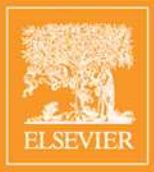

ADVANCES IN CLINICAL CHEMISTRY

Volume 16

Oscar Bodansky &
A. L. Latner

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VOLUME 16

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Advances in CLINICAL CHEMISTRY

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VOLUME 16 • 1973

ACADEMIC PRESS
NEW YORK AND LONDON

A Subsidiary of Harcourt Brace Jovanovich, Publishers

ACADEMIC PRESS, INC.
111 Fifth Avenue, New York, New York 10003

United Kingdom Edition published by
ACADEMIC PRESS, INC. (LONDON) LTD.
24/28 Oval Road, London NW1

LIBRARY OF CONGRESS CATALOG CARD NUMBER: 58-12341

PRINTED IN THE UNITED STATES OF AMERICA

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PREFACE

Problems associated with therapy are becoming of increasing importance to the clinical chemist. Not only must he be in a position to estimate drug levels in the blood and other tissue fluids but must also constantly be aware of the fact that drug administration can interfere with many analytical procedures commonly performed. In this volume of *Advances in Clinical Chemistry,* the Editors have encouraged the inclusion of review articles which deal with such considerations. The aim of the series has always been the description of reliable diagnostic and prognostic procedures, as well as the elucidation of fundamental biochemical abnormalities underlying disease. Therapeutic aspects have now been added. In spite of the considerable advance of technological knowledge in our subject, the Editors consider that this addition will increase the value of the series.

In his review, Schwartz gives a detailed account of factors, including drugs, which interfere in analytical biochemical procedures used for diagnosis. It is only relatively recently that such considerations have been realized to be of great importance and it is now generally accepted that information of this kind will add much to the assessment of laboratory results in regard to reference values, which can be used in the diagnostic process.

Marks, Lindup, and Baylis have presented a full review of the general principles involved in the measurement of therapeutic agents in blood as well as the difficulties which may arise not only in regard to the analytical procedures but also to their interpretation and the actual use they may have in aiding therapy. The relationship of drug blood levels to therapeutic response is still not well understood. Difficulties also arise in regard to such factors as the absorption of therapeutic agents, their binding by serum proteins, and their metabolism.

In the review dealing with the proteins of plasma lipoproteins, Scanu and Ritter have produced a most lucid and detailed discussion of recent advances in this difficult field. An exciting account is given of the isolation of apolipoproteins, their chemical structure, and what is known of their biological functions; lipoproteins relevant to patients with dyslipoproteinemia are also described.

McFarlane has provided a full account of immunoglobulin levels in populations of subtropical and tropical countries, in both health and disease. In the light of current world affairs, it is now highly important

for us to know as much as we possibly can about health hazards in the developing countries. Not only because of this consideration, but also because of its detailed content, this review must, therefore, be considered an important contribution.

The subject of bilirubin conjugation in the liver is becoming increasingly complicated and its biochemical investigation is fraught with many difficulties. Heirwegh, Meuwissen, and Fevery have discussed these and have included an admirable account of the more recently recognized bilirubin conjugates and the clinical application of such investigations. There seems little doubt that more such applications will arise in the not too distant future.

As always, it is indeed a pleasure to thank our contributors and our publisher for their excellent cooperation, without which this volume would not have been possible.

OSCAR BODANSKY
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INTERFERENCES IN DIAGNOSTIC BIOCHEMICAL PROCEDURES[1]

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1. Introduction

There has been great concern recently about interferences in biochemical analysis. In an editorial on the subject it was stated, "aberrations in the results of laboratory determinations caused by drugs clearly set traps that make it possible for all of us to do harm to humans who come for help" (B25). Numerous attempts have been made to collate the effects

[1] This work was supported in part by Grant CA-08748 from the National Cancer Institute, National Institutes of Health.

of drugs on biochemical tests (A5, A7, C5, C8, C10, E1, G1, H2, L9, M14, N1, P2, W12). The most ambitious of these has developed from a 9000-entry computer file based on 1030 references that has been used to assist in the interpretation of abnormal test results in the laboratories of the Clinical Pathology Department of the Clinical Center of the National Institutes of Health (Y2). The task of preparing such lists is formidable since there are over 130,000 drug entries, drug combinations, and drug dosage forms listed in the Physician's Desk Reference (L1). In an appendix to a review on the interpretation of serum protein-bound iodine, over 500 drugs are listed that have been reported to affect, or could potentially affect, tests of thyroid function (A1). A major problem facing the user of collations of laboratory interferences is the fact that drug effects are related to the concentration of the drug and the possible synergistic effects of several drugs. This latter possibility is emphasized by one study of hospitalized patients receiving a new type of penicillin in which it was found that no patient received fewer than 6 other medications, and one patient received as many as 32 additional drugs (L2). In another report, 78 of 100 patients who presented themselves at an outpatient laboratory were being administered a total of 137 drugs (M16). Forty-one of these patients were taking drugs that could possibly create 58 drug-diagnostic test interferences. Two hundred twenty-six biochemical assays were done on these patients, and 18 values were obtained that could have been the result of drug interferences. Of interest is that in 12 of 17 patients in whom the biochemical testing was a part of cardiovascular evaluation, the values could have been related to drug interference (M16).

The metabolic and clinical condition of the patient also plays a role The individual with good kidney and liver function will respond quite differently to a drug than the patient who is not able to detoxify or excrete the compound. The user of laboratory data must be aware of the possible occurrence of drug-mediated enzyme induction, biological variations in the rate of clearance and storage in depot areas (B24), and the genetic factors that play a role in the toxicity of a drug (L2).

In addition to these and analytical problems, users of laboratory data must also be aware of possible changes in laboratory values due to the lability of the substances in the collected specimens, the effect of anticoagulants, dietary and environmental effects, age and sex on the measured concentration.

There are a number of articles in which attempts have been made to define the mechanisms of specific laboratory interferences, the role of sample quality on the analysis, and the effect of biological variation in the reported values (C2, C3, C4, L6, S8, S30, W11). The clinical chemist

will also be interested in a detailed review of drug effects on certain hematological tests including the determination of prothrombin time, bilirubin, folates, serum copper, iron, and ceruloplasmin (S31).

The purpose of this review is to consider these factors and their relationship to each other in the interpretation of the apparent value.

2. Specimen Quality

2.1. Anticoagulants

A universial anticoagulant does not exist, and it is therefore necessary to use, in clinical chemical analysis, serum or plasma prepared with a variety of anticoagulants. The role of the anticoagulant and preservatives on the observed value has been reviewed by Caraway (C2, C4) and by Winsten (W11).

Serum is the preferred sample for clinical analysis, but it must be kept in mind that serum or plasma concentrations may not reflect the amount of a substance in whole blood, because of possible differences in distribution between the erythrocyte and its surrounding fluid and the fact that the water content of plasma is much greater than that of whole blood (C2). Glucose, which distributes itself in the water phase of blood, exhibits plasma values about 13% higher than the equivalent whole blood values (L6). However, the ratio between plasma and whole blood glucose in a group of subjects varied from 0.96 to 1.22. The difference in glucose concentration between venous and capillary blood was small in the fasting state, about 2 mg/ml in 28 normal subjects. Forty-five minutes after a 50 g glucose load, the mean venous-capillary difference in these individuals was 24.8 ± 12.6 mg/100 ml (L6).

The problem of glucose analysis in serum specimens without preservative is an important one since automated multiple analysis instruments usually require a serum sample. In the traditional method of collecting blood for glucose analysis oxalate is used as an anticoagulant and fluoride (10 mg/ml) as an antiglycolytic agent. This fluoride concentration preserves glucose in whole blood for 8–12 hours at room temperature and 48 hours in the refrigerator (W11). It has no effect on glucose oxidase (H11), but does inhibit urease (C4).

The importance of the antiglycolytic agent is accentuated in the busy laboratory, where several hours may elapse between collection of the specimen and laboratory processing, and even longer when specimens are mailed to central laboratories some distance from the hospital or doctor's office where the specimen was collected (R4). In one study, mean glucose values determined in serum obtained and analyzed within 30 minutes after collection were 96 mg/100 ml but they were 86 mg/100 ml in

serums separated after 2 hours on the clot. The greatest change in an individual specimen was 33 mg, and 33% of the samples had a decrease in glucose concentration greater than 3 standard deviations from the mean (H2). Fifty-four percent of the samples showed this extent of change in 4 hours. We have observed marked decreases in glucose concentration in blood collected without an antiglycolytic agent from patients with elevated white counts (Table 1). The specimens were collected and processed in our usual fashion (1–2 hours between collection and analysis).

Oxalate does not interfere with glucose assays, but insulin values determined in oxalate-plasma are lower than those obtained with lithium heparin-plasma or serum (L6). Specimens collected in EDTA demonstrate lower carbon dioxide combining power than those observed with serum or heparin or potassium oxalate plasma (Z1).

Enzymes activities are particularly sensitive to the anticoagulant used in collecting the specimen. Heparin inhibits acid phosphatase (W16) and muramidase (Z5). Amylase activity is inhibited by oxalate or citrate (M10), and lactic dehydrogenase and acid phosphatase lose activity in oxalate (C2). Alkaline phosphatase is stable in oxalate, oxalate-fluoride, or heparin, but 25 m*M* citrate inhibits 50% of the activity, and as little as 50 m*M* EDTA is completely inhibitory (B19). Leucine aminopeptidase is inhibited by EDTA, as is creatine phosphokinase (F3). Amylase activity has been reported to be only 83% of that in serum when oxalate or citrate-plasma is used (M10). Heparin plasma appears to have no inhibitory effect. Despite the fact that clotting factor V is not stable in oxalate or EDTA, these are often used as anticoagulants to obtain plasma for prothrombin determinations (Z2, Z4).

Isoenzymes of 6-phosphogluconate dehydrogenase determined by elec-

TABLE 1
GLUCOSE VALUES IN BLOOD COLLECTED WITH AND WITHOUT FLUORIDE

Subject	Glucose concentration (mg/100 ml)	
	Without fluoride	With fluoride
1	62	112
2	95	160
3	56	78
4	192	222
5 day 1	50	90
day 2	18	77
day 3	18	80
day 4	28	87

trophoresis on starch gel, but not on agarose, differ according to the anticoagulant used to collect the blood. Several additional enzymatic bands of different mobility are observed with hemolysates prepared from heparinized blood, but not blood collected in siliconized tubes, or in the presence of sodium citrate or EDTA (B9). It was postulated that the effect is due to the formation of protein–heparin complexes which affect the molecular sieving of the starch.

2.2. Hemolysis, Leukolysis, or Jaundice

Many constituents, such as magnesium, potassium, phosphorus and enzymes, are present in formed elements of the blood in concentrations many times higher than in the surrounding plasma and therefore lysis of the cells can contaminate the plasma or serum to a measurable amount. There is 25 times more lactic dehydrogenase, 160 times more phosphohexose isomerase, 20 times more potassium or magnesium, 80 times more isocitric dehydrogenase (W14), and 450 times more 6-phosphogluconic dehydrogenase (W14) in erythrocytes than in plasma. In addition, the red cell can release material that interferes in the analytical procedure by contributing color to the reaction, as occurs in the biuret determination of protein, or direct interference in a chemical reaction, as in uric acid assays, which depend on phosphotungstate reduction. In addition to these effects, hemolysis produces an effective dilution of plasma or serum, and observed serum values of constituents, such as sodium or chloride, which are present in low concentrations in erythrocytes will be low.

Brydon and Roberts added hemolyzed blood to unhemolyzed plasma, analyzed the specimens for a variety of constituents and then compared the values with those in the unhemolyzed plasma (B28). The following procedures were considered unaffected by hemolysis (up to 1 g/100 ml hemoglobin); urea (diacetyl monoxime); carbon dioxide content (phenolphthalein complex); iron binding capacity; cholesterol (ferric chloride); creatinine (alkaline picrate); uric acid (phosphotungstate reduction); alkaline phosphatase (4-nitrophenyl phosphate); 5′-nucleotidase (adenosine monophosphate-nickel); and tartrate-labile acid phosphatase (phenyl phosphate). In Table 2 are shown those assays where increases were observed. The hemolysis used in these studies was equivalent to that produced by the breakdown of about 15×10^6 erythrocytes. In the bromocresol green albumin method it has been reported that for every 100 mg of hemoglobin/100 ml serum, the apparent albumin concentration is increased by 100 mg/100 ml (D12). Hemolysis releases some amino acids, such as histidine, into the plasma (A1b).

Insulin concentrations determined by radioimmune assays are lower

TABLE 2
EFFECT OF HEMOLYSIS ON BIOCHEMICAL VALUES[a]

Constituent	Method of analysis	Increase in value (ratio of increase per gram of hemoglobin to value in nonhemolyzed specimen)
Aspartate aminotransferase	Colorimetric (nitrophenyl hydrazine)	2.3
Alanine aminotransferase	Colorimetric (nitrophenyl hydrazine)	2.0
Acid phosphatase	Colorimetric (phenyl phosphate)	6.3
Bilirubin	Diazotization (no blank)	3.2
Creatine phosphokinase	Ultraviolet, coupled	2.0
Lactic dehydrogenase	Ultraviolet (NADH)	5.5
Potassium	Flame photometry	1.7
Hydroxybutyrate dehydrogenase	Ultraviolet	6.1
Phosphate	Phosphomolybdate reduction	1.3
Total protein	Biuret	1.1
Albumin	Bromcresol green binding	1.1
Iron	Tripyridyltriazine	1.2
Magnesium	Atomic absorption	1.1

[a] Ratios calculated from data of Brydon and Roberts (B28).

in hemolyzed than in nonhemolyzed plasma. This has been attributed to the ability of red cell constituents to degrade insulin rather than an effect of hemolysis on the assay (C1). An enzyme in the erythrocyte membrane cleaves the sulfur–sulfur bond between the two peptide chains of insulin and lowers the radioimmune-responsive material (B23). In 10 minutes, the insulin in a freshly hemolyzed specimen was reduced from 50 ng/ml to 10 ng/ml.

Creatine phosphokinase activity has been reported to be minimally inhibited by hemolysis. Hemoglobin concentrations of 1.25 g/100 ml inhibit 5% and 2.5 g/100 ml, 12% (N5). However, in methods utilizing adenosine diphosphate in the reaction mixture, hemolysates containing 100 mg of hemoglobin per 100 ml may have apparent activities of 5–100 units/liter. The activity is presumably related to adenylate kinase in the erythrocyte (S33). In methods utilizing adenosine diphosphate in a coupled enzyme reaction with hexokinase and glucose-6-phosphatase, the inhibitory effect can be eliminated by adding sufficient adenosine mono-

phosphate to inhibit the adenylate kinase activity (W9). Serum lipase activity is stated to be inhibited by extensive hemolysis (Y1).

Chronic *in vivo* hemolysis produces serum lactic dehydrogenase elevations in patients with mitral or atrial valve cardiac prosthesis (J2). In a series of 11 such patients these increases ranged from 1.1 to 1.6 times the upper limit of normal (S29). Blood pH is altered in hemolyzed specimens because carbonic anhydrase is liberated from the erythrocytes and presumably alters the distribution of H_2CO_3 and $NaHCO_3$ (B2). Hemolysis will effect acid phosphatase activity if the substrate is hydrolyzed by erythrocyte acid phosphatase. Thus, hemolysis would be of concern if phenyl phosphate was the substrate, but would have a negligible effect if β-glycerophosphate, which is not hydrolyzed by red cell acid phosphatase, was used (B1).

Hemolysis plays an important role in direct spectrophotometric analysis of bilirubin. In a method where the ratio of unconjugated to conjugated bilirubin is determined by spectrophotometer readings at several wavelengths, the percent of unconjugated bilirubin ranged from 100% in a sample without hemoglobin to 88% in the presence of 38 mg/100 ml of hemoglobin and 62% when 86 mg/100 ml of hemoglobin was added (B21). This is of particular importance in direct spectrophotometric assays of bilirubin in newborn infants where the blood sampling procedure makes hemolysis inevitable. In the colorimetric determination of bilirubin by a modified Malloy and Evelyn procedure, it was observed that hemolysis (2 to 3×10^{-4} g of hemoglobin) reduced apparent bilirubin concentrations from 5 to 15% (M9).

Bilirubin effects depend on the method used for analysis. Interferences in direct serum protein methods are observed at bilirubin levels greater than 5 mg/100 ml (K7). A sample containing 20 mg of bilirubin per 100 ml increased the apparent total protein by 0.2 g/100 ml. Concentrations of bilirubin as high as 20 mg/ml do not effect albumin assays using bromocresol green binding (D12), but have a marked effect on these assays when [2-(*p*-hydroxyphenylazo)-benzoic acid] (HABA) dye is used (A7b).

Bilirubin reacts with the Liebermann-Burchard reagents to form a green chromagen with an absorbance 5–9 times as great as an equivalent concentration of cholesterol. Ferric chloride reagents exhibit this type of interference, but to a lesser extent (B3). In the direct Liebermann-Burchard AutoAnalyzer reaction there is a 4.5 mg/100 ml increase in "cholesterol" for each milligram of bilirubin added to a standard assayed by the Abell method (M13). However, in 19 serums with pathologically increased levels of bilirubin, 8 showed no increase in cholesterol, 6 increases of 6 mg/100 ml in cholesterol per milligram of bilirubin, and 5

increases of from 6.6 to 30 mg/100 ml cholesterol per milligram of bilirubin when compared to the reference method cholesterol values (M13). The addition of 15 mg/100 ml of bilirubin to plasma specimens increased the apparent glucose concentration to 134% of the control when a direct *o*-toluidine method was used, decreased it to 93% when a hexokinase–glucose-6-phosphate dehydrogenase system was used and to 97% with an automated ferricyanide method (W17).

Lysis of formed blood elements other than erythrocytes may produce elevations in serum or plasma constituents. Platelet breakdown during blood collection can introduce enzymes into the plasma (Z3). Aldolase activity is very high in platelets (D1), and elevations of acid phosphatase in myeloproliferative disease are probably the result of platelet lysis (B6).

Serum muramidase (lysozyme) is derived mainly from the degradation of granulocytes (F3). Granulocyte phosphohexose isomerase activity is three times that of leukocytes and accounts for elevations of the serum activity of this enzyme in leukemia (I1). Neutrophile leukocytes possess 10,000 times the serum arginase activity (T1a) and serum glucose-6-phosphate dehydrogenase and glutamate dehydrogenase activity (M2a, W1a).

2.3. Stability of Constituents

The question often arises whether a sample must be analyzed immediately or can be stored, and if so, under what conditions and for how long (B4a, H5a, W9a). Freshly drawn blood maintained anaerobically (A3) at 38°C decreases in pH at the rate of -0.062 unit per hour and in pCO_2, at 4.8 ± 1.3 mg Hg per hour. At 0–4°C, the change is minimal; -0.006 ± 0.004 pH unit and 0.6 ± 0.06 mm Hg. There has been controversy concerning the use of minerol oil to maintain specimens for carbon dioxide analysis (G2). Paulsen found that values of total carbon dioxide in plasma collected in stoppered tubes with and without paraffin oil were identical if the tubes without oil were completely filled to the stopper (P4). The loss of carbon dioxide in tubes stored at room temperature without oil was about 6 mEq/l in 2.5–4 hours. The problem for the laboratory is unfilled tubes and the storage of separated serum or plasma before analysis and in plastic cups during continuous-flow procedures.

Gambino and Schreiber (G3) found that plasma in an open Auto-Analyzer cup lost 1.5 mEq/l of carbon dioxide in 15 minutes; 4 mEq/l in 30 minutes and 5.5 mEq/l in 60 minutes. Thereafter, the loss plateaued and remained at 7 mEq/l. This loss was prevented by alkalization of plasma by the addition of 1 drop (0.035 ml) of 1 *N* ammonium hydroxide

to 1 ml of plasma (G3). Our experience has been that the carbon dioxide content of serum can be preserved by collecting the blood in vacuum tubes containing mineral oil, transferring the serum from the clot to tubes containing oil and during AutoAnalyzer analysis, layering a thin film of oil over the serum in the cup. This amount of oil does not contaminate the pump tubes or interfere in the analysis. The changes in carbon dioxide content of specimens maintained in small tubes with and without mineral oil is shown in Table 3.

The pH of serum, after it is separated from the clot, tends to go up as carbon dioxide is evolved and will eventually reach pH 8.5. This is an important consideration in the determination of acid phosphatase, which is rapidly destroyed at alkaline pH (W16a). Eighty-nine percent of serum acid phosphatase activity remains after 4 hours at pH 7.8 or 1 hour at pH 7.98 (B2). At pH 7.98 only 58% remains after 4 hours (B2). If serum is maintained over the clot, no rise in pH or inactivation of acid phosphatase occurs for as long as 5 hours at room temperature or 24 hours in the refrigerator (B17b).

