started with oral folic acid, 5 mg three times daily. Neurological improvement started within 1 week and over the following 7 months she showed continuous improvement.

Although many of these patients had either a diet poor in folate or malabsorbed the vitamin, a number appeared to have an increased demand suggesting an abnormality in the metabolic pathway of the vitamin. The role of folate in neurological function is not clear; folate is important in DNA synthesis, but little DNA synthesis takes place in nervous tissue. There is a requirement for folate in RNA synthesis and it may be here that a deficiency of folate is able to exert its neurological effect.

Patients with folate deficiency and organic mental changes, polyneuropathy, and depression have been found to have a reduced cerebrospinal fluid level of 5-hydroxyindoleacetic acid. Following treatment with the vitamin the 5-hydroxyindoleacetic acid level was found to return to normal in those patients who showed improvement in their neuropsychiatric signs. However, patients who were folate deficient with neuropsychiatric symptoms which were not responsive to treatment with folic acid showed no change in their level of cerebrospinal 5-hydroxyindoleacetic acid (B17).

5.7. Malabsorption of Folate

5.7.1. *Tropical Sprue*

Tropical sprue is a malabsorption syndrome of unknown etiology affecting the absorption of a wide range of nutrients. Evidence suggests that the primary mucosal lesion may be due to an infectious agent but this has not been confirmed. However, the response to antibiotics (T11, K5) and its epidemiology provides some support for this suggestion. The syndrome is

endemic in southern India, the West Indies, Indonesia, Malaysia, and parts of southern Africa, but it is uncommon in other tropical areas. It is characterized by steatorrhea with large frothy foul-smelling stools, weakness, and weight loss. There may be abdominal distension, glossitis, and stomatitis. Both folate and vitamin B_{12} are malabsorbed. However, because of the relatively small stores of folate, a deficiency becomes apparent often within a few months of the onset of the illness. Vitamin B_{12} deficiency may also occur, but this is likely to be found in association with a more chronic form of tropical sprue (K6).

In one study a low serum folate was found in 87% of patients with tropical sprue (O1), while in others a lower frequency of 55% has been reported (M12). The prevalence of megaloblastic anemia is high and ranges from 64 to 74% (K7). The folate deficiency appears to be due to an inability to hydrolyze the polyglutamate forms, although absorption studies have also shown some impairment of absorption of the monoglutamate (C14). Treatment of patients with folic acid will produce a hematological response and in some it may also bring about an improvement in the intestinal symptoms (S28). Clinical and functional improvement usually follows treatment with broad-spectrum antibiotics which may have to be continued for at least 6 months.

5.7.2. *Gluten-Sensitive Enteropathy*

This is a broad term which has been used to describe celiac disease, nontropical sprue, and celiac sprue. They have in common a failure to absorb many nutrients, particularly folate. Halsted (H26) found that 90% of adult patients with the condition malabsorbed this vitamin. The disease is characterized by an abnormality of the mucosa of the small intestine which appears to be exacerbated by gluten, the protein fraction of wheat rich in glutamine. The cause of the disorder is not known, but it has been suggested that it may be inherited (M13). The proximal portion of the gut is the most severely affected and this is not surprising since it is exposed to the greatest undigested gluten load. Many patients develop a megaloblastic anemia due to folate deficiency but in children an iron-deficiency anemia appears to be more common (D18). Treatment with a gluten-free diet is successful in about 80% of patients and results in a slow return of the gut to apparently normal function (S29).

5.7.3. *Congenital Folate Malabsorption*

This is a rare disorder first described by Luhby *et al.* in 1961 (L9) and since then a total of six patients have been reported in the literature. All exhibited severe malabsorption of folate but no defect in the absorption of fat, glucose, vitamin A, or vitamin B_{12}. Four of the six patients had moderate

to severe mental retardation and one had abnormalities in abstract thinking and had a poor school record. The sixth patient was only 2 months old when reported. All presented with a severe megaloblastic anemia. Some responded to treatment with very large oral doses of folic acid in the order of 40 mg daily (L10), while in others the vitamin was given intramuscularly (P10). Some of these patients have been found to have a very low CSF level of folate while they were being treated with the vitamin and it would appear that they had difficulty in transporting folate from the serum to the CSF. One patient treated with folic acid had a serum concentration of 18 μg/liter and a CSF level of only 2.8 μg/liter (normal is 17 to 40 μg/liter). Treatment with intramuscular 5-formyltetrahydrofolate (folinic acid) raised the CSF folate concentration to 5.5 μg/liter. This inability to maintain a normal concentration of folate in the CSF indicates the presence of a second defect of folate metabolism. The central nervous system requires reduced folates, and it is possible that these infants had difficulty in elaborating 5-methyltetrahydrofolate. Further studies on the forms of folate present in the serum of patients with this most interesting condition are required.

5.7.4. *Giant Hypertrophic Gastritis*

Patients with giant hypertrophic gastritis (Menetrier's disease) have a hypoproteinemia which develops as a result of an increasing loss of plasma proteins into the gastric lumen. Some patients with this syndrome have also been found to have a low serum level of folate. Belaiche (B18) studied the gastric folate loss in three patients with this disease. The three patients, together with five controls, were given an intravenous injection of 15 mg of folic acid and their gastric secretions collected at 15-minute intervals over a period of 3 hours. The folate concentrations of the samples were determined and the patients were found to have a significantly higher ($p < 0.001$) concentration of folate in their gastric secretions than the controls, although the baseline assays were similar. Whether this test reflects a real loss of folate by this route under normal physiological conditions remains to be determined. Folate absorption studies in these patients showed them to have relatively flat curves as compared with the controls.

6. Inborn Errors of Metabolism

6.1. Formiminotransferase Deficiency

Formiminotransferase deficiency syndrome was first described in two patients by Arakawa (A7). It was characterized by mental retardation, a very high serum folate level, and excessive excretion of formiminoglutamic acid following a histidine load. A third patient with this syndrome was later

reported who responded to treatment with both folic acid and pyridoxine (A8). This patient was first seen at 3 months of age because of pallor developing soon after birth. There was nothing significant in the neonatal history except for the pallor of the skin. At 15 days of age he was found to have a low erythrocyte count and hemoglobin concentration. The patient was treated with vitamin B_{12} 500 μg daily for a period of 4 days and was given a daily transfusion of 50 ml of blood for 10 days. This seemed to improve the anemia but a relapse occurred 2 months later. Laboratory studies showed that the patient had a high serum level of folate (80 μg/liter) and also excreted 1.68 mmol/liter of formiminoglutamic acid following a histidine load. As the serum folate level fell so the concentration of the urinary formiminoglutamic acid increased. The patient required further blood transfusions and was then given a course of pyridoxal phosphate, 100 mg daily for 8 days by intramuscular injection, which was then reduced to 50 mg for a further 7 days. This resulted in a good reticulocyte response and a rise in hemoglobin from 51 to 108 g/liter. A course of folic acid was then given, 10 mg intramuscularly daily for 9 days and resulted in a further reticulocytosis and rise in the hemoglobin concentration to a peak of 176 g/liter. The bone marrow morphology, which had always been megaloblastic and sideroblastic, remained megaloblastic but the number of ringed sideroblasts were greatly decreased. A liver biopsy revealed a marked decrease in formiminotransferase activity. The serum pyridoxal phosphate levels were not measured but no increase in the excretion of xanthurenic acid was found following a loading dose of tryptophan.

A total of 10 patients have now been described with formiminotransferase deficiency. Some have had a variable degree of mental retardation and a severe anemia while others have been asymptomatic. Arakana and his colleagues described five patients (two of whom were brothers) and all were severely mentally retarded (A9, A10).

Formiminotransferase deficiency has been reported in a 42-year-old female who presented with early megaloblastic change and mild anemia (H27). She had a raised excretion of formiminoglutamic acid which declined following treatment with folate. Two siblings who excreted massive amounts of formiminoglutamic acid in their urine were studied by Perry (P11). Both had normal serum folate levels and there were no hematological abnormalities. While they could not be described as retarded they were mentally slow, although this characteristic was noted in other unaffected members of the family and may not have been related to the excretion of formiminoglutamic acid.