Enzymes have different degrees of stability after their collection. Alkaline phosphatase demonstrates up to 10% increased activity after a few hours at room temperature (B15). Most enzymes are not stable at room temperature, but can be preserved in the refrigerator for short periods or in the deep freeze for relatively long times. In Table 4 are tabulated the reported stabilities of many serum enzymes. It must be realized that the problem of enzyme stability is complicated by the fact that the isoenzymes of a particular enzyme may have different stabilities and that specimens with high activities may react differently than those with normal activities (K10). Although it is indicated in Table 4 that serum

TABLE 3
CO_2 CONTENT IN SPECIMENS STORED WITH AND WITHOUT MINERAL OIL

	CO_2 content (mEq/l)		
		Without oil	
Specimen	With oil 300 Min	30 Min	300 Min
1	20	18	14
2	28	26	22
3	27	27	23
4	24	23	20
5	27	26	22
6	31	30	27
7	27	26	22
8	22	21	18

TABLE 4
MINIMUM STABILITIES OF SERUM ENZYMES

Enzyme	Stability (days)		
	Room temp., 20°–25°C	Refrigerator, 4°–8°C	Deep freeze, −20°C
Aldolase (F4)	2	21	—
Creatine phosphokinase (K6)	0.15	10	60
Phosphohexose isomerase (S13)	0.33–0.50	21	365
Adenosine deaminase (S12)	—	30	30
Acid phosphatase (D1a)	Rapid with increasing pH	—	115
Alkaline phosphatase (W2)	0.33	7	180
Phosphoglucomutase (B16)	0.33	2	4–7
Pseudocholinesterase (S25)	2	14	Months
Ceruloplasmin (H8)	0	2	14
Amylase (H9)	7	7	150 (W2a)
Glutathione reductase (W5)	3	3	7
Lipase (H9, T5)	7	7	7
Isocitric dehydrogenase (W14)	0.25	Many days	—
6-Phosphogluconic dehydrogenase (W14)	—	50% decrease in 4	—
Aspartate aminotransferase (S11)	2	14	30
Alanine aminotransferase (S11)	—	—	11% loss in 1
Leucine aminopeptidase (K6)	1	21	60
Glutamate dehydrogenase (E2)	7	7	14
Lactic dehydrogenase (S9)	0.33	4	20
Arginase (S10)	1	2–3	60
Ornithine carbamyl transferase (K6)	—	—	365

creatine phosphokinase is relatively stable, it was found that a serum with supranormal activity (113 units) lost 21% of its activity after 24 hours at room temperature, 11% in the refrigerator (4°C), and 5% in the freezer (−10°C) (C9). Serum aspartate aminotransferase was stable at −20° for 10 months when activity was in the normal range, but only for 4 months in specimens with high activity (B17a). Pyruvate transaminase was stable in the normal range for 7 months. However, specimens with high activities lost 20% of their activity in one week (B17a).

Ammonia concentrations increased rapidly in stored blood. Blood that contained 160 ± 103 μg/100 ml when it was collected demonstrated 565 ± 188 μg/100 ml after 24 hours and 1181 ± 21 μg/100 ml in 7 days (P10). Specimens for lipoprotein electrophoresis (cellulose acetate) can be stored at room temperature for 3 days, and in the freezer for 14 days (W10). A second freezing and thawing made the specimen unsuitable. In

such specimens from patients with type II hyperlipidemia the β-band migrated too far and appeared in the pre-β-band (W10).

There are stability problems in urines stored for analysis. Fifty percent of delta-aminolevulinic acid was lost in specimens stored without preservative and exposed to light for 24 hours (V3). The loss increased to 80% in 48 hours, 85% in 72 hours, and 95% in 2 weeks. However, the same specimens acidified with tartaric acid and stored in the dark lost 2% of the aminolevulinic acid in 72 hours and 6% in 2 weeks (V3). The destruction of catecholamines collected in nonacidified urine specimens is well documented (C11). Urinary acid phosphatase was destroyed on freezing (S15). The effect was related to increasing salt concentration during freezing and was prevented by the addition of albumin (S15).

2.4. Pneumatic Tube Specimen Delivery

Shipping specimens through a 1423-meter pneumatic tube system with 67 bends did not affect the concentration of sodium, chloride, carbon dioxide, calcium, phosphorus, urea, uric acid, glucose, creatinine, total protein, fibrinogen, and bilirubin if the tubes were filled completely and foam-rubber inserts were used. The mean increase in potassium was 0.11 mEq/l, hemoglobin 7.88 mg/100 ml, and lactic dehydrogenase 50 units. The increases in lactic dehydrogenase ranged from 9 units to 148 units (M4).

In incompletely filled tubes, the effects were much greater and lactic dehydrogenase increased from 113 units in the control to 172 units in a specimen containing 3 ml in a 10-ml tube. Shipping 5 ml of heparinized blood specimens for pH and blood gas analysis did not affect pH or pCO_2, but pO_2 showed modest increases ranging from 0.5 to 4.5 mm Hg (M4). Other investigators have reported similar findings (D6, S29).

3. Specimen Contamination

3.1. Detergent, Glassware, or Plastics

Detergents left as contaminants in washed glassware can seriously interfere with the analysis of phosphate and with the determination of enzyme activities. A concentration of detergent at a level of 0.6–0.8 mg/ml has been reported to inhibit serum malate dehydrogenase 45%, glutamate dehydrogenase 100%, lactate dehydrogenase 30%, acid phosphatase 10–37%, aspartate aminotransferase 16%, and alkaline phosphatase 17–24% (B10). Detergents can be significant sources of contamination in fluorescent analysis, as can be plastics, glass or rubber stoppers, or stopcock grease (R3).

Calcium was a contaminant in some commercially available vacuum

specimen collection tubes (F7). The amount of calcium was reported to be 0.5–14 μg per tube, or enough to produce spurious elevations of 0.5–2%. Cork stoppers have been reported to contaminate serum specimens with calcium. Serums stored in tubes with cork stoppers were found to have calcium levels as much as 9.9 mg/100 ml higher than the original specimen (S24). Soft-glass Pasteur-type pipettes can add 0.1–2.2 μmoles of hydroxyl equivalent and 0.15–0.32 μmole of sodium to a specimen (M7). Serum protein-bound iodine was elevated in specimens collected through a plastic tube (Bard Intracath) presumably owing to an aliphatic iodine compound washed out of the tubing (S20).

3.2. Skin Contamination

We have experienced complete inhibition of the protein-bound iodine (PBI) reaction in a specimen collected through skin previously swabbed with Merthiolate. The mercury in the Merthiolate is a potent inhibitor of the cerium-arsenious acid reaction utilized in the PBI assay. Intramuscularly administered mercurial diuretics also inhibit PBI. Single injections of 78 mg of mercury as the diuretic drug meralluride resulted in low PBI's for 24 hours after injection of the drug (B18). Bodansky described an elevated serum calcium in a child with all the clinical signs of tetany, who obviously should have had a depressed value (B17). Investigation revealed that the house officer who drew the specimen had been about to inject a 10% calcium gluconate, but as an afterthought, before injecting this drug, he had decided to draw blood for a calcium analysis. A small drop might have been spilled on the skin and then drawn into the syringe with the blood; such a drop would contain 0.5 mg of calcium, an amount equal to the concentration in 5 ml of normal blood. Serious errors can occur in methods based on *in vivo* dilution of an intravenously administered substance if blood taken after injection of the drug is removed from the same vein into which the material was infused. This is an important consideration in BSP determinations and the evaluation of blood volume by either Evans Blue dye or radioactive iodine-tagged albumin.

3.3. Parenterally Administered Substances (Direct Interference)

There are numerous substances that are administered intravenously and have a direct effect on biochemical analysis. Obviously, glucose or electrolyte concentrations will be spuriously elevated if the specimen is taken from the same vein into which these substances are being administered. The presence of sulfobromophthalein dye (BSP) in serum or plasma will interfere with protein determined by the biuret method. The

dye is colorless at the pH of the blood, but blue when made alkaline during the biuret assay. In patients on EDTA therapy, calcium cannot be determined by colorimetric methods based on the chelation of a calcium–EDTA complex. However, in calcium determinations by atomic absorption spectroscopy, organic material is destroyed in the flame and the exact concentration of calcium can be determined in the presence of EDTA.

Serum alkaline phosphatase elevations have been reported following administration of salt-poor albumin (B5). Placenta is very rich in a heat-stable alkaline phosphatase, and albumin prepared from placental blood has a high activity of this enzyme. In one cirrhotic patient who received 1–6 units per day of albumin obtained "from pooled human blood and/or human placenta," the alkaline phosphatase before infusion was 5 Bodansky units and by the thirteenth day of administration had reached a value of 160 units. The physician administering the albumin at first thought the patient was having a severe toxic liver reaction and stopped the therapy. The alkaline phosphatase then started to go down and within 10 days returned to normal levels. Analysis of the albumin indicated that it contained 470 units of alkaline phosphatase activity and was probably responsible for the observed elevations in the serum enzyme activity. Albumin prepared from venous blood did not cause an alkaline phosphatase elevation, but placenta-albumin caused elevations with a half-life of about 8 days (M1).

Blood transfusions can also lead to misleading laboratory values. Stored blood bank blood can appreciably increase the blood ammonia levels (P10). Blood stored 7 days has been found to contain over 1100 μg/ml. A case has been reported of a patient receiving massive blood transfusions whose serum contained an additional lactic dehydrogenase isoenzyme (a splitting of the LDH-1 band). The authors concluded that the extra band was not an artifact, but rather represented an abnormal "H" subunit present in one or more of the transfused plasmas (F6).

4. Direct Physiological Effects

4.1. Rhythmic Variations

Rhythmic changes in biochemical constituents have been known for many years, and the terms daily, diurnal, 24-hour, diel, and mycthemeral have been used to describe them (B26). With the realization that these cycles were not always of 24-hour duration and varied from 18 to 33 hours, the term circadian was used (H3), but most recently, "episodic secretion" has been introduced to emphasize the fact that secretory glands experience periods of quiescence and then spurts of activity (H7).

Rhythmic variation can be important in the interpretation of laboratory data. Although ideally it would be appropriate to obtain specimens from all patients at the same time of the day, this is not practical and there are a number of constituents whose values must be reviewed with the realization that the concentration can vary by 100% or more during a 24-hour period.

A rhythmic variation has been observed in levels of plasma hydroxycorticosteroids (A9, B13, D9) and in the excretion of 17-ketosteroids (P7). As shown in Table 5, urinary excretions of potassium, sodium, chloride, 17-hydroxycorticosteroids and water have been reported to be greatest between 10 AM to noon and lowest between 4 AM and 6 AM (S21). In this study it was shown that within 5 weeks subjects could acclimate to similar patterns for a 21-hour, rather than a 24-hour, day. Hellman and his associates reported that about half of the day's cortisol production is achieved in the early morning hours during sleep and that production is minimal between noon and 10 PM (H7). In one study the plasma cortisol in normal men was 24.6 ± 5.5 μg/100 ml at 7 AM; 13.1 ± 3.4 μg/100 ml at 9 AM; 11.8 μg/100 ml at noon; 9.1 ± 2.3 μg/100 ml at 7 PM and 6.3 μg/100 ml at 10 PM (A9).

Although many workers believe that light is the major controller of these rhythms, it has been shown that social cue, rather than the absence of light, is the inducing factor. Subjects maintained in the dark demon-

TABLE 5
EXCRETORY RHYTHMS DETERMINED IN ADULTS DURING A NORMAL DAILY ROUTINE[a]

Time of day	Urinary excretion				
	K^+ (μmole/min)	17-OHC (μg/min)	H_2O (ml/min)	Na^+ (μmole/min)	Cl^- (μmoles/min)
Midnight–2 AM	37	6.8	0.97	112	115
2 AM–4 AM	28	6.5	0.73	97	96
4 AM–6 AM	23	6.5	0.64	85	91
6 AM–8 AM	35	7.7	0.69	95	105
8 AM–10 AM	75	11.4	0.83	124	156
10 AM–Noon	99	12.3	1.33	237	297
Noon–2 PM	97	11.7	1.15	230	299
2 PM–4 PM	71	11.5	1.24	222	275
4 PM–6 PM	51	10.5	1.32	198	241
6 PM–8 PM	45	9.8	0.97	188	235
8 PM–10 PM	47	8.6	0.45	137	143
10 PM–Midnight	39	7.3	0.96	114	121

[a] Simpson and Lobban (S21).

strated urinary excretions of catecholamines, 17-hydroxysteroids and sodium that were 30–40% of the mean in the early morning hours (5 AM) and at maximum levels in the early evening (8 PM) (A8). The circadian pattern has been demonstrated in a totally blind ansomatic patient (W3). 17-Hydroxycorticosteroid excretion is not affected by illness (S18), night work (M12), or total bed rest (C5a). The excretion pattern of 4-hydroxymandelic acid (VMA) is similar during the antarctic autumn or summer. The maximum absolute excretion occurred between 5 PM and 9 PM, but the maximum percentage of the total daily excretion occurred between 1 PM and 4 PM (D3).

Catecholamine excretion is greater in the afternoon than in the morning (V7). In 12 subjects the mean concentration in 2-hour urine specimens collected in the morning was 8.6 μg/100 ml; and in similar specimens collected in the afternoon, 11.9 μg/100 ml (J1). Serum iron concentrations are higher in the morning than in the evening (B20, H5). Each day for 4 days, specimens were drawn from 11 normal individuals at 8 AM, 11:30 AM, 2 PM, and 4 PM. There was great variation in the individual values; in one patient from 45 to 153 μg/100 ml and in another from 67 to 233 μg/100 ml. However, the general pattern was low values in the evening. In one subject the iron concentration was 174 μg/100 ml at 8 AM, 160 μg/100 ml at 11:30 AM, 134 μg/100 ml at 2 PM and 106 μg/100 ml at 4:30 PM. There was an inverse relationship between the total serum iron and the latent iron-binding capacity so that the total iron-binding capacity remained constant. Serum lipids (H13) as well as creatine and creatinine also demonstrate a diurnal rhythm; 7 PM creatine values were 160% and creatinine values 130% of the 7 AM concentrations (P3).

A circadian variation in serum acid phosphatase has been reported in patients with prostatic carcinoma and phosphatase activity determined with phenyl phosphatase as substrate (D10). The nocturnal values decreased 25–50% of the highest day time activity. The highest values were observed from 9 AM to 3 PM and the lowest between 9 PM and 3 AM. In one patient observations were made at hourly intervals. The peak of 14.2 King-Armstrong units was observed at 11 AM and the lowest activity, 6.4 units, at midnight. Orchiectomy did not eliminate the variation.

The development of sensitive radioimmune assays has permitted extensive studies of blood levels of anterior pituitary hormones. These hormones are released episodically and growth hormone (T1), ACTH (W4), and gonadotropins (K2) are secreted at much higher rates during sleep, particularly during the early morning hours. Plasma prolactin and growth hormone concentrations were observed to be lowest between noon and 5 PM. Peak concentrations occurred between 5 AM and 7 AM with a rapid fall within 1 hour after arising (S3). The rapid fall can be of im-

portance in a hospital environment where patients may be awakened early in the morning to permit specimens to be drawn. Prolactin secretion is stimulated by various stresses including hypoglycemia; strenuous exercise, suckling in postpartum women, and administration of psychotropic drugs (F9).

Seasonal variations have been observed in cholesterol values with generally higher concentration in winter than in summer. Thomas and her associates observed the following mean values in a group of prisoners in Maryland: November, December, and January, 260 mg/100 ml; February, 230 mg/100 ml; April, 225 mg/100 ml; and May through October, 220 mg/100 ml (T3). The diets and schedules of the prisoners were essentially the same throughout the year, and the authors associated the increases in November, December, and January with the stress that may exist for prisoners incarcerated and away from their families during holiday periods (T3). However, studies of healthy individuals in Sweden has indicated a somewhat similar pattern in both men and women with a fairly constant level between January and May, but then a decline with a minimum level in July and an increase from August to a peak in November with elevated values in December and January. The maximal differences were 38 mg/100 ml for men and 29 mg/100 ml for women. In a group of Finns mean cholesterol values reached a minimum in June and had increased about 100 mg/100 ml by January (K5). The pattern was attributed to seasonal differences in the diet and physical activity of the subjects (K5).

4.2. Posture and Bed Rest

Posture and bed rest exert an effect on the blood concentration and excretion of numerous substances. This can be of extreme importance in evaluating data in ambulatory outpatients compared to hospitalized individuals. It has been reported that in pregnant women estrogen excretion was increased by lying down or turning to the left side and was decreased by standing (D8). The changes in estrogen excretion were closely related to changes in renal plasma flow and glomerular filtration and paralleled changes in sodium and creatinine excretion (D8). These findings were compatible with the report of lowered serum estrogen during bed rest (R2). An upright posture increased the excretion of norepinephrine up to 3 times that observed in individuals in the lying position (S32), and PBI levels have been found to change about 0.8 μg/100 ml (S23). Cholesterol levels were elevated an average of 17.4 $\pm$ 1.4 mg/100 ml (49 subjects) when persons changed from a recumbent position in bed to a standing or walking position. The change occurred within 15 minutes (P1).

Plasma volume has been observed to decrease an average of 12% when recumbent individuals arose (F2). The effect was particularly acute in patients with edema or low serum albumin concentrations (F2). In this study subjects were maintained in bed for 12 hours, a blood specimen was taken after 1 hour in a completely flat position and after 1 hour of walking activity. It was found that after walking the protein values rose 10% in normal persons and 10–15% in patients with edema. The average increase in albumin was 0.48 g/100 ml (F2).

In healthy men total plasma proteins were observed to rise an average of 0.53 g/100 ml and albumin 0.42 g/100 ml in specimens drawn 15–30 minutes after arising in the morning, as compared to the concentrations in specimens obtained just before arising (A10). The rise was of the same proportion in each of the plasma protein fractions, and therefore the percentage distribution of the fractions did not change. In another study, increases of as much as 0.75 g/100 ml in total protein were observed in active upright persons as compared to the concentration in a specimen from the subject when he was recumbent and at rest (W6).

Bed rest and complete immobilization are known to bring about demineralization of bone (D5a, H15). During a study of 5 healthy men during 12 weeks of complete bed rest, the urinary excretion of calcium rose from 200 mg/day to a maximum of 375 mg/day during the sixth week. The urinary phosphorus rose from 1000 to 1900 mg/day, and the hydroxyproline from 40 mg/day to 50 mg/day. The serum calcium rose an average of 0.2 mg/100 ml, and although the alkaline phosphatase did not rise during the study, there was an abrupt rise following ambulation (H15). In a 14-year-old boy, immobilized from the waist down following a fracture of the neck of the femur, an observed calcium of 14.6 mg/100 ml resulted in an inappropriate diagnosis of hyperparathyroidism. After ambulation there was a rapid fall in the serum calcium (A1a).

4.3. Fasting—Eating

Dietary effects on laboratory results are extremely important from the standpoint of data evaluation. We are all aware of increases in blood glucose following a meal, and these increases have been the basis for tolerance tests. However, simultaneous decreases in potassium and phosphorus during metabolism of ingested glucose are not as well appreciated. After eating, cholesterol values are variable in the sense that the values are higher by as much as 30–40 mg in some individuals, but lower in others and in some persons there is no change. It has been suggested that nonfasting specimens are adequate for an evaluation of serum cholesterol (H5). The effects of eating differ from individual to individual and particularly between hospitalized ill patients and ambulatory healthy per-

sons. If it is desired to monitor small day-to-day changes, it is essential for the patient to be in a fasting state. A misconception that exists about fasting is that "NPO" means without water. Patients who are fasting prior to drawing of blood for biochemical determinations should be allowed as much water as they desire. This consideration is not as important in normal persons as in a hospital, where many patients are debilitated and dehydration can occur rapidly with elevated results produced by a constriction of the vascular space.

A question often arises as to how soon after eating can a specimen be drawn. It is usually assumed that 12 hours are required. In a study of 11 individuals, triglyceride levels were measured at 4, 8, 10, 12, and 14 hours after a breakfast of eggs, sausages, toast, and a beverage (S4). It was found that lowest levels were observed 10 hours after the meal (Table 6). The cholesterol values measured at the same time showed no significant variations (S4). Changes in daily cholesterol intake of 50% per 1000 calories of diet produced an average change of only 5–10 mg/100 ml in serum cholesterol (K3). In the fasting state there was considerable day-to-day variation in the triglyceride values, and over a 5-day period the coefficients of variation ranged from 2 to 20%. In one individual the range was from 111 mg/100 ml to 178 mg/100 ml and in another patient from 315 mg/100 ml to 203 mg/100 ml. The cholesterol changes were less and ranged from 2 to 15%.

During prolonged dieting by obese individuals on very low-calorie diets, there was a marked increase in sulfobromophthalein (BSP) reten-

TABLE 6
TRIGLYCERIDE CONCENTRATIONS AFTER BREAKFAST[a]

Subject	Triglyceride concentration (mg/100 ml) (hours after eating)				
	4	8	10	12	14
1	172	90	70	65	65
2	112	50	45	53	50
3	82	58	56	58	56
4	170	70	51	46	51
5	82	59	59	56	56
6	50	42	39	40	42
7	244	98	123	132	143
8	150	101	90	98	103
9	312	134	120	132	137
10	64	47	47	50	56
11	140	78	52	70	76

[a] Savory and Sobel (S4).

tion while other liver function tests remained normal (D13). In such a study of 18 obese individuals on fasts lasting 15–66 days, it was observed that maximum BSP retentions were seen within 20–40 days, and in some individuals the retention reached as much as 40–50%. These increases were paralleled by falls in plasma volume, and it was postulated that the BSP changes were related to decreased hepatic blood flow (D12).

Plasma volume and the extracellular fluid space have been observed to constrict 30% during reducing diets (300–600 calories per day) (B22). These changes can be accompanied by functional impairment of glomerular filtration and hepatic perfusion with transient increases up to 2 mg/100 ml in serum creatinine and BSP retention up to 40% (B22). In rare instances a significant fall in serum calcium, magnesium, or potassium was observed. Hyperuricemia was also observed, with concentrations as high as 9 mg/100 ml (B22).

In the normal individual a high protein intake probably does not cause increases in blood urea nitrogen (BUN). However, in the individual with even minimal renal or liver dysfunction, major increases can be observed. In a dehydrated patient with septicemia and pleural abscess after mitral valve surgery, a high-protein diet produced an increase in BUN from normal limits to 100 mg/100 ml (D11). The serum creatinine only rose to 2.9 mg/100 ml (D11). Hemorrhage will also create elevations in BUN. In a patient who experienced gastric hemorrhage and hypotension, the BUN rose to 135 mg/100 ml while the creatinine rose only to 2.5 mg/100 ml (D11).

It has been reported that in normal individuals who eat a standard breakfast there is no significant change in blood urea nitrogen, carbon dioxide content, chloride, sodium, potassium, calcium, phosphorus, total protein, albumin, creatinine, uric acid, cholesterol, and cholesterol esters (A4). It should be emphasized again that the effects of fasting are quite different in normal, healthy persons than in sick people.

The ingestion of Metrecal, which contains iodocasein, has been found to increase PBI levels (T2), and ingestion of foods containing thiocyanates, thiouracil-like compounds, or soybean meal tend to lower thyroid constituents. The effect of bananas on 5-hydroxyindoleacetic acid is well known (M3). In one study ingestion of bananas increased urinary 5-hydroxyindoleacetic acid excretion from 5 mg/24 hours to 54 mg/24 hours—a markedly elevated value (M3). The presence of serotonin-like material in other fruits and vegetables can also interfere with the analysis of these components (Table 7). It is not generally appreciated that fresh pineapple contains as much serotonin as bananas—about 19 μg/g. Canned pineapple juice has 25 μg/g and fresh pineapple juice has

TABLE 7
CONCENTRATION OF SEROTONIN IN FOODS[a]

Food	Serotonin (μg/g)
Banana peel	50–150
Banana pulp	28
Tomato	12
Avocado	10
Red plum	10
Pineapple	19
Canned juice	23–25
Fresh juice	12
Eggplant	2

[a] Adapted from Marley and Blackwell (M3).

12 μg/g (M3). There is at least one case in the literature of an erroneous diagnosis of carcinoid tumor in an individual who drank a large amount of pineapple juice and had an elevated excretion of 5-hydroxyindoleacetic acid. Urinary 5-hydroxyindoleacetic acid rose from 3 to 31 mg in 4 hours in one normal individual after he drank 500 ml of pineapple juice (B27).

Ingestion of citrus fruits, coffee, carrots, and spinach have been reported to interfere with urinary assay of steroids by the Porter-Silber reaction (B11). Citrus fruits also interfere with aldosterone assays, and corn and other vegetables are rich sources of nonsteroidal estrogen bioassayable substances (B11).

4.4. DRUG-INDUCED METABOLIC CHANGES

In many instances administered drugs alter a metabolic pathway and directly produce changes in chemical constituents. Insulin administration results in changes in blood glucose as well as in potassium and phosphorus. Vitamin D may cause changes in calcium concentration, and epinephrine will at first cause an increase in potassium concentration, and then a decrease. Some drugs interfere with an enzyme in a metabolic sequence and alter the concentration of a blood constituent. Allopurinol reduces uric acid production by virtue of its ability to inhibit xanthine oxidase and produces rapid and marked falls in serum uric acid levels (K9). Uric acid levels are also reduced by drugs that interfere with its biosynthesis: azoserine and 6-diazo-6-oxy-1-norleucine (DON) by interference in glutamine metabolism; methotrexate by inhibition of the incorporation of single carbon fragments into positions 2 and 8 of the purine ring (K9).

Uric acid levels are also reduced by drugs that interfere with tubular

reabsorption. These include aspirin, probenecid (Benemid), sulfinpyrazone (Anturane), and phenylbutazone (Butazolidin). It should be emphasized that these effects are related to dosage and there are paradoxical effects. Twenty grains of oral salicylate produced an elevation of serum uric acid with a mean rise of 1.4 mg/100 ml, while 40 grains of salicylate caused a mean fall in serum uric acid of 1.1 mg/100 ml (P9).

Some drugs, such as the two-substituted thiodiazole and acetazolamide (Diamox), increase serum uric acid by stimulating uric acid synthesis (K9). Others, such as chlorothiazide (Diuril), increase uric acid retention by decreasing uric acid excretion (K9). Hydrochlorothiazide inhibits tubular secretion and has been shown to increase pretreatment mean uric acid values from 6.5 mg/100 ml to 10.3 mg/100 ml by the third treatment day. In a patient with gout, the level increased from 8 mg/100 ml to 12 mg/100 ml (H6). In a single case a paradoxical hypouricemia occurred (H6).

The laboratory must be informed when the therapeutic regimens include drugs specifically administered to change the blood level of a biochemical constituent. Cholestyramine resin, a nonabsorbable anion exchange resin administered orally to patients with hyperlipoproteinemia produced a 24% decline in serum cholesterol levels in 14 patients with essential hypercholesterolemia. In these patients the mean cholesterol fell from 414 ± 98 mg/100 ml to 176 ± 21 mg/100 ml (F1). Pectin added to the diet caused a 5% decrease in serum cholesterol values (K4), as did an oral hydrophobic colloid (G4). Levels fell in one case from 220 mg/100 ml to 160 mg/100 ml (G4). Nicotinic acid, neomycin, and *p*-chlorophenoxyisobutyrate have all been used to reduce serum cholesterol (G7).

In addition to drugs administered specifically to produce a metabolic effect, there are drug-related physiological changes that cause laboratory test abnormalities. Many drugs have been associated with the appearance of abnormal liver function tests in a fashion that simulates extrahepatic obstruction. These drugs include, among others, chlorpromazine, cinchophen, methyltestosterone, thiouracil, *p*-aminosalicylic acid, sulfadiazine, reserpine, meprobromate, novobiocin, caffeine, and phenacemide (L7, L8, S6).

5. Indirect Physiological Effects

5.1. Diagnostic Manipulation—Surgical Trauma

Elevations of serum acid phosphatase have been reported in specimens obtained from patients after rectal examination (D4). However, Roubrick and Winsten, in a study of 38 men, found that rectal examination performed on patients without known prostatic disease did not significantly elevate the serum acid phosphatase level (R1). In patients with

benign prostatic hypertrophy there appeared to be a consistent, but clinically insignificant, rise, and in 4 patients with carcinoma of the prostate, the rise was inconsistent despite the fact that in one patient the activity rose from 68 units before palpation to 97.2 units at 30 minutes after the examination (R1).

Elevations of serum acid phosphatase were observed following surgery in 17 of 20 patients with benign prostatic disease and 3 of 6 patients with prostatic carcinoma (W7). In those cases where elevations occurred, preoperative values were regained within 24 hours after surgery. In one patient with benign hyperplasia subjected to suprapubic prostatectomy the value rose from 1.1 to 23.2 Bodansky units within 90 minutes after operation. In another patient with the same diagnosis and the same operation the change was from 0.12 units to 0.72 unit (W7).

Elevations of serum aspartate aminotransferase have also been observed after surgical trauma (Table 8). These effects are probably due to cutting of muscle and liberation of enzyme, as well as anesthesia-induced spasm of the sphincter of Oddi, creating elevations by regurgitation. Not all patients subjected to surgery demonstrate these increases, but in those who do the values, in some cases, do not return to normal until the ninth postoperative day. In patient 5 (Table 8) in whom the preoperative level was slightly elevated to 59 units, there was a 3-fold increase in activity to 165 units on the day of surgery. The elevations are probably related to the severity of the surgery and the extent of trauma.

Blodgett and his associates studied aspartate aminotransferase values in 33 patients before and after transurethral prostatic resection (B14). In only two patients was there an elevation. Forty-seven percent of 51 patients who were in accidents involving bodily trauma without any

TABLE 8
EFFECT OF SURGICAL TRAUMA ON SERUM ASPARTATE AMINOTRANSFERASE

	Serum aspartate aminotransferase (units)			
		Days post-op		
Patient	Pre-op	1–2	6–7	9–10
1	29	61	53	32
2	36	82	44	—
3	14	54	25	19
4	44	118	47	44
5	59	165	117	66
6	18	29	36	24

evidence of cardiac injury had elevations in aspartate aminotransferase (L5). The activities in most patients reached maximum levels within 2 days after the trauma and returned to normal over variable periods of time. In some cases, the maximal value was not reached until 3–6 days after the injury (L5). Creatine phosphokinase is also elevated after surgery. Penneys and Wilkinson have reported its elevation following amputation of a leg (P5).

5.2. Intramuscular Injections

Intramuscular injections have been shown to produce elevations in serum enzyme activities presumably due to either inflammatory areas in the muscle or actual breakdown of cells and release of enzyme. In one study, preinjection values of creatine phosphokinase were in the normal range of 24–100 units. Multiple intramuscular injections of penicillin, diuretics, and narcotics every 6 hours caused the creatine phosphokinase values to rise to levels between 160 to 240 units, or up to 2.5 times the upper limit of normal. When the injections were stopped, the creatine phosphokinase values returned to normal within 48 hours (B7). Similar observations of aspartate aminotransferase activities were made in patients receiving intramuscular injections of penicillin every 4 hours. Activities rose to values as high as 200 units. Other workers have reported injection related serum creatine phosphokinase elevations following intramuscular administration of chlorpromazine and suxamethonium (H10, M11, T6).

5.3. Opiates, Narcotics, and Anesthesia

Opiates can effect serum levels of enzymes and other substances whose homeostatic control depends on clearance through the liver (F8, G12, M15, N4, S19). In one reported case, the aspartate aminotransferase was within normal limits before the administration of codeine, but within 2 hours after the drug, the enzyme activity had risen to two times the normal value; by 8 hours to eight times the normal activity, and within 24 hours it had returned to normal (F8). Increases in transaminase to levels 5–85 times the control value have been reported in 6 of 16 patients with disease of the biliary tree following the administration of codeine phosphate (2 grains) (B7, F8). Gross has shown that morphine, codeine, or mepheridine administration produce elevations of serum amylase or lipase (G12). These elevations have been attributed to constriction of the sphincter of Oddi and increased intraductal pressure on the pancreatic duct (G12, N4).

The role of anesthesia in affecting liver function tests has been appreciated for a number of years (L8). In a study of 100 patients undergoing

abdominal surgery, half of whom received halothane and half diethyl ether, minor, but significant liver function abnormalities (transaminase, alkaline phosphatase, bilirubin, albumin, and globulin) were observed in 30 patients: 17 after halothane and 13 after diethyl ether anesthesia (D5). In three patients marked elevations of transaminase and alkaline phosphatase with depressed albumin were noted on the fifth postoperative day. Both halothane and ether anesthesia induce increases in blood glucose (G9, S17). In one study the average increase with ether was 50 mg/100 ml and with halothane 25 mg/100 ml. Elevations of lactate and pyruvate were also observed (S17).

5.4. Muscular Activity

Muscular activity effects the concentration of many biochemical constituents. Mild exercise, walking at a rate of 5.6 km/hour produced a 20% increase in creatine clearance (K1). However, severe exercise (jogging at 10.5 km/hr) produced a 40% decrease in clearance (K1). Mild exercise (a 3-hour march) produced a mean fall of 18.2 mg/100 ml in haptoglobin levels from pre-exercise concentrations of 84.5 mg/100 ml (H14). Similar observations have been made for thyroxine and hormone-binding proteins (D7).

An intriguing study has been reported by Griffiths (G11) of medical students participating in an annual "London to Brighton Stroll." Specimens for creatine phosphokinase analysis were drawn before the march, at the midway point (25 miles) and at the end of the march (53 miles). At the 25-mile point, the mean creatine phosphokinase activities were increased 7-fold, and at the end of the march there was a 24-fold increase. In Table 9 are shown the values in several representative subjects in the study (G11).

Soldiers on a 2-hour strenuous march experienced statistically significant increases in lactic dehydrogenase ($p < 0.001$), malic dehydrogenase

TABLE 9
Creatine Phosphokinase Activity after Exercise[a]

Subject	Extent of walk		
	Start	25 mile	50 mile
2	35[b]	213	1018
5	45	107	575
6	35	325	1240
9	57	243	243

[a] Griffiths (G11).
[b] Values are expressed as units of creatine phosphokinase activity.

($p < 0.01$), and sorbital dehydrogenase ($p < 0.01$). No significant differences were observed in aspartate aminotransferase or aldolase (H4). The greatest elevations were observed for lactic dehydrogenase. Two hours before the start of the hike the values were 244 ± 11.9 units; at the end of the march, 394 ± 27.2 units; and 2 hours later, 291 ± 18.4 units. Poortman and his associates noted, in a group of athletes during a cross-country run, a mean increase in aspartate aminotransferase activity from 33.8 ± 10.3 units to 52.2 ± 8.7 to 48 ± 12.4 units (P8). Assuming an upper limit of normal of 55 units, 4 of the 10 subjects had values outside the normal range (62, 64, 62, and 62 units).

Catecholamine excretion was increased after strenuous exercise, but not after mild or moderate exercise (V5). Climbing stairs for 10 minutes or a 1-mile walk produced no clinically significant increase in catecholamine output (J1).

5.5. Emotion, Stress

Emotion or stress may bring about abnormal concentrations of biochemical constituents. In a study of 52 male freshman medical students who had blood specimens drawn at an emotionally comfortable time and again at the time of their final anatomy examination, the average serum cholesterol at the time of the examination was 276 ± 5.6 mg/100 ml and at the less tense time, 205 ± 4.3 mg/100 ml (T4). There were increases of 7.5 to 67.5 mg in 39 students, a fall in the cholesterol value in 9 students, and no change in 4. Following the stress of surgery, Guravich and Venegas observed 300–400 mg/100 ml drops in serum cholesterol in 3 patients with familial hypercholesteremia (G13). In 30 of 31 subjects exposed to severe psychological stress for 3 days, Levi observed increases in PBI ranging from 0.5 to 5.8 μg/100 ml (L4).

Epinephrine and norepinephrine secretions are directly related to emotion and stress (S5). Hockey players demonstrate increases in excretion of norepinephrine during active competition (E3), as do pilots during moderately stressful situations (V4, V6). Increased excretion of catecholamines has also been observed in subjects watching emotionally evocative motion pictures (L3). Muscle pain stimulated increased excretion of vasopressin and epinephrine, but not of norepinephrine (K8).

The stress of cold produced increased urinary excretion of norepinephrine but not of epinephrine or vasopressin (K8). Cold in the form of accidental hypothermia also resulted in increased serum creatine phosphokinase (M2). Mental stress (problem solving) resulted in increases of urinary vasopressin from 33 to 47.6 units, epinephrine from 5.5 to 11.3 mg, and norepinephrine from 17 to 21 mg (K8).

A medical student who received a saline injection was informed that

he had received a large dose of insulin and would experience a severe hypoglycemic reaction. His plasma glucose remained unchanged, but both plasma cortisol and growth hormone levels increased. The growth hormone increased from 1.1 ng/ml to 5.2 ng/ml in 30 minutes and 10.5 ng/ml in 60 minutes (G10).

5.6. Oral Contraceptives

It has been estimated that more than 18.5 million women in the United States are regular users of oral contraceptives. The widespread use of these drugs has created numerous problems in the interpretation of laboratory data. Blood glucose concentrations may be elevated in such persons and they may exhibit abnormal glucose tolerance curves (W1) and fatty acid levels (W18). Insulin and growth hormone levels are also increased (S26). In the case of human growth hormone, the mean fasting level in 26 women during a control period was 8 ng/ml and while on the drug, 28 ng/ml (S27). Sunderman has reviewed the many reports of estrogen–progestin oral contraceptive-related increases in serum iron, iron binding capacity, transferrin, ceruloplasmin, and copper. These effects are probably related to the estrogen portion of the drugs (S31).

Oral contraceptives have also been reported to produce increases in sulfobromophthalein retention and other liver function tests, as well as in prothrombin time, clotting factors VII, VIII, IX, serum thyroxine, and protein-bound iodine (B8). In a group of 48 women the mean cholesterol value was 206 ± 41 mg/100 ml while they were receiving a variety of oral contraceptives and 179 ± 28 mg/100 ml when they were not receiving the drugs (W19).

Oral contraceptives have their most significant effect on endocrine parameters. Blood cortisol, thyroxine, protein-bound iodine, T3 uptake, and urinary free cortisol are elevated. Urinary 17,21-dihydroxy steroids, 17-ketosteroids, and estrogens are decreased. There is no effect on urinary catecholamines or VMA (Table 10) (L10). The effect of thyroid functions tests is due to the administered hormone stimulating an increase in the production of thyroid-binding globulin which in turn binds *l*-thyroxine. The lowering of free thyroxine stimulates the anterior pituitary to produce thyrotropin, which in turn stimulates the thyroid to produce more thyroxine. Since the additional thyroxine is bound to the extra protein, there is an equilibrium and the patient remains clinically euthyroid, but the protein-bound iodine and the thyroxine are elevated.

5.7 Endogenous Metabolites

The possible interfering effect of an endogenous metabolite in biochemical analysis is exemplified by the problems in the assay of aspar-

TABLE 10
HORMONE LEVELS IN WOMEN ON ORAL CONTRACEPTIVES[a]

Hormone	Level in physiologically menstruating women	Level in women on oral contraceptives
Blood cortisol (AM)	14.6 ± 4.0 μg/100 ml	39.6 ± 10.9 μg/100 ml
Serum thyroxine	6.6 ± 1.4 μg/100 ml	9.4 ± 1.7 μg/100 ml
Serum PBI	6.4 ± 1.0 μg/100 ml	8.0 ± 1.2 μg/100 ml
T3 Binding	All patients, 91–120 units	14 of 40 patients, above 120 units
Urinary free cortisol	56.8 ± 23.7 μg/day	79.5 ± 36.5 μg/day
Urinary 17-OH corticosteroids	4.6 ± 1.4 mg/day	2.6 ± 0.9 mg/day
Urinary pregnanediol	0.8 ± 0.6 mg/day	0.3 ± 0.2 mg/day
Urinary pregnanetriol	0.7 ± 0.3 mg/day	0.3 ± 0.1 mg/day
Estrogens	40 ± 17.8 μg	25.9 ± 7.4 μg
Catecholamines	37.1 ± 14.6 μg	37.1 ± 18.9 μg
VMA	3.5 ± 1.6 μg	3.3 ± 1.6 μg

[a] Compiled from Lucis and Lucis (L10).

tate aminotransferase in patients with diabetic ketosis. Cryer and Daughady reported elevations in activity in 69% of 61 patients with diabetic ketosis (C13). These assays were performed on the SMA-12 by a colorimetric procedure in which enzymatically formed oxaloacetic acid is coupled with a dye in a nonspecific diazo reaction. Acetoacetic acid has been shown to interfere in this reaction.

Addition of ethyl acetate to a specimen having a transaminase activity of 47 units was responsible for the following increases in enzyme activity: 10 mg/100 ml, 60 units; 20 mg/100 ml, 77 units; 40 mg/100 ml, 107 units; and 80 mg/100 ml, 150 units. Transaminase activity in these specimens determined by another method ranged from 32 to 34 units (C7). Thus, when serum from patients with ketosis is assayed for aspartate aminotransferase activity by the diazo method, false elevations of activity may be recorded due to reaction of acetoacetic acid. In Table 11 are shown some values obtained by the diazo method and by an ultraviolet NADH $\rightleftharpoons$ NAD aspartate aminotransferase technique (B12). Examination of the medical records of these patients indicated that they were either diabetics who were in ketosis or individuals who were eating very poorly and had some degree of starvation ketosis. Similar elevations have been observed in patients receiving *p*-aminosalicylic acid (G6).