It is not possible to say if all of these patients are true examples of formiminotransferase deficiency. A degree of clinical variation could be expected and this would depend on how much of the active enzyme was available. The patients described by Perry excreted much more formimino-

glutamic acid than other patients who had a severe megaloblastic anemia, and it seems likely that these patients have a different or an additional metabolic defect. The patient responding to both pyridoxine and folate is more difficult to understand. He may be a variant or he may also have an abnormality of one of the enzymes in the pyridoxine pathway in addition to the formiminotransferase deficiency.

Another variant has been found affecting two sisters (N5, N6). This was characterized by the urinary excretion of large quantities of hydantoin-5-propionic acid and formiminoglutamic acid, and was unresponsive to treatment with folic acid. One had retarded speech, but this was the only defect, and the other child was normal.

6.2. Impaired Folate Uptake by Bone Marrow Cells

What appeared to be a new inborn error of metabolism was described by Branda in 1978 (B19). The patient was a young man who came from a family in which hematological disease had contributed to, or caused the death of 18 of 34 affected members of the family. He was found to have a severe anemia and an aplastic bone marrow and required regular transfusions. A further bone marrow biopsy 2 years later showed continuing severe aplasia but a megaloblastic change was noted in the few remaining erythrocyte precursors. His serum vitamin B_{12} was normal but his serum folate was minimally depressed at 3.4 μg/liter. Treatment was started with folic acid, 5 mg daily, and he immediately noted an improvement in his general sense of well being and within a month transfusion was no longer required. His folate dosage was increased to 20 mg daily and his hemoglobin rose to 150 g/liter and has remained at that level for 3 years. However, his leucocyte and platelet counts continued to be slightly below the lower limit of the normal range. While he was receiving 5 mg of folate daily the erythrocyte folate was distinctly abnormal, being described as one-third of normal. When the folate was increased to 20 mg daily the erythrocyte folate returned to normal. The patient appeared to have a defect in his ability to maintain a normal concentration of intracellular folate, and it was suggested that this may have been due to a failure to form polyglutamates. Folate in the form of 5-methyltetrahydromonoglutamate is able to enter cells, but if it is not changed to a form which prevents its movement across the cell membrane it can just as easily move out again (B20).

6.3. Methylenetetrahydrofolate Reductase Deficiency

This disorder was first described in 1972 and so far nine patients have been reported who appear to have a deficiency of this enzyme (M14, F6).

Most of the patients have been females with severe mental retardation, few surviving beyond the second decade of life. The disease is characterized by the urinary excretion of large quantities of homocysteine. Treatment with large doses of folic acid may reduce the amount of homocysteine excreted, but, with the exception of one patient (F6), there was no intellectual improvement. In two families, four children were affected in one and two in the other.

Methylenetetrahydrofolate reductase activity was found to be 20% or less than that present in controls (M14). The enzyme is required for the reduction of methylenetetrahydrofolate to methyltetrahydrofolate; however, the lack of methyltetrahydrofolate did not result in any anemia and the blood picture has been normal in the patients so far described.

6.4. N^5-Methyltetrahydrofolate Transferase Deficiency

One patient has been found with this deficiency (A11). The patient, an infant, was mentally retarded, had a megaloblastic anemia and abnormally high levels of serum and erythrocyte folate. In spite of the high serum folate concentration there was a marked rise in the reticulocyte count when the patient was treated with folate. It was thought that the patient had impaired utilization of N^5-methyltetrahydrofolate. Assay of liver N^5-methyltetrahydrofolate transferase showed it to be reduced. It was suggested that folate accumulated at the N^5-methyltetrahydrofolate block and could therefore not be further utilized. Treatment with pteroylglutamic acid provided a means of producing active folate up to the point of the block. Unfortunately this patient was also treated with pyridoxine, and it is not clear which vitamin was responsible for the reticulocyte response. Further studies are required to determine the precise nature of this metabolic disorder.