These differences emphasize potential metabolite interference related to methodology. An interference can occur when one method of analysis is used, but not with another. These possible differences are well appreciated for glucose analysis. Caraway (C4) has shown that the apparent

TABLE 11
METHODOLOGY DISCREPANT ASPARTATE AMINOTRANSFERASE VALUES IN PATIENTS WITH KETOSIS

Patient	Aspartate aminotransferase activity (units)	
	Ultraviolet NADH ⇄ NAD	Colorimetric diazo
1	10	70
2	80	163
3	38	102
4	68	146
5	65	159
6	24	61
7	28	140

mean glucose concentration in 20 urine specimens as determined by 6 different methods varied from 5 mg/100 ml to 162 mg/100 ml (Table 12).

In uremia there are present in the serum a variety of known and unknown metabolites that can produce aberrant laboratory results. Significant differences in glucose concentration have been observed in such specimens analyzed by ferricyanide (F2a) or Fe(II)-5-pyridylbenzodiazepin-2-one reduction methods compared to glucose oxidase procedures (K7a). In a patient with elevated creatinine (15 mg/100 ml) and uric acid (10 mg/100 ml), the glucose value determined by the automated alkaline ferricyanide procedure was overestimated by 20 mg/100 ml (C4). In uremic patients undergoing chronic hemodialysis there is a decrease in transaminase activity. In 11 of 19 such patients, there was

TABLE 12
APPARENT GLUCOSE IN 20 URINE SPECIMENS AS DETERMINED BY SIX DIFFERENT METHODS[a]

Method	Apparent glucose (mg/100 ml)	
	Mean	Range
Ferricyanide (automated)	162	52–368
Folin-Wu	116	30–250
Somogyi-Nelson	49	7–109
Dinitrosalicylic acid	19	5–45
o-Toluidine	18	3–39
Fermentation	5	0–10

[a] Caraway (C4).

little or no measurable transaminase activity (W13). The effect was attributed to either presence of a nondialyzable inhibitor or depletion of pyridoxal phosphate (W13).

6. Direct Analytical Effects

As mentioned earlier, there are numerous listings of drugs which interfere directly in biochemical analysis (A5, A7, C5, C8, C10, E1, G1, H2, L9, M14, N1, P2, W12). It would serve no purpose to list them here once again. Perhaps the most publicized area of direct interference in clinical chemical analysis is in the assay of protein-bound iodine or thyroxine. These effects have been subject to extensive review (A1, D2, H1, P6, S7). The most serious and long-lasting interferences are those caused by intravenously administered radiopaque agents that contain iodine. In Table 13 are listed substances reported to affect protein-bound iodine, but not thyroxine, determined by column (A7a). Orabilix and Dionosil are negative on thyroxine analysis when the concentration is no greater than 100 μg, but are positive at 1000 μg. Some materials listed in the table will adversely affect the thyroxine reaction even at low concentrations.

Thyroxine determined by protein binding (M17) is not subject to interference by iodinated radiopaque agents. However, when this technique is used, elevated values are observed in pregnancy and in persons with elevated thyroid-binding globulin or albumin and in persons receiving estrogens or oral contraceptives. Spuriously low values will be obtained in persons with low thyroid-binding globulin or albumin or patients with nephrotic syndrome or those receiving dilantin or triiodothyronine (B12a).

Recent restrictions in the use of hexachlorophene preparations for antisepsis and the recommendation that povidone-iodine (Betadine) be used in its place will undoubtedly lead to increased thyroid function assay

TABLE 13
EFFECT OF IODIDES ON THYROID FUNCTION TESTS[a]

1. Those affecting PBI, but not T_4, determined by column
   a. Cholecystography—Cholographin, Orabilix
   b. Bronchography—Dionosil, Lipiodol
   c. Pyelography—Hippuran, Hypaque, Urokan
   d. Intravenous urography—Miokan, Skiodan
   e. Salpingography—Salpix
   f. Inorganic iodides—Lugol's expectorants, etc.
2. Those affecting both
   Cholecystography—Telepaque, Priodax

[a] Anonymous (A7a).

interference. It has been reported that absorption of Betadine from broken or denuded skin can result in increased PBI values (A6).

The use of multitesting instruments for screening of large numbers of apparently normal persons has made understanding of interference with analytical procedures used in these assays of extreme importance (V1). In one study of 547 persons, a diagnosis could not be made in 46 of 53 patients with abnormal alkaline phosphatase activity, 17 of 20 with elevated bilirubin, 4 of 7 with abnormal aspartate aminotransferase, and 45 of 51 with elevated blood urea nitrogen (S6a). The role of drug interference in these patients must be considered. In the Kaiser-Permanente medical care program, 4637 of 8446 patients had at least one test abnormality in a 20-test battery (F10). Singh and his associates have studied the *in vitro* effect of 45 common drugs on the 12 automated biochemical tests usually available on the Technicon SMA 12/60 (S22). At drug concentrations that would be observed in the blood at maximum therapeutic dose, only 8 drugs demonstrated significant interferences. *N*-Acetyl-*p*-aminophenol caused an increase of 10 mg/100 ml in glucose, 0.5 mg/100 ml in uric acid, and 245 units in aspartate aminotransferase activity. The other drugs (*p*-aminosalicylate, ascorbic acid, 1-dopa, α-methyldopa, hydralazine, isoniazid, 6-mercaptopurine, and sulfathiazole) caused interferences in glucose, uric acid, bilirubin, and transaminase activity. Ingestion of phenacetin caused increases in uric acid from 6.6 to 7.7 mg/100 ml within 4 hours of the administration of 2 g of the drug. The elevations of transaminase were observed with the diazocolorimetric procedure. Aberrant elevations with this method have been described in patients with ketosis and also in patients given erythromycin (S1) or *p*-aminosalicylic acid (G6). These interferences would not be observed in ultraviolet transaminase methods.

The determination of 17-ketosteroids is most often determined in the clinical laboratory by the Zimmerman reaction, in which the ether-extracted material is allowed to react with *m*-nitroaniline to yield a colored product. Thus, any compound with the 17-keto basic structure such as reserpine, morphine, ascorbic acid, or their metabolites will interfere. The Porter-Silber reaction used in the determination of 17,21-dihydroxysteroids is also not specific, and the reaction requires a dihydroxyacetone side chain. Paraldehyde, chloral hydrate, meprobromate, and potassium iodide have been found to interfere, and patients should be maintained free of these drugs for 24–48 hours before the urine collection (B11).

Enzyme activity measurements are greatly affected by buffer systems used in analysis. In measuring alkaline phosphatase activity under optimal conditions, ethylaminoethanol buffer yielded activity 3.8 times

that with 2-amino-2-methyl-1,3 propanediol buffer, almost 9 times that observed when carbonate buffer was used and 3.5 times the activity measured in a Tris buffer system (M5). When compared to activities in Tris-HCl buffer, sulfate ion in phosphate buffer inhibited the cationic isoenzyme of aspartate aminotransferase and accelerated the activity of the anionic isoenzyme (N3). This factor can be of importance in the assay of serums with high activity and a large proportion of cationic isoenzyme, particularly in coupled assays where malic dehydrogenase dissolved in ammonium sulfate is used.

Inhibitors of lactic dehydrogenase have been reported in commercial preparations of NAD^+ and NADH (B4, M6, S28). The concentration of inhibitory substances varied from lot to lot. In a serum lactic dehydrogenase study with NAD^+ from 8 sources, activities were found to vary from 145 to 75 units (B4). Inhibitors of lactic dehydrogenase activity have also been observed in dialyzates in uremic patients (W8) and in human urine (G8). The purity of available substrate can also effect enzyme activity. Schwartz and Bodansky observed that, in 6 batches of fructose 6-phosphate, all weighed to a 0.5 m*M* concentration, the actual concentration varied from 0.13 m*M* to 0.55 m*M* (S14).

An important area of direct biochemical interference is that caused by fluorescent or fluorescent quenching materials in the blood or urine after the administration of a drug. These interferences may be observed during catecholamine analysis in urine from patients receiving α-methyldopa, tetracyclines, chlortetracyclines, oxytetracycline, erythromycin, chlorpromazine, or quinidine (A2, G5).

The administration of spironolactone (Aldactone) interferes in the determination of 11-hydroxycorticosteroid by methods that depend on formation of fluorescence in strong sulfuric acid (W15). In 5 patients, the administration of the drug produced as much as a 5-fold increase in the apparent plasma cortisol levels. Aspirin interferes in the determinations of homovanillic acid (HVA) by a fluorometric method. The HVA fluorophore occurs at 320 nm and 420 nm, and acetylsalicylic acid produces fluorescence at 305 to 405 nm (H12).

Colorimetric assays used in endocrinological procedures are also often subject to drug interference. We have observed an interesting interference in a patient with carcinoid. The patient excreted 400 mg of 5-hydroxyindoleacetic acid (5-HIAA) and when a vanillylmandelic acid (VMA) determination was performed by a nonspecific diazo method, the value was reported to be 375 mg. The catecholamines were just above normal. There was an immediate suggestion that the patient also had a pheochromocytoma. However, when a specific chromatographic VMA method was used, the value was found to be within normal limits. Subse-

quent studies indicated that 5-HIAA reacts on an almost equal basis with VMA in the nonspecific diazo reaction, and the reported VMA was in fact an interference by 5-HIAA (F5). VMA assays based on diazo reactions are particularly subject to interferences and possible erroneous diagnosis. Sapira and his associates, in a report entitled "Non-Pheochromocytoma," have reported that only 2 of 14 patients with elevated VMA proved to have pheochromocytoma (S2). Methocarbinol and *p*-aminosalicylic acid have also been shown to interfere in the diazo-VMA reaction (S2). Chloropromazine has been shown to interfere with the Porter-Silber 17-hydroxycorticosteroid assay (M8). Phenothiazine and its derivatives greatly inhibit the color development of the 5-HIAA test (C2). Urinary 17-hydroxycorticoids and 17-ketosteroids were depressed by ingestion of prophoxyphene (Darvon) as was 17-hydroxycorticoids by pentazocine (Talwin) (C12). The mechanism for these depressions was not clear since the addition of the drug to urine had no effect.

7. Conclusion

The phenomenon of biochemical interference is a complicated one. No attempt has been made to list all drugs reported to interfere in any test, for these listings are available. Rather, emphasis has been placed on pointing out the difficulties in assessing the problem in the individual patient. Most patients receive a variety of drugs, some of which may induce enzymes that affect the rate of clearance or metabolism of the drug (N2, V2), and synergistic effects of several drugs are in most instances not known. Attention to these problems is a responsibility of the laboratory, the pharmacist, and the clinician, who is faced with the dilemma of using laboratory data in arriving at his diagnosis or in following the patient's course. These responsibilities are clearly focused by an editorial relating to the diagnosis of diabetes: "In view of these prodigious problems in establishing a proper diagnosis, the increasing willingness of some physicians to accept a couple of glucose levels as the certain arbiter of diabetes should be discouraged. When enshrined on a hospital chart or in a physician's files, the diagnostic label is difficult to remove and leaves the patient with heightened anxieties that are not allayed when he learns of limitations in obtaining life insurance or in changing his job. His stigma extends even to his children who are now burdened with a family history of diabetes" (S16). The plight of the physician in making a diagnosis of diabetes is made more difficult by the fact that the oral glucose tolerance test is affected by thiazides, diuretics, oral contraceptives, exogenous steroids, oral hypoglycemic agents, and salicylates (C6).

Laboratory medicine of the future will require that submitted specimens be accompanied by the drug history of the patient to permit

computer-assisted evaluation of drug interferences. In addition, as new drugs are introduced, the pharmaceutical industry must assume responsibility to conduct studies on both analytical and physiological interferences.

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MEASUREMENT OF THERAPEUTIC AGENTS IN BLOOD

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1. Introduction

Patients generally seek medical help because they want relief from disease. They have little interest in diagnosis—except in so far as it helps the doctor treat them more efficiently—and still less in the mechanism of their illness. Nevertheless, clinical chemistry traditionally has been more concerned with diagnosis and the elucidation of the mechanism of disease than with treatment. In only a few circumscribed areas, such as management of water and electrolyte imbalance, diabetic coma, and renal dialysis, has clinical biochemistry proved indispensable for treatment, the overall *raison d'être* of the health industry.

Drugs have always played an important part in treatment even when few were of proved efficacy and their mode of action was poorly understood. The introduction, during the present century, particularly the past thirty years, of selective, powerful and potentially dangerous drugs, is

ensuring that therapeutic practices that were adequate in a bygone age are no longer applicable or acceptable. Drug dosage regimes that take no account of differences in the way that different individuals absorb, metabolize, excrete, and react to drugs belittles their value on the one hand, and exaggerates their toxicity on the other. The clinician, in order to steer a median course between these two hazards must, in many cases, "tailor" his treatment of disease to each individual patient's requirements. The value of clinical biochemistry in this process becomes clearer as more examples are discovered of drugs to which the therapeutic response correlates better with its concentration in plasma than with the daily dose (B25, B26, K10, P12, V7, V9).

For some drugs the therapeutic response can be assessed, quickly and simply, by observing or measuring one or more of its biological effects. Anticoagulant therapy and the control of diabetes by hypoglycemic agents, for example, have long been regulated by reference to plasma prothrombin times and blood glucose concentrations, respectively. For many diseases, however, there are no objective methods. This is particularly true for those illnesses where the major manifestations are subjective or where the drug is used to prevent rather than to treat a disease.

While the study of the absorption, disposition, metabolism, and excretion of drugs, and their therapeutic applications falls properly within the province of the clinical pharmacologist, it is upon the skills and knowledge of the clinical biochemist that accurate, and hence clinically meaningful, measurements of blood drug concentrations depend. The clinical biochemist must therefore be familiar not only with the methodology, but also with the value, and limitations, of such measurements.

The importance for therapy of the blood drug concentration rather than the size of the daily dose was first appreciated, and utilized clinically, in the case of the antibiotics and antimalarials, but in this review little attention will be given to this important group of drugs which affect the metabolism of parasites rather than that of the host. Suffice to mention briefly the importance of controlling the blood concentration of streptomycin and some of the newer antibiotics, especially in patients with impaired kidney function, so as to maintain bactericidal levels of the drug in the blood and tissues without, at the same time, producing serious and irreparable damage to the host.

Consideration will be given mainly to the principles of pharmacokinetics and methods of measuring drugs whose effectiveness derive from their ability to alter the patient's own metabolism, either locally or generally, and for which there is reasonable evidence that therapeutic responsiveness and/or toxicity is related to the steady-state blood drug level.

2. Pharmacokinetic Considerations

2.1. Steady-State Concept

The steady-state concept of therapeutic agents is a useful if somewhat naive one (W1). Drugs are, by their very nature, administered intermittently. Consequently their concentration, even after "equilibration" throughout the one or more body compartments in which they distribute either rises as input into the system exceeds total output by combination of excretion and metabolic degradation, or falls when the reverse obtains. Nevertheless providing the biological half-life of the drug is relatively long compared to the interval between successive doses of the drug, a more or less steady state may be considered to exist. It is clear, however, that in any study of blood drug concentrations, particularly insofar as it relates to an individual patient in a clinical situation, it is essential to define the temporal relationship between the last dose of the drug and time of collection of the sample upon which the measurement is made. It is surprising how often this important aspect of the problem is ignored or overlooked in practice and even in the scientific literature.

For some agents, such as reserpine, monoamine oxidase inhibitors, and alkylating agents, to which the descriptive label of "hit-and-run drugs" has been attached (B25), the biological effect persists long after the drug has been completely eliminated from the body. In this case no correlation between the drug's therapeutic effectiveness and its concentration in the blood exists, and plasma measurements cannot be expected to provide useful information.

Toxic symptoms may be dose-dependent and merely an exaggeration of the therapeutically desirable response, e.g., the coma of barbiturate overdosage and persistence of muscular paralysis after succinylcholine administration, or an unpredictable effect of the drug upon an organ or tissue remote from that upon which the therapeutic effect is manifested.

Even in the ideal situation in which the statistical correlation between the concentration of a drug in the blood and its biological effectiveness is extremely high, other determinants of therapeutic responsiveness preclude the *uncritical* use of blood drug levels as the sole guide to treatment. While this is self-evident, and no truer for this class of measurements than any other, it has, nevertheless been advanced as an argument against the utility of blood drug measurements in the clinical situation.

That blood drug measurements can sometimes provide valuable additional information is, however, not seriously in doubt. For some drugs the intensity of the pharmacological action and severity of side-effects correlates much better with the plasma steady-state concentration than with

the daily dose. It is with drugs of this type that this review mainly deals even though, at the present time, the number of therapeutic agents for which such a relationship has been sufficiently well established to be clinically useful is pitifully small.

2.2. Relationship of Blood Level to Dosage

For some drugs there is a more or less linear relationship between the daily dose and the plasma steady-state concentration. This is particularly true for drugs, such as aspirin, that are largely or wholly eliminated unchanged by excretion in urine and have a relatively simple pattern of distribution within the body. For most drugs, however, no such clear-cut correlation exists, and it becomes apparent only when a very large population has been sampled (C1). Reasons for this include (a) differences in rates and completeness of absorption of drugs from the gut or other site of administration, (b) volume of distribution, and (c) inherited and acquired differences in metabolic conversion and elimination of drugs from the body.

2.3. Rates and Completeness of Absorption

Most therapeutic regimes tacitly assume that a drug is absorbed unchanged from its site of administration and, moreover, that if absorption is less than complete the proportion absorbed is similar in all individuals. Some drugs are ineffective orally because they are rendered unstable or destroyed by physical or chemical conditions prevailing in the gut. Changes in these conditions as a result of disease, changes in diet or for other reasons may have a profound effect upon the amount of drug entering the body. The formulation of the drug, its rate of disintegration, dissolution, and passage through the gut and temporal relationships to ingestion of other constituents of the diet also exert their influence. The drug may be unable to cross the intestinal mucosa or may be metabolized there. Perhaps more commonly, having entered the portal blood stream the drug is so rapidly removed by a single passage through the liver that its concentration in the peripheral blood never achieves therapeutically significant values.

Even when administered by intramuscular or subcutaneous injection the rate of absorption of drugs from their site of administration varies, not only from individual to individual, but from site to site in the same subject (B13). For the few drugs studied in this way absorption and distribution was quicker and higher blood levels achieved when injections were given into upper rather than lower limbs and proximal to the trunk than distally.

Most drugs are deactivated or eliminated at a rate proportional to their plasma concentration. This means, all things being equal, that the

more rapidly a drug is absorbed, the faster it is eliminated and the shorter its duration of action. This is particularly true of drugs with a short biological half-life; insulin, with a half-life of only a few minutes, is a good example (S2). It is with drugs of this type that preparations and formulations designed to slow down absorption and extend the period of distribution, are most useful. The other situation where such formulations are useful occurs when, despite a long biological half-life, the therapeutic index of the drug is so low that with rapidly absorbed formulations blood concentration may temporarily exceed the toxic limit even though the steady state achieved is therapeutically desirable.

2.4. Volume of Distribution

The importance of body size, and consequently the volume of distribution, upon which blood levels of drugs given in fixed dosage depends, would be too obvious to mention were it not for the fact that, except in children, it has received virtually no cognizance, either by clinicians or formulators of drug dosage regimes. It is perhaps because of its relatively small magnitude *vis-à-vis* other more recently recognized determinants of drug distribution and metabolism such as pharmacogenetics and acquired differences due to enzyme induction that it has been possible in the past to ignore it with such apparent indifference to the therapeutic outcome.

2.5. Binding of Drugs to Plasma Protein

Drugs are transported in the bloodstream in two forms: (1) simple solution or (2) bound to plasma proteins or cellular components. The proportion of unbound to bound drug varies with the total concentration of drug and its relative binding affinities to various plasma constituents. The plasma proteins are generally considered the most important regulator of the unbound drug concentration, but since the binding of drugs to the cellular components of the blood has not been widely investigated this view may have to undergo some modification. Salicylates and phenobarbitone, for example, have been shown to bind to human erythrocytes as well as to albumin (M1).

The binding of small molecules to plasma proteins is a fairly nonspecific process, and most drugs are transported in the bloodstream at least in partial association with plasma proteins. With the majority of weakly bound drugs this association is probably not therapeutically significant, but for highly bound drugs it may be of direct importance (M16).

2.5.1. *Qualitative Aspects*

The interaction between a drug molecule and a molecule of plasma protein may be considered as a reversible equilibrium. Goldstein (G10)

and Meyer and Guttman (M16) have reviewed the extensive *in vitro* experimental evidence, which indicates that most drug–protein interactions are reversible. Direct *in vivo* evidence that drug–protein interactions are reversible is more difficult to obtain, but several common observations point to this conclusion. In most instances a drug has only a limited and finite duration of action. After the administration of a single dose, the drug and/or its metabolites are completely eliminated within a few days indicating that the association with tissue components is readily reversible. Further evidence that drug-receptor binding is reversible is provided by the availability of competitive inhibitors of drug action.

Plasma contains a large variety of proteins, all of which have the potential to interact with drugs. Albumin is generally considered to be the most important contributor to the plasma protein binding of drugs (G10, G11, M16), but for some, e.g., steroids, other proteins play a major role.

Bovine and human serum albumin which are well characterized and readily available in crystalline form (P15, S9) have been widely used for *in vitro* studies of drug–protein interaction. Human albumin has a molecular weight of about 69,000. At its isoelectric point, around pH 5, each molecule carries about 100 positive and 100 negative charges. Consequently, although albumin carries a net negative charge at the pH of plasma *in vivo*, it can nevertheless interact strongly with anions as well as with cations. Indeed, acidic drugs are generally more avidly bound. Phenylbutazone ($pK_a = 4$) for example, is bound to plasma albumin to the extent of 98% at therapeutic plasma concentrations (B24). The total number of binding sites per albumin molecule is lower than the total number of charged groups but is variable depending upon the molecular structure of the drug.

There is evidence that nonionic or hydrophobic interactions between albumin and the bound molecule are important in the binding process. The binding of phenothiazines and their derivatives to plasma albumin is thought to be mediated by hydrophobic interactions between the protein and the benzene rings of the drug (J2). O'Reilly (O8) concluded that the binding of warfarin was largely nonionic in character, and similarly Lukas and De Martino (L17) suggested the formation of a hydrophobic bond between digitoxin and albumin.

The α- and β-globulins of the plasma also form an important group of binding proteins and a number of them have been isolated and found to have a high affinity, but relatively low capacity, for a variety of endogenous and chemically related synthetic compounds. Westphal (W7) has reviewed the extensive literature on the interaction of corticosteroids

with the plasma proteins, particularly the corticosteroid binding globulins. Binding globulins have been defined for other endogenous compounds including testosterone (R9), thyroxine (P5, R2, S1), vitamin B_{12} (R6), and vitamin A (K2). The physiological importance of these high affinity binding globulins is not clear, but they may act as transporters and regulators of biological activity (K6, S1).

So far the plasma γ-globulins have not been found to interact significantly with drugs (G11) except where they occur as specific antibodies to them. The production of antibodies to protein hormones, such as insulin and gonadotropins, is well known as a cause of lessening therapeutic efficiency in cases where these drugs have been used for a long time. With growing recognition that many chemically simpler drugs are capable of behaving as haptens or antigens the possibility that some patients can develop circulating antibodies capable of binding drug avidly and extensively must once again be seriously considered.

2.5.2. *Quantitative Aspects*

There are two components to consider in the interaction of a drug with a protein. One is the *capacity* of the protein for binding drug molecules and this is related to the number of binding sites (n). The other is the *affinity*, or strength of binding, which is usually expressed as an apparent association constant (k).

Most experimental investigations have been concerned with measuring drug-protein interactions at equilibrium, and since the binding is a reversible process the law of mass action has been the basis upon which several equations have been developed for exploiting experimental data.

If the drug (D) is reversibly bound to plasma protein (P) then the interaction can be described by the equilibrium:

$$\mathrm{P} + \mathrm{D} \rightleftharpoons \mathrm{PD}$$

and from the law of mass action,

$$\frac{[\mathrm{P}] \times [\mathrm{D}]}{[\mathrm{PD}]} = K \tag{1}$$

where [D] = molar concentration of unbound ligand, [P] = molar concentration of protein, [PD] = molar concentration of combined drug-protein molecules, and K = equilibrium or dissociation constant.

Klotz (K8) put forward the following equation, based on the law of mass action, to describe reversible binding:

$$r = \frac{nk[\mathrm{D}]}{1 + k[\mathrm{D}]} \tag{2}$$

where r = moles of drug bound per mole of total protein, k = apparent

association constant (i.e., $k = 1/K$), and n = number of binding sites on each protein molecule. Equation (2) is based on the assumptions that (a) activities can be represented by concentrations, (b) all sites have equal affinities, (c) no interactions occur between the binding sites.

Two further equations have been derived from Eq. (2), and these are frequently employed to plot experimental data in order to obtain an estimate of the number of binding sites (n) and the apparent association constant (k). The reciprocal plot (K9) involves the following rearrangement of (2).

$$1/r = (1/n) + 1/nk[\mathrm{D}] \tag{3}$$

and $1/r$ (ordinate) is plotted against $1/[\mathrm{D}]$ (abscissa). Estimations of n and k are made from the slope and intercept values of the plot (Fig. 1).

Scatchard (S5) proposed another form of Eq. (2):

$$r/[\mathrm{D}] = nk - rk \tag{4}$$

and in this case $r/[\mathrm{D}]$ (ordinate) is plotted against r (abscissa) [Fig. 2; see (Pla)]. The intercept on the abscissa gives n and the intercept on

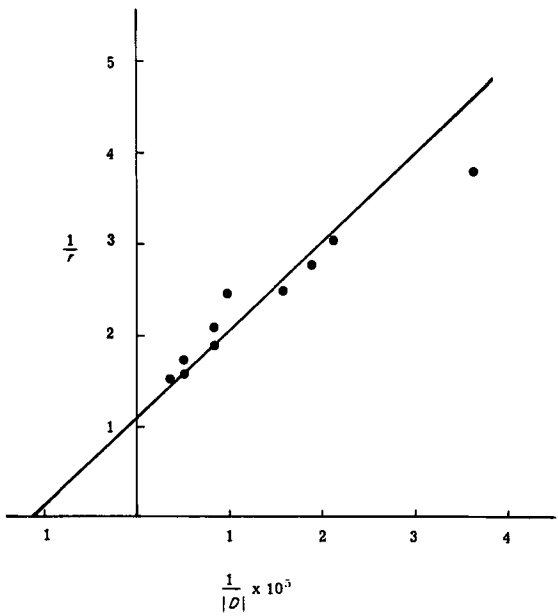

FIG. 1. Reciprocal plot: the binding of [^{14}C]warfarin to human albumin. r = moles of warfarin bound per mole of protein; $[D]$ = molar concentration of unbound warfarin. After Solomon and Schrogie (S22), reproduced by courtesy of Microforms International Marketing Corporation.

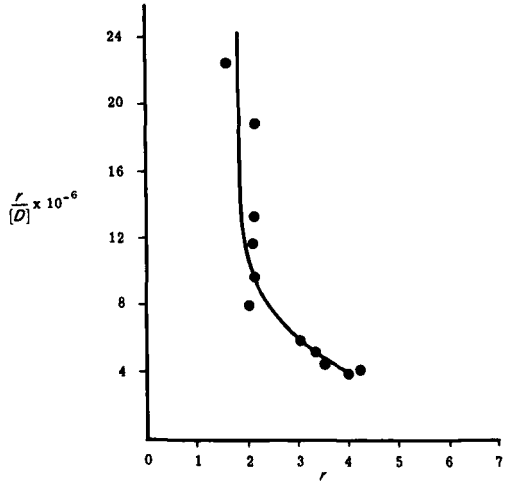

FIG. 2. Scatchard plot. The binding of [^{14}C]carbenoxolone to human albumin. r = moles of carbenoxolone bound per mole of protein; $[D]$ = molar concentration of unbound carbenoxolone. Data obtained by ultrafiltration; each point represents the mean of six experiments.

the ordinate gives nk. Graphic presentation of experimental results by the reciprocal plot (Eq. 3) puts greater emphasis on the data obtained at low unbound drug concentrations and can therefore be misleading (K9). The Scatchard plot gives a more balanced weighting to the individual results and is generally considered to be the graphic method of choice (M16). The extent of extrapolation of the data by these two graphic methods has been well demonstrated by Davison (D2).

Curvature of Scatchard plots is frequently encountered, and this produces difficulties of interpretation. Deviations from linearity may result from one or more of the following causes: (i) binding sites are not, in fact, independent, and interactions occur, for example, from electrostatic forces, (ii) binding sites are not equivalent, and there are different n and k values; (iii) curvature may result from a ligand-induced conformational change in the protein; (iv) a ligand having several groups involved in binding may bind in different ways to the protein. Extrapolation of a curved Scatchard plot, when more than one class of binding sites is present, gives $\Sigma n_i k_i$ and Σn_i.

The reciprocal (Eq. 3) and Scatchard (Eq. 4) plots cannot be applied to experimental data obtained from experiments with whole plasma as it is necessary to know the molecular weight and the amount of protein in the experimental system. However, Sandberg and co-workers (S3) and Rosenthal (R8) have proposed a Scatchard-type plot based on Eq. (5).

$$D_b/[D] = nkP_t - kD_b \tag{5}$$

where D_b = concentration of bound drug and P_t = total molar concentration of protein. A plot is made of $D_b/[D]$ (ordinate) against D_b which is independent of protein concentration, and this allows estimation of nkP_t, nP_t, and k, from the ordinate intercept, abscissa intercept, and slope, respectively. Curvature of the plot results when more than one species of binding protein is present or when there is more than one class of binding site on the binding protein.

It has been shown (G10) that the fraction of total drug bound to protein (B) can be calculated from Eq. (6)

$$B = \frac{1}{1 + (1/nkP) + ([D]/nP)} \tag{6}$$

which shows that the fraction bound depends upon (1) the protein concentration, (2) the drug concentration, (3) the apparent association constant of the drug-protein complex, and (4) the number of binding sites on each protein molecule. Thus, for any given plasma drug concentration, the higher the values for n and k, the tighter the binding will be and the greater the fraction bound. It also follows that protein binding can never be complete, however strong the drug–protein interaction, and that changes in the nature of the binding protein—either as a result of inherited or acquired abnormalities—can have a profound effect upon the ratio of total to unbound drug in the plasma. This effect is not necessarily the same for all drugs (R5). Moreover, meaningful reports of the percentage of a drug that is protein-bound should always specify either the total or "unbound" drug concentration.

A number of useful reviews of small molecule–protein interaction have appeared (D2, E4, S26, W7), and these contain detailed treatments of the mathematical analysis of binding data as well as information on experimental methods.

2.5.3. *Consequences of Plasma Protein Binding*

2.5.3.1. *Absorption.* Extensive plasma protein-binding may enhance absorption of a drug by rendering the concentration gradient favorable for absorption. It is particularly important with a drug poorly soluble in water, since binding will affect the equilibrium between drug already absorbed and drug remaining in the gut, both dissolved and undissolved. It has been shown (N6) that dissolution is often the rate-limiting step in the absorption of drugs, and it can be seen from Fig. 3 that avid protein-binding tends to move the equilibrium from left to right. Indeed it has been suggested (B21) that drugs with poor solubility character-

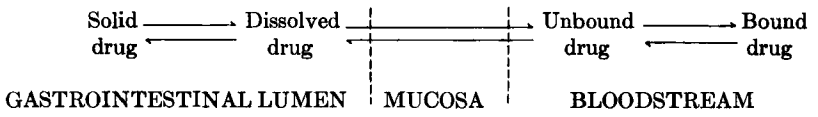

FIG. 3. Effect of binding on absorption.

istics, such as dicoumarol, would not be well absorbed unless they were bound to plasma proteins.

2.5.3.2. *Distribution and Pharmacological Action.* Plasma protein binding can influence the distribution and pharmacological action of a drug. Most drugs distribute through body water and tissues by passive diffusion down a concentration gradient. It is widely held, therefore (B21, G10) that only the unbound drug is available for transport to the extravascular or tissue sites where they exert their pharmacological action (Fig. 4). Experimental observations lend support to this concept, particularly with regard to the activity of antibacterial agents, such as sulfonamides (A7, R7) and penicillins (K17).

Providing they do not bind preferentially or irreversibly to tissue sites, drugs that are highly protein-bound are likely to be located mainly in the plasma compartment, at least at low doses when the high affinity binding sites are unsaturated (M9). In such cases, the protein-bound drug serves as a depot from which it releases unbound drug as it is removed from the body pool by metabolism and excretion. In the steady-state the concentration of unbound drugs in the tissues, and tissue compartments of the ECF, such as the synovial fluid, equals that in the plasma (H13). Martin (M9) has discussed in a simple model the binding of four hypothetical drugs ranging from weakly bound to highly bound (Fig. 5). At low doses, a strongly bound drug ($k \geqslant 10^5$)

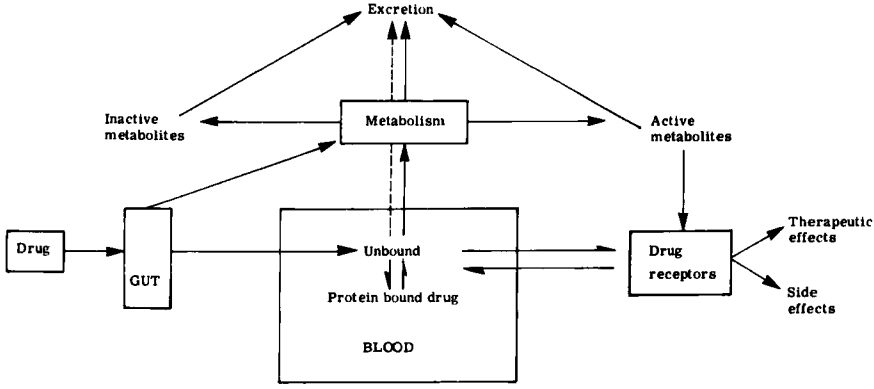

FIG. 4. Factors affecting drug concentration at receptor sites.

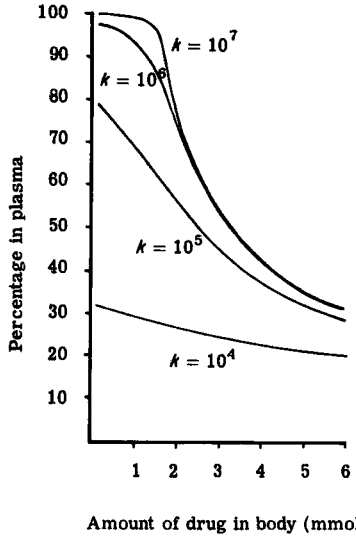

FIG. 5. Effect of apparent association constant (k) on the distribution of four model drugs. After Martin (M9), reproduced by courtesy of *Nature*.

locates mainly in the plasma, but as the dose increases the available plasma binding sites become fewer, with the result that more of the drug diffuses into the tissues and the fraction of drug remaining within the vasculature is reduced. For drugs of this type, there is, therefore, a fairly narrow range over which small dose changes can exert a profound change on the distribution of the drug within the body.

The plasma concentration of chlorpromazine fluctuates after intravenous doses both in dogs and in man (C18). Since chlorpromazine is a highly protein-bound drug (C16), the suggestion has been made that movement of the drug between tissue and plasma stores could result from small changes in protein binding by plasma and tissues (C17). Although tissue binding has not yet been intensively investigated, it seems likely that some highly bound drugs have even higher affinities for certain tissue proteins which may or may not be the receptor proteins. The cardiac glycosides for example have been found, *in vitro*, to have a higher affinity for cardiac actin and myosin than for bovine serum albumin (G5) and *in vivo* their concentration in the heart is about twenty to thirty times that in plasma. But, since this ratio remains constant, the plasma digoxin level provides a useful index of the concentration of the drug at its active site (S20).

Martin's model (M9) for the effect of plasma protein binding on distribution is highly simplified but illustrates that the therapeutic dose

range may be critically narrow for highly bound drugs that undergo large dose-dependent changes in distribution. There are other factors, such as inhibition of metabolism by drugs, that can lead to redistribution of a drug, particularly if toxic or irreversible accumulation occurs. Several pathological conditions predispose to accumulation and redistribution; among them are hypoproteinemia and hypoalbuminemia (L10). The frequency of prednisone side effects, for example, doubles when serum albumin concentrations are less than 2.5 g/100 ml.

Impaired liver function, either due to primary disease or secondary to cardiac failure, may cause drug accumulation (T1). Renal insufficiency may have two effects; changes in the binding properties of plasma protein secondary to uremia can occur (R5, S16), or the drug and its metabolites may accumulate owing to impaired urinary excretion.

Competition between drugs for plasma binding sites occurs and is responsible for some of the clinically most important changes in drug distribution. Phenylbutazone and oxyphenbutazone, for example, potentiate the action of warfarin by displacement (A2) and trichloroacetic acid, a major metabolite of chloral hydrate has a similar effect (S12) and is the cause of hemorrhagic complications during coumarin therapy (A2).

Numerous drugs have been shown *in vitro* and in animal experiments to compete for plasma protein binding sites (M16), and the many potential drug interactions of this sort have been extensively reviewed (H17, P11, S33). Data relating to human subjects are limited and largely anecdotal. Potential and actual drug interactions must be clearly distinguished since competitive phenomena that are readily demonstrable *in vitro* may not be of sufficient magnitude to have a clinical effect or may be obscured by other factors *in vivo*.

It is difficult to predict displacement of one drug by another merely from a knowledge of binding data and plasma concentrations. A drug with a higher association constant (k) does not necessarily displace a drug of lower affinity unless both share a common binding site.

2.5.3.3. *Metabolism and Excretion*

2.5.3.3.1. *Metabolism.* It is generally believed that only unbound drug is available for metabolism and filtration at the glomerulus. The limited number of studies on the kinetics of plasma protein binding have been discussed by Meyer and Guttman (M16), who concluded that in most cases rates of association and dissociation are very rapid, and, although it seems unlikely that protein binding is a limiting factor in determining the rate of metabolism and excretion of most drugs, there is a remarkable lack of data.

Newbould and Kilpatrick (N3) found that addition of plasma to the fluid perfusing a rabbit liver preparation reduced the rate of acetylation of two "long-acting" sulfonamides and that the rate of metabolism was dependent on the concentration of unbound drug. Anton and Boyle (A8) and Wiseman and Nelson (W15) using data from both *in vitro* and *in vivo* techniques reported a correlation between the rate of metabolism of a sulfonamide and the extent of protein binding.

The higher affinity of digitoxin ($k = 10^5$) for serum albumin compared to digoxin ($k = 10^3$) has been held responsible for the higher plasma concentration, lower urinary excretion rate, and longer plasma half-life of the former drug in man (L17). O'Reilly (O9) has suggested that the binding of warfarin to albumin, and its nonpolar character account for its presence in the plasma and the absence of unchanged drug in urine. On the other hand, hydroxylated metabolites of warfarin which are more weakly protein bound are virtually absent from plasma but are present in urine.

In contradistinction to the above, there are some highly bound drugs, such as sulfobromophthalein, with an association constant ($k = 10^7$) that are rapidly metabolized (B3). Phenylbutazone is a highly plasma protein bound drug (B24) and is rapidly metabolized in several species, although not in man. Investigation of the effect of protein binding on the metabolism of drugs is complicated because changes in chemical structure that can affect the extent of protein binding may also affect the nature and rate of metabolism.

2.5.3.3.2. *Excretion.* Several authors have considered the elimination of drugs possessing high binding affinities for plasma protein from a theoretical point of view but without complete agreement (K4, K15, M10). Considering the importance of this subject remarkably few experimental studies have been made. In the case of sulfonamides, a study showed no correlation between plasma protein binding and the rate of disappearance of the drug from plasma or its appearance in the urine. Indeed, no such effect would be expected if significant tubular secretion and reabsorption of the drug occurred, but it might if elimination occurred exclusively through glomerular filtration. Under these circumstances, the amount of drug appearing in the urine would be related to the unbound fraction present in the plasma.

In another experiment, the ranking of urinary excretion rates of salicylic acid and four of its derivatives paralleled, both in rabbits and in man, the extent to which the drug was bound to plasma proteins. The most extensively bound salicylate had the longest plasma half-life; and the least extensively bound, the shortest (K1).

Many drugs are eliminated unchanged (M18) or as metabolites in the

bile (P1, W13), and this route of excretion has received increasing attention in recent years, but so far the effect of plasma protein binding on biliary excretion is largely conjecture. For some drugs the rate of biliary excretion is a function of the ratio of their affinity for liver proteins to that for blood (P13), but the study upon which this conclusion is based is open to criticism on grounds of technique.

Biliary excretion of certain anions (S25) and cations (N2) may be by an active transport process, and many drugs are excreted into bile against a bile:blood concentration gradient exceeding 50:1 (G11).

2.6. Drug Metabolism

2.6.1. *Metabolic Pathways*

On the basis of the reactions and the biological activity of their products, the metabolism of drugs can be conveniently regarded as occurring in two phases (W11). Phase one reactions consist of oxidation, reduction, or hydrolysis, and these result in (1) deactivation, (2) activation of an inactive compound into an active drug, (3) conversion of one active drug into another.

The second phase of metabolism consists of synthetic reactions that convert active compounds into inactive excretory products. Drugs that contain suitable chemical groups, such as —OH, —NH_2, or —COOH, can undergo these synthetic conjugation reactions; if not present in the parent compound, such drugs may be introduced during phase one reactions. Phase one and phase two metabolic reactions occur sequentially.

Most phase one reactions are catalyzed by the drug-metabolizing enzymes (mixed function oxidases, oxygenases) located in the endoplasmic reticulum of liver and, to a lesser extent, in intestine, kidney, and lung. These enzymes have been the subject of intensive research (G7, G8, L1).