6.5. Other Defects of Folate Metabolism

There have been other single reports of megaloblastic anemia associated with an apparent abnormality of folate metabolism. One child had a normal serum folate of 6 μg/liter, an erythrocyte folate of 1480 μg/liter, and a megaloblastic anemia which responded to treatment with folic acid (V1). Lampkin (L11) described two sisters with a severe megaloblastic anemia and normal vitamin B_{12} and folate levels. Absorption of vitamin B_{12} was normal and both patients excreted an increased amount of formiminoglutamic acid. It was thought that they required both vitamin B_{12} and folate to restore normoblastic hemopoiesis.

7. Folate Metabolism and Renal Failure

Folate deficiency in patients with chronic renal failure is not uncommon and may be the result of a poor diet, vomiting, or dialysis (H28, W20, S30). Less frequently, this may be severe enough to cause a megaloblastic anemia. Swainson and Winney (S31) studied the loss of folate in patients on dialysis and found that it was only slightly greater than that of normal subjects. Over a 12-month period they found no fall in the serum or erythrocyte folate and concluded that there was no need for folate supplementation in patients having regular hemodialysis. However, Siddiqui and his colleagues (S32) found that 52 μg of folate was lost with each hemodialysis. In another study it was found that in patients receiving folate supplements up to 37% of the dose could be lost with each hemodialysis (S33). Although the serum folate concentration may fall following dialysis, it has been found to return to the predialysis level within 4 days (W21). As would be expected, hemodialysis has no effect on erythrocyte folate.

Patients with serious renal disease may have difficulty in consuming a diet containing enough folate and other vitamins, and where a deficiency occurs a dietary cause should first be sought. Many of these patients are given folate and other vitamin supplements and this seems to be a good preventative measure.

Patients who have received a renal transplant have sometimes been found to have an increased MCV, and in one study 74% of 69 patients were found to have a macrocytosis together with a megaloblastic bone marrow (W22). All of these patients were taking azathioprine and it is known that this drug causes an increase in the MCV (W23). The majority of patients with renal allografts do not appear to have any marked change in their MCV and the erythrocyte folate remains normal in these patients and also those with an increased MCV. It is likely that a fall in the concentration of serum folate may occur following renal transplantation, independent of any effect that may be associated with the use of immunosuppressive drugs. The level of serum folate was found to fall in baboons (*Papio ursinus*) following allotransplantation but it was noted that it also fell in humans after a variety of surgical procedures such as cholecystectomy, mastectomy, and thyroidectomy. These patients also had a temporary rise in urinary folate over the 5 days following the surgical procedure (R12). The reason for this is not known.

8. Folate Deficiency during Intensive Therapy

A rapid depletion of folate may occur in critically ill patients. Beard (B21) described four patients with a ruptured abdominal aortic aneurysm who

developed thrombocytopenia and marked megaloblastic changes within 3 to 10 days following surgery. In two of these patients there was direct evidence of folate depletion within marrow cells on the basis of the deoxyuridine suppression test. All of these patients had previously consumed an adequate diet, they only occasionally took alcohol and they were not taking any drugs which interfered with folate or vitamin B_{12} metabolism. It was suggested that one possible explanation for the rapid onset of folate deficiency was that the patients were septic and had pneumonia which led to an increased demand for granulocytes. Their marked bleeding would have led to marrow hyperplasia and tissue hemorrhage would lead to an increased consumption of platelets. The increased demands made on the marrow for granulocytes, red cells, and platelets would in turn increase the demand for folate. The folate deficiency appeared to be confined to the bone marrow. Liver concentrations were unchanged and it seems that these patients may lack the ability to mobilize folate from the liver quickly enough to satisfy the increased demand.

This type of acute folate deficiency is not restricted to elderly patients. Ibbotson (I2) reported a 28-year-old man who had sustained an extensive crush injury to the lower limbs and developed acute renal failure requiring peritoneal dialysis. On admission his hemoglobin concentration and blood count were normal but after 17 days the hemoglobin had fallen to 74 g/liter and the platelets to 60×10^9/liter. The bone marrow was megaloblastic. Microbiological measurement of serum folate could not be made because the patient was receiving antibiotics, which inhibited growth of the test organism. Treatment with folic acid produced a rapid response with an increase in platelets and white cells. A bone marrow aspiration repeated 9 days later was normoblastic.