Eight different types of conjugation or phase two reactions are known to occur in man (W12). Conjugation with glucuronic acid, sulfate, or acetylation are among the most common. Both phases of drug metabolism, especially the conjugation mechanisms, increase the polarity of drugs and facilitate their excretion in the urine and/or bile.

The age of a patient can be important with regard to drug-metabolizing capacity. The newborn baby often has deficient drug-metabolizing capacity (G11). In the elderly, drug metabolism has not been carefully studied, but it is probably reduced (G11).

2.6.2. *Pharmacogenetics*

The striking genetic differences in the response to a small number of drugs is the basis of pharmacogenetics (V8). Succinylcholine was the

first drug to attract attention, but since then other drugs have been added to the list (L2). Genetic differences in receptor sensitivity may be responsible for some differences in response to drugs but are difficult to detect and evaluate clinically. It seems likely that most important differences are due to variations in the rate of metabolism, which is more amenable to clinical investigation. Measurement of plasma drug concentrations may often reveal hitherto inexplicable differences and aid rational therapy.

2.6.3. *Stimulation and Inhibition of Drug Metabolism*

Many foreign compounds, including drugs, induce microsomal drug-metabolizing enzymes (C9). Except in those cases where drugs are activated by phase one reactions, the extent and duration of their action is shortened in patients with induced enzyme activities, necessitating an increase in dose to achieve the same therapeutic effect. But the situation is complicated. Barbiturates, for example, increase the rate of metabolism of coumarin anticoagulants but also reduce their absorption (O10). The proved clinical importance of stimulation of drug metabolism is limited. The most important concern anticoagulants (D3, O10), cardiac glycosides (M22), and anticonvulsants.

Inhibition of drug metabolism may be clinically more important than stimulation, but it has not been so widely investigated (D3). There is evidence, for example, that both allopurinol and nortriptyline and possibly methylphenidate, oxyphenbutazone, methandrostenolone, and phenyramidol inhibit the metabolism of several drugs (V10). A rise in serum phenytoin concentration coupled with a doubling of plasma half-life occurred in three patients given chloramphenicol presumably due to inhibition of phenytoin metabolism (C5). Similar observations have been made in patients with steady-state phenytoin levels given a second anticonvulsant drug (B7). The danger of accumulation resulting from reduced metabolism may be diminished by plasma monitoring of drugs likely to be affected by this process.

There are genetic differences in the degree to which different individuals respond to a given dose of a drug inhibiting or stimulating drug metabolism (V10), and this cannot be predicted from a knowledge of the dose regimen.

2.6.4. *Plasma Half-Life*

Until recently it was assumed that with certain exceptions, such as ethanol and benzoic acid, the capacity of the enzyme system was not a rate-limiting factor in drug metabolism and the biological half-life of a drug could be represented by its plasma half-life and was independent of

the dose (L9). It is now apparent however, that the metabolism of many drugs is limited by the capacity of the enzyme system involved (L9), and the plasma half-life is consequently dose-dependent (L9, V9). Examples include dicoumarol, phenylbutazone, and phenytoin (V9), for which the larger the dose, within the therapeutic range, the longer the plasma half-life.

The plasma half-life of a drug is useful in determining the frequency of dosing for maintenance of the optimum therapeutic effect. When the dose interval is equal to the plasma half-life of the drug, four to seven doses are required to reach 95% of the steady state (W2). From a pharmacokinetic point of view an ideal dosing schedule is one in which the initial loading dose, equal to twice the maintenance dose, is followed by maintenance doses given at time intervals approximately equal to the average plasma half-life of the drug.

2.6.5. *Timing of Blood Samples*

Plasma drug concentrations can be interpreted properly only if the time elapsed since the last dose is known. There is no ideal time. Koch-Weser (K10) has suggested that for many drugs it is most informative to measure the plasma drug concentrations 1–2 hours after an oral dose when both absorption and distribution are nearly complete. For some drugs this is unsatisfactory, and most investigators prefer to sample immediately before the next dose of the drug is given or after an overnight fast.

3. Biological Response to Drugs

The rationale for measuring blood drug concentrations in the clinical milieu is that it provides an additional parameter for regulating and monitoring the response to therapy. There are, however, a large number of drugs that seemingly produce their beneficial effect indirectly through the agency of one or more biologically active substances, which may not only be more amenable to measurement, but provide more useful information. A classical example is the coumarin type of anticoagulants (K12). These potentially dangerous agents have a low therapeutic index and are able to be used extensively clinically only because they are believed to exert both their therapeutic and major toxic effects indirectly through their ability to influence blood coagulation mechanisms. So firmly is this view entrenched, that virtually no attention has been given to the possibility that the major beneficial effect, and at least some of the toxic manifestations, of coumarin-type anticoagulants could be directly related to the concentration of the drug in the blood and independent of its effect upon blood coagulation. Nevertheless because of the

ease with which both coumarins and their effects upon blood coagulation can be measured, this group of drugs have been among the most intensively investigated (K12). They have served as the model upon which much of the current teaching of pharmacokinetics and drug interaction is based.

The oral hypoglycemic agents—particularly the sulfonylureas—and hypolipidemic drugs share the same advantages, but one of them throws an interesting sidelight on the whole rationale of the measurement of changes in chemical parameters as an index of therapeutic efficacy. For almost a decade, clofibrate has enjoyed popularity as an hypocholesterolemic agent. On the unproved supposition that hypercholesterolemia predisposes to morbidity and mortality from myocardial insufficiency, it has been used extensively for the treatment of patients with hypercholesterolemia irrespective of its association with angina pectoris or other manifestations of coronary artery insufficiency. In 1972 the results of two large multicenter therapeutic trails of clofibrate in the treatment of patients with myocardial disease were published (C3, N4, O6). They established that clofibrate is indeed effective in reducing deaths from myocardial infarction, but only in one small group of subjects—and in them the beneficial effect was independent of, and unrelated to, any effect of the drug upon plasma cholesterol concentration.

These examples are given to show that a simplistic approach to therapy, which is based upon clinically useful but unproved hypotheses of disease causation, can lead to the neglect of studies into the relationship between blood drug levels and clinical response, as determined by morbidity and mortality, rather than by some easily measured but possibly irrelevant chemical parameter.

4. Clinical Applications of Blood Drug Measurements

It is difficult to overestimate the potential contribution to the understanding of the mode of action, pharmacological effects and therapeutic usefulness of drugs that can be made by measuring their concentration in blood and tissues. Nevertheless, at the present time the practical value of such measurements for monitoring and regulating drug therapy in individual patients is limited and does not fully justify the more optimistic forecasts of their potential usefulness. Some of the reasons for caution have already been mentioned, and others are discussed later in this chapter.

It should be possible, however, to predict those drugs for which plasma measurements are likely to prove most useful. (1) The drug should show a more or less close correlation between its concentration in the plasma and its therapeutic effectiveness and/or toxicity (see below); (2) the disease for which the drug is used must be of sufficient duration to make

multiple dosing, and consequently the achievement of a steady state, essential (see above); (3) the plasma half-life of the drug must be sufficiently long for differences in rates of absorption and distribution, and minor differences in timing in the collection of specimens to have only an insignificant effect upon the clinical interpretation of the analytical results; (4) a method capable of yielding accurate and precise analytical data sufficiently rapidly and economically to be practicable must be available.

4.1. Methodology

This subject has recently been extensively reviewed (B22, B23, L1), and only the most salient points will be discussed here.

4.1.1. *Extraction Procedures*

Most methods for measuring plasma drug levels depend upon an initial extraction procedure wherein the drug is separated from proteins and other substances likely to interfere with its assay (T2). The tenacity with which many drugs bind to protein and its variability at different drug concentrations may cause less than complete recovery, the extent of which may vary from one sample to another. Thorough denaturation of the plasma proteins, together with efficient extraction, overcomes the problem in most cases, but even established and well tried procedures have sometimes been shown to yield valid information over only a limited concentration range. Some reasons for this have been discussed by Nagashima *et al.* (N1).

4.1.2. *Colorimetry and Spectrophotometry*

Many methods for measuring the concentration of drugs in blood use the principles of colorimetry or spectrophotometry for quantitation (M3). With few exceptions, none are sufficiently specific, sensitive, and precise to be useful at plasma drug levels within the therapeutic, rather than the toxic range in patients whose drug history is not known with certainty (B10). An additional problem, seldom encountered in the analysis of biological fluids for endogenous substances is interference by metabolites which may or may not themselves be therapeutically active. The frequent failure of investigators to appreciate the limitations of their analytical methods accounts for many of the conflicting data relating blood drug levels to dosage, therapeutic response and toxicity, with which the literature abounds.

4.1.3. *Spectrofluorimetry*

Because of its greater sensitivity many drugs are amenable to measurement by spectrofluorimetry at the low concentrations at which they occur

in plasma when used therapeutically (A1, R11, U1). However, relatively few of the assays have been adopted for routine use by clinical biochemists often because of nonspecificity arising from interference by metabolites and other substances present in biological material especially that obtained from patients whose drug history is not known with certainty.

4.1.4. *Chromatography*

Methods for measuring blood drug levels based upon gas–liquid chromatography (GLC) have become commonplace. They often have the dual advantage of sufficient sensitivity and specificity to qualify them for monitoring treatment (B4, B18, B19, G3). Indeed separation of the native drug from its metabolites and their independent quantitation may, as in the case of the tricyclic antidepressants provide more information than measurement of the parent compound alone. Moreover, GLC techniques sometimes permit simultaneous measurement of two or more drugs used together and which may vary independently of each other. GLC methods, however, are currently still heavy consumers both of human and instrumental time and, despite the availability of solid microsample injectors, not readily amenable to automation or handling in large numbers in the clinical situation.

The introduction of GLC–mass spectrometry as an analytical tool (G14) and latterly of GLC–mass fragmentography as a quantitative technique (H4, K3, K14, S32) has reduced the limit of detection and extended the range of substances amenable to measurement in microsamples of blood to include virtually every simple organic compound used as a therapeutic agent in man. Experience with this technique is at present limited and, because of its enormous expense, is likely to remain so for many years. Nevertheless, the potential of GLC–mass fragmentography as a method for measuring the concentration of drugs and their derivatives in microsamples of blood and tissue obtained through a biopsy needle is so great that the possibility of its being available for clinical purposes within the next decade, at least in special centers, is far from remote (L4).

It can be expected that high-pressure liquid chromatography, which has already proved valuable in separation and measurement of mixtures of organic compounds (W10), will eventually equal, and possibly surpass, GLC for measuring blood drug concentrations. Currently the technique does not possess sufficient sensitivity to make it practicable for measuring drugs at the concentration they normally achieve in blood during therapy, and to date no method suitable for monitoring drug treatment has been described.

Thin-layer chromatographic (TLC) methods for measuring drugs in

blood (W10) do not ordinarily have sufficient sensitivity or precision to qualify them for serious consideration as suitable for regulating therapy except in the special situation of distinguishing signs and symptoms of drug intoxication from those of untreated or undertreated disease.

4.1.5. *Radioimmunoassay and Protein-Binding Methods*

These techniques, originally introduced for the measurement of hormones in blood (B12, E5), have already been adapted for use with a number of drugs (C2, F1, S19, S24, V4, V5), one of which, digoxin, has already become a routine determination in many laboratories. The principles upon which these methods depend have been extensively reviewed (K7). The advantages of radioimmunoassay for analysis are that (1) it is extremely sensitive so that even drugs present at a concentration of less than 10^{-9} w/w can be measured in microsamples of plasma with an acceptable degree of precision, (2) reagents can be prepacked, (3) it is amenable to large-batch handling and at least semi-automation, and (4) the technique has general applicability so that the number and type of drugs that can be measured by it are potentially limitless.

Disadvantages of the method include (1) the necessity for meticulous technical competence; (2) the unpredictability and difficulty of preparing the primary reagents, including antisera of appropriate specificity and avidity and isotopically labeled tracer material of sufficiently high specific activity to yield a practicable assay; (3) the short shelf life of isotopically labeled tracers, which necessitates either frequent preparation or repurification; and (4) the intrinsic and unpredictable nonspecificity of the technique, which may fail completely to distinguish biologically distinct but chemically closely related compounds. Methods for overcoming this latter disadvantage are available and include prior separation by extraction or chromatography, but they are tedious.

Two recent developments of immunoassay, namely the use of free radicals and enzymes (M17, R10) as markers instead of isotopic nuclides, can be expected to increase the scope and application of the technique. Both have already been applied to the detection and measurement of drugs in biological fluids but have not, so far, achieved the requisite specificity and precision necessary for clinical use.

4.1.6. *Miscellaneous Techniques*

4.1.6.1. *Radioactive Isotopes.* The administration of isotopically labeled drugs is now commonplace in the experimental pharmacology of every new therapeutic agent (K18, M4) but has little or no clinical application. It often provides much of the initial information about the pharmacokinetics upon which subsequent clinical practice depends, but

health hazards and economic considerations clearly prevent its extension outside the confines of experimentation.

The sensitivity and specificity conferred by making isotopically labeled derivatives of drugs *in vitro* can be used clinically (K18, K19), as can isotope dilution methods (H2, O7). The great imposition upon time, skills, and resources that such techniques demand, limits their application to selected research studies. They nevertheless provide a basis against which other more practicable methods can be assessed.

4.1.6.2. *Bioassay.* Bioassay is the principle upon which most methods for measuring plasma concentration of antibiotics are based and for this purpose has proved extremely sensitive, reliable, and useful. Only rarely have bioassays proved sensitive enough, however, to be practicable for measuring other types of drugs in blood, and even here the inherent technical difficulties have limited their usefulness to the gathering of scientific information in an experimental situation (G13).

4.1.6.3. *Flame Photometry, Atomic Absorption, and Neutron Activation.* Comparatively few substances amenable to measurement by these techniques are used therapeutically; chief among those that are being sodium, potassium, lithium, calcium, magnesium, zinc, copper, and iron, for all of which one or other of the techniques is the method of choice.

4.1.7. *Comparison of Methods and Quality Control*

The enormous interest shown by clinical chemists in the comparison of different methods of assay and quality control has had little impact upon the field of blood drug measurements. The complexity and laboriousness of establishing a therapeutic range—and the impracticability of deriving one locally—makes the importance of quality control in this area of clinical biochemistry probably greater than in any other. Preliminary data from a small informal quality control scheme between laboratories using different analytical techniques for measuring plasma phenytoin levels in which the authors have participated have revealed disconcertingly disparate results (see below).

In a study of 18 patients treated with phenytoin, Berlin *et al.* (B10) found that a widely used colorimetric method gave values for plasma phenytoin approximately 8% higher than those obtained using GLC. In two patients, however, the results were very different. In one patient the values obtained were twice as high, and in the other patient six times as high, with the colorimetric as with the GLC method. This may have been due to the presence of other drugs taken by the patient unbeknownst to the investigators, but which nevertheless interfered with the assay, and if so highlights the necessity for ensuring specificity of analytical techniques when used in a clinical situation. Lack of specificity is not always

obvious when a method is developed and evaluated under controlled experimental conditions.

4.2. Lithium

The clinical value of monitoring drug therapy by measuring plasma levels is probably best exemplified by reference to lithium (F6). It is a useful drug, which has a narrow therapeutic index, and treatment without reference to plasma levels is probably not ethically justified. Toxic side effects are predictable and severe. It has an acceptably long plasma half-life, and its measurement both in blood and urine is comparatively simple. Moreover, there is no problem of interference from either active or inactive metabolites.

Lithium has been used clinically for many years in the treatment of mania (D1, S27), and latterly as a prophylactic in recurrent depression (B1, B2, C10, H16). For a short while, about 1948, lithium salts were sold as a "common salt" substitute for patients on low sodium intake diets, but its high toxicity, culminating in a number of deaths, led to its discontinuance for this purpose.

Plasma lithium measurements are usually made on blood collected before the morning dose (F6), but some authors prefer a fixed time of around 12–18 hours after the last intake (S8). Lithium is usually administered orally as the carbonate, but other salts have also been used. Unless deliberately formulated to give a delayed release pattern, absorption from the gut is rapid and virtually complete. After about 8 days on a constant daily dose, excretion of lithium in the urine equals the amount ingested, but the exact time taken to reach this equilibrium varies markedly from patient to patient. Some remain in positive lithium balance for as long as 12 days whereas others achieve equilibrium after only a few days (S11, T5). The reasons for these differences are not known. But, contrary to some expressed views (S13), they are of no diagnostic, prognostic, or therapeutic significance (S27) except insofar as they affect the plasma lithium level produced by a standard daily dose. Lithium equilibration and distribution in the body during therapy is both slow and complicated (M20, R1, S6). In the steady state the ratio of intracellular to extracellular lithium concentration is 2.15 in striatal muscle but only 0.36 in liver (S6). The presence of large, slowly equilibrating pools of lithium explains why lithium removal by peritoneal or hemodialysis in cases of lithium toxicity, though the best treatment currently available, is nevertheless both difficult and often unrewarding. It also provides the rationale for continuing dialysis long after the plasma lithium concentration has returned to acceptable levels (A6, H9, W14).

Lithium can be measured in plasma, and other biological fluids, either

by emission flame photometry or atomic absorption. Despite their apparent simplicity, both techniques require high quality instrumentation and strict attention to detail if the results obtained are to be of sufficient accuracy and precision to be clinically useful. Some older, simpler flame photometers were inadequate for this purpose, but with modern equipment and lithium standards of similar sodium and potassium composition to diluted plasma, measurements can be made even on microsamples of plasma with acceptable accuracy and precision (A5, L8, V11). It may or may not be necessary, depending on the design of the flame photometer and of the atomizer in particular, to deproteinize plasma samples prior to analysis (A5). Atomic absorption offers no material advantages over emission spectrophotometry for the measurement of lithium in plasma (L8), but is more suitable for measurements on urine and other biological fluids of uncertain electrolyte content.

Lithium excretion is exclusively via the urine, but it is complicated and does not obey first-order kinetics. Nevertheless, for practical purposes, lithium can be considered to have a plasma half-life of between 24 and 48 hours, which is decreased by salt feeding and increased by salt deprivation or kidney damage (T5).

Steady-state plasma lithium levels correlate well with dosage in the same individual providing that salt intake does not change drastically or kidney damage develop. On the other hand, they correlate poorly with the daily dose of lithium in different individuals (S11). This may help explain why some clinical trials of lithium given in fixed dosage as a prophylactic in recurrent depression failed to demonstrate its superiority over placebo, whereas others, in which dosage was adjusted so as to maintain the plasma steady-state lithium level within a comparatively narrow and well defined limit, were unequivocally favorable (B1, B2, C10, H16). Undoubtedly toxic side effects can occur at almost any plasma lithium level, but they are seldom serious at plasma lithium levels below 1.5 mmoles/liter and only very rarely give rise to concern until steady-state plasma levels exceed 2.0 mmoles/liter (S7, S8, T5). Steady-state plasma lithium levels above 3.0 mmoles/liter are potentially dangerous and, unless promptly reduced, constitute a serious threat to life. If the plasma lithium level exceeds 4.5 mmoles/liter, it almost invariably portends a fatal outcome except when it is the result of acute intoxication due to deliberately large intake with suicidal intent. Under these circumstances a patient has been known to survive a plasma lithium level of 8.2 mmoles/liter following ingestion of 22.5 g of lithium carbonate (H12), probably owing to the slowness with which plasma lithium equilibrates with the brain (M20).

What constitutes optimal therapeutic plasma levels for lithium, and

whether they are the same both for the treatment of mania and prevention of recurrent depression, is not known for certain. Schou and his colleagues (B1), in their classic study of the use of lithium for prevention of recurrent depression, considered patients with steady-state plasma lithium levels of between 0.6 and 1.5 mmoles/liter as receiving acceptable treatment. Nevertheless, they believe that for optimum effects plasma lithium concentration in blood collected at least 10 hours after the last dose should be maintained between 0.8 and 1.2 mmoles/liter (S8). Other authors who have adopted these recommendations have found them satisfactory. In clinical practice, however, relatively few patients receiving long-term lithium therapy are maintained within these limits (F6). This may, in part, be due to the tendency of all patients to default on drug taking (P10), but, more important, reluctance by clinicians using lithium to increase the daily dose of the drug beyond an arbitrary upper limit regardless of the plasma concentration achieved.

4.3. Anticonvulsant Drugs

4.3.1. *Phenytoin*

Phenytoin, introduced as an anticonvulsant drug in 1938, remains one of the drugs most frequently prescribed for convulsive disorders. The precise mode of action is unknown, but it appears to inhibit the accumulation of sodium in nerve cells, thus stabilizing hyperexcitable cell membranes (A13)—a property also utilized in the treatment of cardiac arrhythmias.

The success of drug therapy in the treatment of epilepsy is singularly difficult to assess owing to the extreme variability of the disorder, both in type of seizure pattern and the frequency with which attacks occur. The natural history of the disorder is also notoriously variable, further increasing the difficulties of assessing the contribution made by therapeutic agents. Moreover, the therapeutic aim varies from patient to patient. In one subject, for example, "successful" therapy may mean complete seizure control, while in another a reduction in seizure frequency from daily to weekly attacks may be considered adequate.

The potential benefits of regulating therapy on a precise biochemical basis are obvious. However, lack of suitable chemical methods of analysis has, until comparatively recently, limited biochemical monitoring of anticonvulsant therapy to a few specialized centers. Methodological problems still exist but routine phenytoin determinations now lie within the scope of any moderately well equipped clinical biochemistry laboratory.

The earlier methods of measuring phenytoin, including colorimetric

(D5, W4, W6), ultraviolet (O1, S31), spectrophotometric procedures, and thin-layer chromatographic methods (H14, O2), have been superseded largely by more sensitive and specific gas–liquid chromatographic methods (B4, E7, F5, G3, K20, M2, M14, S4, T4). The final choice of method depends in each laboratory on factors such as the availability of apparatus and the decision as to whether simultaneous determination of other anticonvulsant drugs is contemplated. Fewer problems arise if phenytoin is the sole drug, and in these circumstances modified versions of some of the older techniques (D6) may have advantages over GLC methods.

After an oral dose of the sodium salt, peak plasma phenytoin levels occur from one (H5) to between 4 and 8 hours (D5) later. After either oral or intravenous administration, phenytoin accumulates rapidly in organs such as the liver, kidney, and salivary glands, and maximum levels are reached within 30 minutes even in tissues such as the brain and skeletal muscle, which accumulate phenytoin more slowly. Uptake by fat is delayed (N5).

Phenytoin is distributed equally between plasma and red cells (D5), and in the steady state the ratio of the concentration in blood and brain remains constant (N5). Biphasic curves after oral or intravenous phenytoin have been reported (H5, N5) but have not been found by all workers (S17).

Normally, approximately 70% of blood phenytoin is protein bound, and under steady-state conditions the unbound and protein-bound blood fractions and tissue fractions are in equilibrium. Decreased protein binding in uremic states has been described (R5).

The principal metabolite of phenytoin is 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (parahydroxy phenytoin) which, as the glucuronate, forms over 70% (65–81%) of the urinary excretion products. Free phenytoin accounts for less than 5% of the total (G9). Little phenytoin is excreted in the feces although free and hydroxylated phenytoin are excreted into the saliva and bile (N5).

Phenytoin has a long half-life, 18–24 hours, after oral administration (G9), which may be prolonged further at high dosages (V1). Twice daily prescriptions of phenytoin should result in relatively constant blood concentrations once steady-state conditions have been achieved, a process taking 5–15 days (K21). A limited amount of experimental data supports this prediction (B30, C1, D5, H11).

In most subjects, an increasing dose of phenytoin results in an increasing capacity to hydroxylate the drug until a maximum dose of approximately 10 mg/kg body weight is reached. However, genetic variations in hydroxylating ability appear to exist, and in a small minority of subjects unbound drug accumulates, precipitating toxicity, with doses of only 1–3 mg/kg (K22). Other factors influencing phenytoin metabolism

include liver function, the availability of cofactors and drugs taken in addition to phenytoin.

Sulthiame (H7, O4), phenacemide (H15), chloramphenicol (C5), dicoumarol (H6), antituberculous drugs (K23), disulfiram and phenyramidol (S23) have been described as inhibitors of phenytoin metabolism, while alcohol and possibly phenobarbitone have the reverse effect. Clinical and experimental evidence regarding the latter are confusing (B16, B29, C11, C12).

It is hardly surprising that blood phenytoin levels bear little relation to the total daily dose (B4, B5, C1, G4, G6, L12), or that the concept of the steady-state blood levels frequently does not hold in clinical practice (B7, M15). This indicates the necessity for regulating phenytoin therapy on the basis of blood concentrations, assuming that this bears a reasonably close correlation to the therapeutic effect. Conclusive evidence of the validity of this basic assumption is lacking (Table 1).

Buchthal *et al.* (B31) demonstrated improved seizure control in patients suffering from grand mal epilepsy when the dose of phenytoin was adjusted to achieve blood concentrations of over 10 μg/ml, and significant improvements in electroencephalographic recordings were also observed. No toxic side effects were apparent with plasma phenytoin levels below 14 μg/ml and were only mild in association with levels below 30 μg/ml. However, severe phenytoin intoxication was observed in 50% of all patients with blood levels above this. Kutt *et al.* (K22) described a detailed relationship between blood levels and the appearance of various manifestations of toxicity, and it has even been suggested that observation of these phenomena could be used as a guide to blood phenytoin levels (H1).

Buchthal *et al.* (B31) defined a therapeutic range for plasma phenytoin levels of 10–20 μg/ml. This, or the narrower range of 10–15 μg/ml described by Kutt and McDowell (K21), has been generally accepted into clinical practice, although further confirmatory evidence is inadequate and is obtained mainly from retrospective comparisons of plasma phenytoin levels and seizure control in random samples of patients—many of whom were receiving additional anticonvulsant drugs (H1, L19). Not uncommonly, higher blood phenytoin levels are observed in patients in whom seizure control is poor, reflecting the greater drug dosage prescribed in these circumstances (B29).

Seizure control is improved in many patients when the dose of phenytoin, and blood concentration, is increased, although the occurrence of toxic symptoms may limit this process. Baylis (B7) and co-workers examined the monthly fit frequency in 47 children with severe epilepsy in whom the only change in medication was in the dose of phenytoin, which in each case was sufficient to produce a rise in blood phenytoin

TABLE 1
THERAPEUTIC AND TOXIC PLASMA PHENYTOIN LEVELS

Reference	Analytical methods	Number of cases	Plasma phenytoin (μg/ml)		Comments
			"Therapeutic range"	Toxicity	
Buchthal *et al.* (B31)	UV	80	10–20	Onset 15–30 Severe > 30	Prospective study correlating blood levels and EEG data
Kutt and McDowell (K21)	Colorimetric	(Review)	10–15 (20)	Onset 15–25 Severe > 30	—
Haerer and Grace (H1)	UV	166	Good control, mean 11.3 Poor control, mean 9.4	Onset 15–19 Severe > 30	Out-patients
Buchanan and Allen (B29)	GLC	Well controlled = 108 Poor control = 15	0–45.5, mean = 6.7 1.2–24.0, mean = 10.1	Onset (mean) 11.9	Single determinations on out-patient children
Lund (L19)	Colorimetric	294	Complete control, mean = 12.6 ± 8.8 Poor control, mean = 8.6 ± 4.6	Onset 27.5	Single determinations on out-patients
Baylis (B7)	GLC	177	20–25	Severe > 25	Prospective study on in patient children

level of at least 5 μg/ml. Twenty-nine of the children improved, four were the same, and fourteen were worse. Of these latter, six had increased seizures when the blood phenytoin concentration rose above 20 μg/ml—possibly owing to drug intoxication. Plasma phenytoin levels exceeded 25 μg/ml in 41 other children (also with severe epilepsy), and 70% of these were free from grand mal seizures. However, many showed signs of phenytoin intoxication.

A note of caution must be sounded with regard to the absolute values of therapeutic or toxic blood phenytoin levels, for these may differ markedly depending on the method and place of assay. A recent survey revealed large variations between results obtained by several laboratories, which on occasions, were as great between those employing the same type of analytical process (for example GLC) as between laboratories using other techniques.

The conclusion seems inescapable that random measurements of plasma phenytoin levels are useless apart from detecting suspected drug defaulters or confirmation of gross toxicity. The optimum concentration for any individual patient must be determined and regular monitoring carried out thereafter to check that this level is maintained. Thus it should be possible to improve seizure control even in severely affected patients without precipitating toxicity.

4.3.2. *Phenobarbitone, Primidone, and Carbamazepine*

While regular monitoring of plasma phenytoin levels can result in improved seizure control, the benefit derived from measuring other commonly prescribed anticonvulsant drugs is difficult to assess. Phenobarbitone, primidone, and carbamazepine will be discussed briefly.

4.3.2.1. *Phenobarbitone.* Phenobarbitone was introduced in 1912 and forms one of the mainstays of anticonvulsant therapy. Methods for determining phenobarbitone include colorimetric (C14) or ultraviolet (B27, O1, S31) spectrophotometric procedures (L15), thin-layer chromatography (G2, H14, O3), and gas–liquid chromatography (B4, E7, G3, K20, L5, M13, M14, T4, V2).

Phenobarbitone has a long half-life of about 3 days (M7) and plasma levels correlate moderately well with the daily dose (B4, B32, S30). A therapeutic range (Table 2) of 8–21 μg/ml has been described (S30), and severe toxic symptoms are rarely encountered in association with plasma levels below 50 μg/ml (L11).

Buchanan and Allen (B29) failed to demonstrate a relation between plasma phenobarbitone levels and seizure control in 128 children managed on an out-patient basis, but the significance of their findings is difficult to assess. Their results referred to single determinations carried

TABLE 2
THERAPEUTIC AND TOXIC PLASMA PHENOBARBITONE LEVELS

Reference	Analytical method	Number of cases	Plasma phenobarbitone (μg/ml) "Therapeutic range"	Plasma phenobarbitone (μg/ml) Toxicity	Comments
Svensmark and Buchthal (S30)	UV	13	8–21	—	Prospective study correlating blood levels and EEG data
Huisman *et al.* (H15)	TLC	—	30–40	mean = 70	Levels refer to total plasma phenobarbitone (including Primidone metabolite)
Gardner-Thorpe *et al.* (G4)	TLC	121	0–54 mean = 12	—	Unselected patients; mainly single determinations
	GLC	47	1–77	—	

out before any attempt had been made to adjust the therapy to improve seizure control and performed on subjects receiving additional anticonvulsant therapy. Essentially similar results were found by Baylis (B7) and co-workers, who compared plasma phenobarbitone levels and seizure control in 200 children with epilepsy of sufficient severity to need care in a residential hospital school. They found no correlation between seizure control and plasma phenobarbitone at concentrations up to 60 μg/ml.

4.3.2.2. *Primidone and Carbamazepine.* Even less is understood about the significance of blood levels of drugs such as primidone or carbamazepine and seizure control. Primidone differs from phenobarbitone by only one carbonyl group on the pyrimidine ring (in primidone the oxygen atom is replaced by 2 hydrogen atoms), and rapid bioconversion of primidone to phenobarbitone takes place in subjects receiving the drug on a long-term basis (B17, O5). Plasma levels of phenobarbitone and primidone in subjects receiving normal "therapeutic" doses of primidone (Table 3) have been reported (B17, G4, H15). The phenobarbitone: primidone ratio is usually greater than one, and the phenobarbitone levels correlate moderately well with the daily dose of primidone (B7).

The relation between the drug levels and seizure control is not clear, and opinion is divided whether primidone exerts anticonvulsant activity solely as the metabolite phenobarbitone (B15, O5) or as native primidone, as suggested by experimental (G1) and clinical (B7, B17) studies.

TABLE 3
THERAPEUTIC AND TOXIC PLASMA PRIMIDONE LEVELS

Reference	Analytical method	Number of cases	Plasma primidone (μg/ml) "Therapeutic range"	Toxicity	Comments
Booker *et al.* (B17)	GLC	30	0–23, mean = 9.2	>10	Unselected patients. Toxic subjects were all receiving additional anticonvulsant therapy
Gardner-Thorpe *et al.* (G3)	GLC	23	4–48, mean = 19	—	Unselected patients
Baylis (B7)	GLC	47	3–14, mean = 6.2	—	Children in-patients
		21	2–17, mean = 6.7	—	Unselected adult out-patients

Toxic side effects in association with primidone levels of 10 μg/ml or more have been reported (B17).

Primidone has a comparatively short half-life of 10–12 hours (B17). Therefore, the time relationships between blood sampling and dose regimes acquire greater importance when interpreting the results than with either phenobarbitone or phenytoin.

Little is known as yet about the pharmacokinetics of carbamazepine in humans although preliminary reports suggest slow absorption (M21) and marked variations in blood levels during a day in some subjects (M15). Blood levels varying from trace quantities to 12 μg/ml have been found in patients taking 400–1000 mg daily (P3), but no relation between the level observed and the dose was apparent. In the studies so far published carbamazepine blood levels have not correlated with seizure control (P3), but all the subjects were receiving additional anticonvulsant drugs.

Regular monitoring of blood phenytoin levels provides a valuable contribution to the management of patients with epilepsy. The value of determining the blood level of other anticonvulsant drugs is unconfirmed. At present such work should be undertaken only as prospective research procedures combining clinical and biochemical methods of assessment and patient-management.

4.4. CARDIAC GLYCOSIDES

Although 200 years of clinical experience has been gained since William Withering published his "Account of the Foxglove and Some of Its

Medical Uses," difficulties are still encountered in the use of the cardiac glycosides, of which digoxin is the most important. Among the problems are those of assessing "adequate digitalization" clinically, and the virtual impossibility of measuring end-organ effectiveness.

Myocardial sensitivity to cardiac glycosides is influenced by a variety of factors including the concentration of potassium, calcium, and magnesium in the plasma. The frequency and severity of toxic side effects to digoxin is affected by sodium balance, thyroid status, the presence of severe heart disease and chronic pulmonary disease. Although their nature varies, some such as nausea, vomiting, and cardiac arrhythmias may be indistinguishable from those for which cardiac glycosides are themselves prescribed. It may be impossible, therefore, to distinguish clinically between signs and symptoms of under- and overdigitalization (L6).

Only three of the metabolically active cardiac glycosides, namely digoxin, digitoxin, and lanatoside C, are commonly used in therapy. Digoxin is the form most frequently prescribed in the United Kingdom and the United States.

The clinical pharmacology of both digoxin and digitoxin have been extensively investigated using tritium-labeled drugs (B14, D8, D11, M5, M6, R3) in patients and human volunteers; they exhibit several important differences.

Digoxin is rapidly absorbed from the gut in the fasting subject and reaches a peak concentration in plasma after 30–60 minutes. When taken with food absorption of digoxin is delayed and peak plasma levels occur around 120 minutes after ingestion (W8). Steady-state levels are reached 4–6 hours after the last oral dose. In patients receiving long-term therapy plasma digoxin levels fall slowly with a plasma half-life of about 34 hours (D8).

Digoxin in the blood is virtually confined to the plasma where, in the therapeutic range, only a relatively small percentage (30%) is protein-bound (P7). After a single intravenous injection of digoxin only 3% remains in the blood after 1 hour in normal subjects (M6). Since only 30% is excreted during the first 24 hours after injection, the data indicate a large volume of distribution.

Digoxin is selectively concentrated in the kidneys, liver, and myocardium. In the latter its concentration is, on average, almost 30 times that of the plasma, but there are large individual differences (C8, S20).

Digoxin is excreted in the urine largely unchanged, and in patients on long-term therapy approximately 30% of the total body content of digoxin is excreted by this route each day (M5). The proportion is decreased in patients with impaired renal function (D8). Biliary and fecal

excretion of digoxin is small, and scarcely any is destroyed by metabolic conversion (D10, M5).

Digitoxin is also rapidly and completely absorbed from the stomach and upper small intestine (B14), but unlike digoxin is predominantly (90–97%) protein-bound in plasma (D8, L18). The plasma half-life of digitoxin is longer than that of digoxin and has variously been reported as 50 hours (D8), 4–6 days (L18) or 8 days (B14). Moreover in contrast to digoxin, digitoxin is excreted in the urine primarily as its metabolites, including digoxin (D8). Biliary and fecal excretion is of relatively minor importance (B14) and, as would be expected of a drug that is eliminated largely by metabolism, the plasma half-life of digitoxin is not materially influenced by impaired renal function (R3).

Although valuable information about the parmacokinetics of digoxin and digitoxin was gained using isotope-labeled drugs, investigation into the significance of tissue and blood cardiac glycoside levels under clinical conditions has had to await the development of simpler but equally sensitive methods with greater general applicability. Many such methods have been introduced during the past decade, but, apart from radioimmunoassay (E8, S19, S20, S21), none, including those techniques based upon double-isotope derivatization (L18), inhibition of erythrocyte ^{86}Rb uptake (G13, L16), and enzyme inhibition (B9), have fulfilled these conditions or found wide clinical acceptance.

Radioimmunoassay procedures, some of which are available commercially in kit form, generally employ antibodies to digoxin raised in rabbits; these antibodies cross-react with both digoxin and digitoxin. Consequently they can be used for measuring either one of these two drugs. The affinity of antibodies for cardiac glycoside is so much greater than that of plasma proteins that preliminary extraction is unnecessary.

Cross reactivity between the different cardiac glycosides is of little importance when pure drugs are employed for treatment, but its potentially dangerous implications for patients receiving uncertain or mixed digitalis therapy has been discussed by Edmonds *et al.* (E3).

Tritiated digoxin has been used as a tracer in most of the published procedures, but recently techniques employing [^{125}I]tyrosine-labeled digoxin have been described. Such techniques not only are more sensitive, but have the added advantage of dispensing with the necessity to use scintillation counting.

4.4.1. *Digoxin*

Most clinical studies published relate to plasma digoxin levels. In many the time relationship between collection of the sample and the last dose of digoxin was neither controlled nor specified (Table 4). However, for a

TABLE 4

CARDIAC GLYCOSIDES: THERAPEUTIC AND TOXIC PLASMA DIGOXIN LEVELS

Reference	Analytical method	Number of cases	Plasma digoxin (ng/ml)		Dose: blood sampling time relationship
			Therapeutic range	Toxic range	
Lowenstein and Corrill (L16)	Rubidium 86	—	0–5	—	Not specified
Grahame-Smith and Everest (G13)	Rubidium 86	50	0.8–4.5	4.0–>8.0	Not specified
Chamberlain *et al.* (C4)	Radioimmunoassay	139	0.25–3.2, mean = 1.4	2.0–5.2, mean = 3.1	8–24 Hours
Evered *et al.* (E8)	Radioimmunoassay	67	0.4–5.0, mean = 1.6	—	Not related
Smith and Haber (S20)	Radioimmunoassay	227	0.3–3.0, mean = 1.4	1.6–13.7, mean = 3.7	8–12 Hours
Beller *et al.* (B8)	Radioimmunoassay	93	Mean = 1.0	Mean = 2.3	6–48 Hours
Fogelman *et al.* (F2)	Radioimmunoassay	104	Mean = 1.4	Mean = 1.7	8–24 Hours
Redfors (R4)	Rubidium 86	11	0.5–1.4, mean = 1.1	0.7–1.9, mean = 1.6	24 Hours; *N.B.* Both optimum and toxic levels were determined on each subject

drug with such a long plasma half-life this is comparatively unimportant, provided blood sampling is performed at least 6 hours after the last dose when steady-state levels will have been reached.

Correlation between the daily dose of digoxin and plasma digoxin levels in randomly selected patients (E8) is poor, but it is improved if only those with normal renal function are considered (C4, R4). The difference is consistent with the primary role played by the kidneys in the excretion of digoxin and the likelihood that many patients treated with digoxin are elderly and infirm and have impaired renal function (B6).

The majority of authors agree that plasma digoxin levels in adequately digitalized but nontoxic patients are lower than those in whom toxic symptoms are present and prominent, but there is considerable overlap. Moreover, precise limits for therapeutic effectiveness and toxicity are, for reasons already discussed difficult or impossible to define. Few patients derive much benefit from digoxin at plasma levels below 0.7 ng/ml, and toxic symptoms do not become unduly frequent until plasma digoxin levels exceed 2 ng/ml.

In contrast to the majority of studies, Fogelman *et al.* (F2) could not discriminate between plasma digoxin levels in toxic (1.7 ± 1.3 ng/ml) and nontoxic digitalized (1.4 ± 1.1 ng/ml) patients. The discrepancy between this study and others probably derives from the difficulty of defining precisely either "therapeutic" or "toxic" plasma digoxin levels. There is, for example, little correlation between the plasma digoxin level and its therapeutic effects when this is measured objectively in terms of resting heart rate in patients with atrial fibrillation (C4, R4). Measurement of the systolic portion of the cardiac cycle with which plasma digoxin (and digitoxin) correlate well (S14) is not possible on a routine clinical basis, and no single type of cardiac arrhythmia is pathognomonic for digitalis intoxication. With these qualifications, however, a "therapeutic" range for plasma digoxin levels of 0.7–2.0 ng/ml in blood, taken at least 6 hours after the last dose, is suggested, with the proviso that there exists an important degree of overlap between therapeutic and toxic blood levels.

4.4.2. *Digitoxin*

Fewer comparable studies (B8, B9, S19) to those reported for digoxin have been carried out on patients receiving digitoxin, but they permit of similar conclusions. Plasma digitoxin concentrations are about 15 times higher than those of digoxin owing mainly to its higher binding affinity for plasma proteins (Table 5).