8.1. Parenteral Nutrition

Many patients in intensive care units receive intravenous nutrition, which is a mixture of various amino acids, sorbitol, and ethanol. In a study of 30 patients with normal preoperative folate levels who were operated on for gastrointestinal disease, the serum folate fell within 48 hours by 60–95% in 20 patients receiving intravenous nutrition (W24). Seven patients had a megaloblastic bone marrow. Daily treatment with 0.5 mg of folic acid given intravenously prevented any clinical signs of folate deficiency. These patients received between 100 and 150 g of ethanol daily as part of their parenteral nutrition, and this may have played a significant role in the development of folate deficiency. However, Tennant (T12) examined this possibility and found that acute depression of the serum folate concentration occurred with both alcohol-free and alcohol-containing preparations used for parenteral nutrition. It was also noted that only one particular brand of

preparation for parenteral nutrition appeared to cause this acute form of folate depletion, and, although there is yet no strong evidence, it seems likely that it is influenced by the composition of the amino acids in the preparation (T13). Methionine and its *S*-adenosyl derivative are known to be involved in the regulation of 5-methyltetrahydrofolate (K8) and the oral administration of methionine has been shown to result in acute depression of serum folate (C15). However, this was shown not to be the cause of the folate depression in patients receiving parenteral nutrition (T13). The problems associated with this acute form of folate deficiency can be avoided by giving appropriate supplements.

9. Folate and Malignant Disease

Patients with malignant disease, particularly in relapse, have been found to have an abnormality of folate metabolism. This may be due to an increased demand such as might occur in the presence of a rapidly growing tumor, or to interference with the production of enzymes required in the processing of folate. The folate concentration within the tumor tissue may also be important.

Saleh (S34) found that patients with malignant disease excreted less radioactivity in their urine following an oral dose of radiolabeled folate than did control patients without malignant disease. It was thought that the remaining radioactivity was taken up into the tumor tissue (S35) and this was supported by the finding of an inverse correlation between the approximate tumor mass and the level of urinary radioactivity. These patients also appeared to incorporate more of the administered folic acid into the reduced folate pool. These changes became more pronounced in the patients with the more advanced malignant disease.

Studies of the differing patterns of folate coenzymes in tumor tissue have shown that there tends to be more 10-formyltetrahydropteroyltetraglutamic acid than 5-methyltetrahydropteroyltriglutamic acid (P12). This may explain the increased level of formyltetrahydrofolate said to be present in the plasma of patients with malignant disease (R13).

Malignant disease may also be accompanied by an increase in total and unsaturated folate-binding capacity. In a study of 77 patients with solid tumors, it was found that 31 with lung cancer had an increased serum level of folate-binding protein and a similar pattern was found in patients with breast cancer. However, patients with cancer of the gastrointestinal tract had no increase in folate-binding capacity (C16). Surgical removal of the tumors did not significantly change the total folate-binding capacity. The folate-binding capacity of lung tumor tissue has previously been found to be higher than

normal tissue (D19), and this may be the source of the raised level of serum binder in these patients.

Impaired absorption has been found in some patients with malignant disease, but this is probably related to active disease close to or actually involving the small intestine (P3, K9). Little information is available on folate metabolism in patients with hepatic carcinoma and its effect on the recycling of folate in bile.

Most of the folate abnormalities observed have been associated with active disease. Patients in remission usually have a normal serum folate provided their dietary intake is adequate. Folate depletion associated with active neoplastic disease is in the main due to a poor diet and this may be made worse by an increased demand due to rapidly growing tissue. Although up to 85% of patients with malignant disease have been found to have a low serum folate, most have had a normal erythrocyte concentration of the vitamin (M15).

10. Other Causes of Disordered Folate Metabolism

10.1. Malaria

Serum folate is often low in malaria and this probably results from a combination of pyrexial illness and associated hemolysis. In a few instances the antimalarial drugs may also have an effect. Antifolates such as pyrimethamine which are being increasingly used in the treatment of malaria do depress folate metabolism, and in patients already depleted this can result in the development of a megaloblastic anemia. Usually, however, the dose used is not sufficient to cause problems in the majority of people. Folate supplements have been shown to have a beneficial effect with higher reticulocyte, white cell, and platelet counts than were seen in patients treated only with antimalarial drugs (T14). Treatment with folate does not interfere with the effectiveness of the antimalarial therapy since the parasites are in the main only able to use endogenous folate. For this they require paraminobenzoic acid, and sulfadoxine may be used to inhibit the incorporation of paraminobenzoic acid in the early stages of folate synthesis.

10.2. Rheumatoid Arthritis

Serum folate levels have been found to be significantly reduced in patients with rheumatoid arthritis. In a study of 77 patients, 42 with rheumatoid arthritis and 35 with osteoarthritis, Sanderson (S36) found the serum folate to be lower in the group with rheumatoid arthritis to a significant degree

($p < 0.001$) and in eight the level was below the lower limit of the normal range. Other workers have reached similar conclusions. Omer and Mowat (O2) found a low serum folate in 24 of 37 patients with rheumatoid arthritis and 38% of these also had a low red cell folate. Gerber and Gerber (G7) noted an abnormality of histidine catabolism in patients with rheumatoid arthritis and it has been suggested that an increased excretion of formiminoglutamic acid together with a low serum folate may indicate a primary abnormality within the histidine pathway. This is unlikely and it seems that there is an increased frequency of folate deficiency in this disease which may occasionally be severe enough to result in the development of a megaloblastic anemia, although this complication is rare. The drugs used to treat this disease may also have an effect on folate metabolism. Alter (A12) observed that patients with rheumatoid arthritis who were being treated with aspirin had an abnormally rapid plasma clearance of radiolabeled folate and a significant reduction in the binding of serum [^{3}H]folic acid. A similar effect was obtained by giving normal subjects aspirin and repeating the experiment.

10.3. Folate and Infection

Many patients with infection have a reduced serum level of folate, particularly those with chronic bacterial infections. However, the development of a megaloblastic anemia is uncommon and when it does occur is perhaps more often associated with the treatment. It is probable that the folate deficiency is the result of a combination of factors including poor dietary intake, low reserves, an increased demand due to an increased cell turnover, impaired absorption, vomiting, and impaired metabolism due to the toxic state of the patient (C17, M16, W25). Pyrexia may also inhibit the reduction of folate. Panders and Rupert (P13) found that if folic acid was incubated with a chicken liver enzyme preparation at an elevated temperature the reduction of folic acid to tetrahydrofolic acid was inhibited.

Folate deficiency has been shown to depress cell-mediated immunity, and in deficient rats the antibody response to inoculated human red cells was found to be severely depressed (L12, W26). Youinou and his colleagues (Y2) studied the phagocytic activity of polymorphonuclear leukocytes taken from a group of critically ill patients. They found that the phagocytic activity was significantly lower in the cells from the folate-deficient patients and this could be corrected by the addition of plasma. There appeared to be a correlation between the level of folate in the plasma and the phagocytic activity of the leukocytes. This was an interesting result because one would not have thought that folate could have had a direct effect on a defective cell. Kaplan and Basford (K10) also studied the effect of folate on neutrophil function and found that folate deficiency had no effect on neutrophil phagocytic activity.

Whether folate deficiency predisposes to infection is not clear. Certainly many folate-depleted patients develop infections and there is little doubt that infection tends to place further demands on folate reserves.

10.4. Folate and Sickle Cell Anemia

Reduced serum folate concentrations have been reported in patients with sickle cell anemia. In a study of 22 patients the concentration was found to be significantly lower than in hematologically normal controls and in five of these patients it was below the lower limit of the reference range. However, none had any other signs of folate deficiency such as hypersegmentation of the neutrophils or a megaloblastic bone marrow (P14). Liu (L13) measured the serum and erythrocyte folate concentrations in 61 patients with homozygous sickle cell disease; he found that the mean erythrocyte folate was raised (521 μg/liter) but that eight patients had an abnormally low level. The high level could be expected in a young cell population.

Patients with homozygous sickle cell disease (SS) had a mean serum folate of 5.8 μg/liter compared with 7.2 μg/liter in patients with sickle cell trait and 7.9 μg/liter in healthy controls. However, there was no correlation between the serum folate and the hematocrit or reticulocyte count. Since reticulocytes may have a higher folate concentration than mature erythrocytes, Liu found that the erythrocyte folate, measured before and after removal of the reticulocytes, was a reliable indicator of the folate status in patients with sickle cell disease despite the variable degree of reticulocytosis. Using this technique only one of nine patients was found to have a low erythrocyte concentration of the vitamin. Treatment with folic acid resulted in higher hematocrits in three of four patients with low serum and erythrocyte folate concentrations, but only one of 12 patients with a normal folate concentration showed any improvement when treated with folate.

There appears to be an increased requirement for folate in some patients with sickle cell disease, but this depends on the degree of hemolysis present and many patients seem able to maintain a normal folate profile.

10.5. Thalassemia

The demand for folate is increased in people with thalassemia because of a more rapid cell turnover due to ineffective erythropoiesis. Where this demand cannot be met, a megaloblastic anemia may be superimposed on the thalassemia (R14, G8). This may be difficult to recognize because the abnormal synthesis of the globin moiety of hemoglobin may prevent the development of characteristic megaloblasts. A similar situation may occur in iron-deficiency anemia where characteristic megaloblasts will not develop until

the patient has been treated with iron. Castaldi (C18) examined the plasma and erythrocyte folate concentrations in 41 symptom-free β-thalassemia heterozygotes. It was found that both the serum and erythrocyte folate were significantly lower in the thalassemics as compared with 21 controls. No correlation was found between the hematocrit and plasma or erythrocyte folate levels. It would seem from this that even in symptom-free patients with thalassemia there is an increased demand for folate. There may be a case for treating these patients, some of whom appear prone to chronic folate deficiency, with prophylactic folate (V2).

11. Folate Nutrition in the Elderly

There have been a number of studies on folate nutrition in the elderly and in this article they have been separated from the nutritional folate-deficiency anemias because in the elderly folate deficiency is a special problem. The elderly are particularly prone to folate deficiency because, not only do many have only a moderate to poor diet which may result from poor circumstances or a lack of interest in food, but they appear to utilize the vitamin less efficiently. As a result they may have an increased requirement.

Absorption of the vitamin may be a problem in the aged. Baker and his colleagues (B22) measured the absorption of folate, vitamin B_6, pantothenate, and riboflavin from a natural food source (yeast), and synthetic pteroylmonoglutamic acid in 24 elderly sugjects all over 70 years of age and in 12 younger subjects under 42 years of age. All subjects showed good absorption of riboflavin, vitamin B_6, and pantothenate. However, the pteroylpolyglutamates present in yeast proved to be a poor source of folate for the elderly subjects, although synthetic pteroylmonoglutamate was well absorbed. The yeast was a satisfactory source of folate for the younger subjects.

Baker suggested that this inability to release and absorb conjugated folates present in food may induce changes in the epithelial structure and enzyme secretions in the intestine which may further exacerbate folate malabsorption.

Varadi and Elwis (V3) found low erythrocyte folates in 10 of 81 hospital patients over the age of 70 years. They were suffering from a variety of disorders which included senile dementia, cerebral thrombosis, and arthritis. None had a megaloblastic anemia. Thirty nine percent of aged people admitted to the hospital in the London area were found to have a low serum folate (H29). Read (R15) in another study found that 40 (78%) of 51 people admitted to an "old peoples' home" were folate deficient with a serum concentration below 6 μg/liter and in three the concentration was below 3 μg/liter. In an apparently healthy control group of similar age all had levels

above 3 μg/liter and in 70% it was greater than 6 μg/liter. After 6 months in the home the formiminoglutamic excretion returned to normal in 17 of 23 patients but there was no increase in the serum folate levels.

Elderly people living at home were found to consume approximately 145 μg of folate daily compared with 223 μg consumed by healthy young controls. The serum folate concentrations in the two groups were similar (H30). Nevertheless, the lower intake in the elderly is likely to make them more susceptible to stress. Some old people living at home have been found to have a diet which consisted mainly of tea and biscuits or one or two slices of bread daily. Some are mentally disturbed and others may have been recently bereaved and are seriously depressed. These people may present for the first time with a profound megaloblastic anemia.

Not all studies have found a deficiency of folate in the elderly. MacLennan (M17) concluded that folate deficiency was rare in the elderly people that they studied and occurred in only 1% of their subjects.

Mental dysfunction in folate-deficient elderly people has been reported, but it is not easy to determine cause and effect. Folic acid has been found to correct the dementia in some patients (M18, S37). Sneath (S38) found no correlation between mental assessment score and serum or erythrocyte folate activity. However, they did find a correlation between mental assessment scores and erythrocyte folate in those patients who had low erythrocyte folate values.

12. Toxicity of Folic Acid

There are only a few reports of folic acid having a toxic effect in man, and this is surprising in view of its very widespread use. A number of patients having a hypersensitivity reaction to the vitamin have been described. Woodliff and Davis (W27) saw three patients with such a reaction. One was a housewife who had been told in 1955 that she had pernicious anemia and had been treated with liver preparations and vitamin B_{12}. Investigation showed that she did not have pernicious anemia but her serum folate was at the lower end of the normal range. Because of this a course of folic acid was recommended. Immediately after taking one tablet the patient began to feel peculiar and within a few minutes had developed generalized erythema and pruritus. Within 15 minutes she attended her doctor with generalized erythema and urticaria and was given adrenaline by injection and an antihistamine by mouth. The reaction took several hours to subside. On questioning she stated that she had a similar, but less severe reaction several years previously after taking a multivitamin preparation. Intradermal skin tests using 10 μg of sodium folate, formyl tetrahydrofolate, and aminopterin

produced a marked reaction and within 5 minutes the patient complained of itchy arms. After 20 minutes a weal surrounded by an area of erythema 40 mm in diameter had developed at the site of the folic acid injection. The reaction to folinic acid and aminopterin was similar but less marked. The patient showed no reaction to methotrexate or saline. A severe generalized urticarial rash was seen in a 9-month-old Indian baby who was given a 5 mg tablet of folic acid and further studies confirmed that the child was sensitive to the synthetic form of the vitamin. The child's mother had a history of urticaria (M19). Sesin and Kirschenbaum (S39) described a 36-year-old anephric man who began taking 1 mg of folic acid but had to discontinue because of pruritus. In a subsequent admission to hospital he was again given a 1 mg tablet of the vitamin and became pruritic and febrile, with a temperature spiking to 39.7°C. In spite of his reaction, treatment with folic acid was continued for some time. He was eventually shown to be sensitive to the vitamin on the basis of intradermal skin testing.

In a study on the effect of pharmacological doses of folic acid, 15 mg was given orally to 14 volunteers for a period of 1 month. They complained of gastrointestinal and sleep disturbances, malaise, excitability, and vivid dreaming (H31). The trial was without controls and repeating the study using a double-blind technique failed to show adverse effects in any of the volunteers (H32).

Methyltetrahydrofolate has been reported to have a neurotoxic action on rat cerebellum. Spector (S40) found that methyltetrahydrofolate had kainate-like neurotoxic properties. Roberts and his colleagues (R16) observed that following the intracerebellar injection of 250 nmol of methyltetrahydrofolic acid in rats there was a substantial loss of Purkinje cells and a reduction in associated markers for functions mediated by γ-aminobutyric acid. The time course of degeneration following methyltetrahydrofolic acid was much slower than occurred with kainate and other neurotoxic amino acids. It has also been found that pteroylglutamic acid and 5-formyltetrahydrofolic acid are substantially more powerful than methyltetrahydrofolate in producing a limbic seizure/brain damage syndrome (O3). Since folates are required for normal cerebral function it appears that its brain-damaging effect is a dose-related phenomenon. It has been shown by others that folates can have a convulsant action (O4), and it is known that epileptics given folate may suffer an increase in their fit frequency, although little work has been done to determine whether folates have a role in human epilepsy.

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