The conclusion is inescapable that neither plasma digoxin nor digitoxin levels are infallible guides to drug-induced toxicity, but must be

TABLE 5
THERAPEUTIC AND TOXIC PLASMA DIGITOXIN LEVELS

Reference	Analytical method	Number of cases	Plasma digitoxin (ng/ml)		Dose:blood sampling time relationship
			Therapeutic range	Toxic range	
Lowenstein and Corrill (L16)	Rubidium 86	—	10–50	—	Not specified
Bentley *et al.* (B9)	Enzymatic	233	10–40, mean = 25	Overlap with higher therapeutic levels: >45 frequently highly significant	3 Hours
Smith (S19)	Radioimmunoassay	58	3–39, mean = 17	26–43, mean = 34	Nontoxic subjects 6–12 hours; toxic subjects 12–48 hours after last dose increment
Beller *et al.* (B8)	Radioimmunoassay	37	Mean = 20	Mean = 30	6–48 Hours

interpreted in the full clinical setting. Nevertheless in certain clinical situations a knowledge of the plasma drug level can be of particular value. Examples include occasions when information about therapy is inadequate or deemed to be inaccurate, as is so often the case in elderly forgetful patients; in patients with gastrointestinal disease and secondary malabsorption of digoxin (H10); those with primary or secondary reduction in glomerular filtration and in hemodynamically unstable patients in whom intercurrent illness may precipitate digitalis toxicity. Plasma digoxin assays have also been useful in determining the cause of digitalis toxicity following changes in drug formulation (S15, V12). Providing the results are intelligently applied (D9), measurement of plasma digoxin or digitoxin levels can play an important part in the clinical management of patients (R4).

4.5. Lignocaine (Lidocaine)

Lignocaine, originally introduced as a local anesthetic, is now widely used for the treatment and prevention of ventricular arrhythmias. When used for this purpose, it is usually administered either by intramuscular injection, or as a bolus intravenously, or, more commonly, by constant intravenous infusion. For clinical purposes, lignocaine measurements are usually carried out on plasma collected either while the patient is receiving a constant intravenous infusion or at a specified time after the last intramuscular injection. Colorimetric methods have been used in the past (S29), but, because they lack both sensitivity and specificity, may yield false and misleading results. They have largely been replaced by GLC techniques (A3, E1, K5).

Despite reports to the contrary (P2), lignocaine is not therapeutically effective when given orally and does not achieve satisfactory blood levels when these are measured by specific techniques. Moreover, since the oral route of administration is associated with an unacceptably high incidence of side effects, which are probably due to metabolites of lignocaine rather than to the drug itself, this mode of administration has not gained widespread acceptance.

Even when given by the parenteral route, blood lignocaine levels correlate poorly with the dose of drug given (S10). Lignocaine is rapidly metabolized in the body—mainly in the liver—and only a clinically insignificant amount of the unchanged drug is excreted in the urine even at acid pH. In the "steady-state" the plasma half-life of lignocaine at therapeutic blood levels in healthy subjects is 108 ± 7 minutes (T1). It is substantially greater in patients suffering from heart failure and more than trebled by liver disease, and is the reason why patients with these conditions may tolerate usual doses of lignocaine poorly (T1).

Ventricular arrhythmias are well controlled when the plasma lignocaine concentration lies between 2 and 6 μg/ml (H8, S10). The efficacy of the drug is low at blood levels of less than 2 μg/ml and side effects, predominantly affecting the nervous system, begin to increase in frequency as plasma lignocaine levels exceed 6 μg/ml.

Plasma steady-state levels of up to 10 μg/ml are occasionally necessary for control of ventricular arrhythmias but cannot always be tolerated without serious toxic effects. Of thirteen patients referred to a specialist coronary care unit because of reputed refractoriness to the therapeutic effect of lignocaine, in only four was the diagnosis substantiated by demonstrating failure of therapeutic response to blood lignocaine concentrations in excess of 10 μg/ml (H8). In four patients a therapeutic response was observed at blood lignocaine levels between 5 and 10 μg/ml, and another five patients were responsive to lignocaine blood levels within the usual therapeutic range.

Animal experiments have shown (A3) that equilibration of lignocaine between blood and brain occurs relatively slowly. This may explain why plasma levels of lignocaine that can readily be tolerated, without cerebral side effects, after intravenous bolus injection nevertheless are associated with serious toxic symptoms when produced by constant intravenous infusion or when resulting from impaired metabolic degradation.

4.6. Procainamide

The therapeutic effectiveness of this valuable anti-arrhythmic agent is closely related to its plasma concentration (K11, K13). The drug is effective orally, but may also be given by intravenous infusion. Blood levels bear little relationship to the amount given, which is consequently a poor predictor of therapeutic response. Plasma procainamide concentrations within the range 4–8 μg/ml correlate well with clinical effectiveness. Evidence of toxicity is uncommon at plasma concentrations less than 12 μg/ml. Only a relatively small proportion (15%) of procainamide present in the plasma is protein bound, and its concentration in red cells is about two-thirds that of plasma. The plasma half-life of procainamide is in the region of 3.5 hours, but there are wide individual variations (range 1–6 hours). Koch-Weser *et al.* (K11) have stressed that with such a short plasma half-life procainamide should be administered about every 3 hours in order to prevent fluctuations of more than 50% in its plasma concentration, instead of at 4 or 6 hours as is common practice. Approximately one-half of the dose administered is excreted unchanged in the urine (M8)—the remainder is metabolized.

Methods available for measuring the plasma concentration of procainamide are well within the capabilities of most clinical laboratories.

Results obtained by spectrophotometric and spectrofluorometric methods agree closely with each other (K11) and are sufficiently sensitive and specific for general use. GLC methods are also available (A12) but do not offer any obvious advantage over older methods for clinical use.

Using plasma procainamide levels as a guide to therapy, it has been demonstrated (K13), contrary to evidence derived from uncontrolled trials (E2), that procainamide is valuable for preventing as well as controlling ventricular arrhythmias which develop in the immediate post-myocardial infarct period and are one of the main causes of death at this time.

4.7 Phenylbutazone and Oxyphenbutazone

Phenylbutazone and its derivative oxyphenbutazone have analgesic, anti-inflammatory, and antipyretic properties and are used mainly in the treatment of rheumatic disease. Both drugs are readily absorbed from the gastrointestinal tract, and peak blood concentrations occur after 2 hours. Phenylbutazone is bound to plasma proteins to the extent of about 98% at therapeutic plasma concentrations, ranging from 50 to 150 μg/ml (B33). Phenylbutazone has a long biological half-life in man; one report indicates a mean value of 3.3 days (L7), and in an investigation of monozygotic and dizygotic twins by Vessel and Page (V8), plasma half-life values ranged from 1.2 to 7.3 days with a mean of 3.0 $\pm$ 0.3 days. Vessel and Page (V8) concluded that the variability in the rate of phenylbutazone disappearance from human plasma was a genetically determined trait, and it has been suggested (W9) that phenylbutazone metabolism is genetically controlled in a similar way, and to a similar degree, as is body height.

Phenylbutazone is metabolized by the liver at a rate of about 15–25% per day (B33), but plasma levels do not increase proportionately with increasing doses of the drug. The work of Burns *et al.* (B33) indicates that above a certain level plasma phenylbutazone concentrations plateau. The concentration at which this occurs varies among individuals and is probably a reflection of the level at which "saturation" of high-affinity plasma protein binding sites occurs.

The plasma concentration of phenylbutazone is not proportional to the dosage, probably owing to genetic variations in its rate of metabolism (V8, W9), but in any individual patient on a constant dosage the plasma concentration remains constant over prolonged periods.

The plasma concentration of phenylbutazone is related to its therapeutic effect, but not to the daily dose, which may need to vary several-fold from patient to patient in order to achieve similar blood levels.

In one series of patients with rheumatoid arthritis, a plasma phenyl-

butazone concentration of 80–100 μg/ml was required initially for suppression of the symptoms but lower levels of 46–80 μg/ml were adequate to maintain remission (C13). Meanock (M13), using whole blood, suggested a therapeutic concentration range of 50–100 μg/ml whereas Pemberton (P4), who used serum, considered concentrations of 120–160 μg/ml desirable. Bruck *et al.* (B28) reported maximal suppression of symptoms of rheumatoid arthritis at serum phenylbutazone levels between 50 and 100 μg/ml, but once adequate therapeutic levels were reached any marked reduction in plasma phenylbutazone often resulted in relapse. In another study (T3) similar results were obtained, but in several cases a therapeutic effect was obtained at whole blood levels as low as 25 μg/ml. In gout, a plasma phenylbutazone concentration of about 100 μg/ml appears to be useful in the relief of symptoms as well as producing a uricosuric effect (Y1).

Side effects and plasma phenylbutazone levels correlate, and according to Bruck *et al.* (B28) the critical level is about 100 μg/ml. Above this value the incidence of toxic effects increases markedly without further clinical improvement (cf. phenytoin). The suggestion by Bruck *et al.* (B28) that ideally phenylbutazone dosage should be adjusted to maintain optimum blood therapeutic levels has not been generally adopted, partly because of the tediousness of assays on a routine basis, and partly because their value as an aid to regulation of treatment has not been well appreciated.

While most authors agree that an optimum therapeutic plasma concentration for phenylbutazone exists, its precise limits are ill defined. Reasons for this include differences due to plasma protein-binding secondary to the disease process, and the use of whole blood by some workers and plasma or serum by others. In many studies the time of sampling with regard to the last dose of drug was not strictly controlled. The method used for measuring phenylbutazone in these studies was in all cases based on the spectrophotometric procedure of Pulver (P14), the precision and reproducibility of which has been criticized by Moss (M23). This and other methods commonly used for measuring phenylbutazone in plasma in the past did not distinguish between the native drug and its metabolites, but since its major metabolite, oxyphenbutazone, has similar therapeutic properties to the parent drug, this may not be as serious a disadvantage in practice as it is in theory (B33, F4, F7, M23, V3). A recently developed micromethod is said to be free from interference by a number of acidic and basic drugs (J3, W3) including oxyphenbutazone.

GLC methods capable of measuring phenylbutazone in serum and urine are available with a sensitivity of 1 μg/ml of serum or urine (P6,

S28), but in view of the relatively high therapeutic levels of the drug under normal circumstances, such methods offer no practical advantage over spectrophotometric techniques.

The therapeutic effects of phenylbutazone and its toxic effects are related to the plasma concentration. Its measurement in plasma—at least in selected patients, such as those who fail to give an adequate response to the drug or who exhibit toxic symptoms at low therapeutic doses—may be clinically useful and enable treatment with the drug to be continued when it would otherwise be stopped.

4.8. Tricyclic Antidepressants

Several drugs sharing a number of chemical features and therapeutic properties are included in this category. The most widely used at the present time are imipramine, desipramine, amitriptyline, and nortriptyline. Not all authors have distinguished clearly between them, and some have uncritically equated results obtained with two different drugs as though they were identical (B34).

As a class, all the tricyclic antidepressants demonstrate marked pharmacokinetic heterogeneity—largely due to genetic factors (A4, H3, S18). Up to 40-fold differences in plasma levels are observed in subjects receiving identical daily dosage regimes. This should put the tricyclic group of drugs among those for which blood drug measurements would be most useful clinically. At the present time, however, concrete evidence in support of this contention is sparse and conflicting.

Analytical methods suitable for measuring one tricyclic antidepressant have often been useful, after slight modification, for measuring the others (H2). Thin-layer chromatographic and spectrophotometric methods (W5) suitable for measuring the concentration of tricyclic antidepressants in biological fluids for forensic or toxicological purposes, have proved to be too insensitive and imprecise to be useful for monitoring therapy with these agents. Spectrofluorimetric methods (D7, M19) are more sensitive, but still not sufficiently so for clinical use. Nevertheless they have found considerable application in pharmacokinetic studies.

4.8.1. *Amitriptyline and Nortriptyline*

These two drugs can conveniently be considered together, as nortriptyline is an active metabolite of amitriptyline. The possibility that nortriptyline is the major, or even sole, agent through which amitriptyline exerts its therapeutic effect is unlikely since the two drugs have different effects upon adrenergic nerves.

The average biological half-life of both drugs in man is comparatively long—in the region of 1–2 days—but large pharmacogenetic differences in

metabolism make any general statement of dubious value in the clinical situation. For practical purposes, the daily dose of the drug bears no relationship to the plasma "steady state," nor to the speed with which it is achieved (B34).

Many of the published data relating to the concentration of these two drugs in blood during pharmacokinetic and clinical studies in man have been obtained using fluorometric techniques of limited sensitivity or adaptations of the *in vitro* isotope derivative technique (H2). In skilled hands, this has a sensitivity of 5 ng/ml in plasma and an acceptably low coefficient of variation (SD ± 5%) but is too time-consuming and technically demanding to find widespread routine clinical application. Recently developed gas-chromatographic methods (B19, E6) not only enable one tricyclic antidepressant to be measured accurately and precisely in the presence of another, but bring such measurement within the capacity of most clinical laboratories.

Studies designed to investigate the clinical usefulness of plasma nortriptyline and amitriptyline measurements are both few and conflicting. Asberg *et al.* (A10) studied 29 patients treated with variable doses of nortriptyline for endogenous depression over a 2-week period. Plasma nortriptyline was measured by an isotope derivative method on blood collected 7 hours after the last dose of the drug given by mouth. After 2 weeks' treatment, plasma nortriptyline levels bore a nonlinear relationship to clinical improvement. "Amelioration of depressive symptoms was more pronounced in the intermediate plasma range (50–139 ng/ml) and was slight both at lower and higher plasma levels" (A10). On this basis the authors concluded that "monitoring plasma levels may be a way to increase the efficiency of treatment with these drugs." A contrary view was expressed by Burrows *et al.* (B34), who studied 32 patients over a 6-week period. These authors found "no significant correlation between the plasma level and clinical response at 4 or 6 weeks." Steady-state nortriptyline levels were, on average, much higher than those reported by Asberg *et al.* (A10) despite the use of an almost identical therapeutic protocol and analytical methods. The reasons for these differences do not emerge from the published data.

Braithwaite *et al.* (B20) treated fifteen patients with a fixed daily dose of 150 mg of amitriptyline by mouth for 6 weeks. Plasma amitriptyline and nortriptyline levels were measured, by GLC, in venous blood collected 19 hours (±SD 4.3 hours) after the last dose of the drug. Steady-state blood levels were achieved within 2 weeks, on average, but there were large between patient differences.

The ratio between amitriptyline and nortriptyline levels at "equilibrium" also varied widely. Good clinical response to the drug after 6 weeks' treatment, correlated with the presence of a total plasma tricyclic

drug concentration (i.e., nortriptyline and amitriptyline combined) of 120 ng/ml or more. In this small group of subjects there was no evidence of a lessening of therapeutic response at high plasma tricyclic levels. Nevertheless the failure of many patients on a fixed dosage regime to achieve therapeutically effective plasma drug levels led the authors to conclude that "there seems to be a strong case for monitoring plasma drug levels in patients receiving tricyclic antidepressant therapy" (B20).

Plasma nortriptyline levels have been reported as correlating with the frequency and severity of toxic side effects both in normal volunteers (A11) and in depressed patients (A9). The correlation was, however, neither close nor sustained beyond the first 3 weeks of treatment and in other studies no correlation between plasma nortriptyline levels and toxic side effects was observed (B20, B34). All authors agree, however, that side effects tend to diminish with time. This has also been observed with other psychotropic drugs (C18). Some authors have, however, observed, in individual patients, an association between very high plasma tricyclic antidepressant concentrations and the appearance of toxic side effects during what would generally be considered "normal" or "subnormal" daily drug dosage regimes (A9, H3).

4.8.2. *Imipramine and Desipramine*

These two drugs, one of which—desipramine—is a pharmacologically active metabolite of the other, have been extensively studied. GLC methods, sensitive to 1 ng/ml and suitable for use in ordinary clinical laboratories, are available (E6), but so far no systematic studies to determine the relationship between steady-state plasma levels and therapeutic response have been published. Both drugs have widely variable plasma half-lives ranging from "a few" to 54 hours in different individuals. Uncontrolled trials (H3) suggest that therapeutic response depends upon achieving and maintaining a minimal plasma level the exact value of which has still to be determined by clinical trial. It seems likely, but so far unproved, that the failure to achieve these minimal levels with what have become conventionally accepted as usual therapeutic doses, explains the resistance to the antidepressant effects of tricyclic drugs observed in some families.

Thus, while there is suggestive, but conflicting, evidence that therapeutic response is related to plasma "steady-state" levels of tricyclic antidepressant drugs, the position as regards toxic side effects is uncertain.

4.9. Gold

Gold salts have been used for the treatment of rheumatoid arthritis for more than 40 years. Despite their proved efficacy in a large proportion

of cases, the unpredictable appearance of severe, and occasionally fatal, side effects has limited their usefulness. Several organic salts of gold are available commercially, including sodium aurothiomalate, sodium aurothioglucose, gold keratinate, and sodium aurothiosulfate. They are usually given in 50-mg doses by intramuscular injection at weekly intervals up to a total dose of 1 g, equivalent to approximately 500 mg of elemental gold. Thereafter similar amounts are given at monthly intervals up to a total dose of 5 g.

Many methods, including classical wet chemistry, ultraviolet emission spectrography, polarography and, latterly, neutron activation, have been used to measure the concentration of gold in plasma, but have not found general acceptance in clinical laboratories. The availability of methods using atomic absorption (D12, L13) puts the assay within the scope of any well equipped clinical laboratory.

The pharmacokinetics of gold salts are complex and poorly understood. Gold is rapidly absorbed from its site of injection and reaches its peak plasma concentration within 4 hours. After a single injection the concentration of gold in the plasma falls slowly to reach basal levels after several days (M12). A second injection, before this stage is reached, produces a larger increment in plasma gold than the first and the effect persists for longer. Succeeding injections produce a gradual stepwise increase in plasma gold concentration which, in any one individual, bears little relationship to the total dose of gold salts administered (M12).

Distribution of gold within the body is complicated and does not permit of simple compartmental analysis (G12). The plasma "steady state" is usually achieved after 6–8 weeks' treatment. Mean plasma gold levels measured at about this time, and 1 week after the last injection, vary according to the total amount of gold injected. With a weekly dose of gold salts of 25 mg, the mean plasma gold concentration is 2 μg/ml: the corresponding figure for a weekly dose of 50 mg is 3.3 μg/ml (D4). After treatment is stopped, plasma gold levels fall slowly but are still measurable after 84 days (D4).

Gold is excreted in both urine and feces but the total amount and proportion excreted by each route bears no constant relationship to each other nor to the plasma gold concentrations. The exact chemical form in which gold circulates in the blood is unknown and may not be identical in every case, even when the same salt is used. Up to 95% of the gold in plasma is protein bound, but whether to albumin (M12), α_2-globulin (C7), or α-lipoprotein (L3) is uncertain. The proportion of unbound to protein-bound gold varies in different individuals and in the same individual at different stages of treatment.

None of the many studies made so far has examined the relationship

between plasma gold levels and therapeutic effectiveness in more than a small number of cases, and definitive conclusions are premature. Most authors (F3, J5, M12) have failed to observe the correlation between plasma gold concentrations, therapeutic response and the severity of toxic side effects reported by others (K16, P9). This is not altogether surprising if, as seems likely, gold is preferentially deposited in synovial tissue, where it exerts its major therapeutic effect.

Adverse reactions to gold, especially dermatitis, can occur at plasma gold levels as low as 1 μg/ml but impaired creatinine clearance, which is indicative of severe kidney damage, is rarely seen at plasma gold levels less than 4 μg/ml (L14). The clinical value of plasma gold measurements must still be considered unsettled, despite the optimism of many investigators.

4.10. Miscellaneous Drugs

In addition to the drugs already discussed, and about which there is a general agreement (K10, P12, V6), there is a further small group for which there is suggestive evidence, from one or more studies, of a clinically useful correlation between plasma concentration and the therapeutic response and/or toxic symptoms.

4.10.1. *Chlorpromazine*

Chlorpromazine was the first, and probably is still the most widely used, major tranquilizer. Although 95–98% of the drug in plasma is bound to protein, it is extensively and rapidly metabolized within the body into a large number of metabolites. Much of the metabolism may indeed take place in the gut before, or in the gut mucosa during absorption.

Although normally administered in comparatively large doses the concentration of unaltered chlorpromazine in plasma is usually too low to be measured except by sensitive GLC methods (C6, C15). Using such a technique, Curry *et al.* (C18) have shown that "the optimum antipsychotic effect appears to occur at plasma levels which are lower than those causing side effects in *chronically* treated patients but higher than those causing these effects in *acutely* treated patients."

Because of the small number of patients studied in detail, no valid conclusions could be drawn about the precise limits of plasma chlorpromazine concentration for optimum therapeutic effect. Nevertheless, failure to respond to chlorpromazine was shown in one patient to relate to the extremely low plasma concentrations achieved—even by larger than average daily doses of chlorpromazine—whereas in another patient symptoms were aggravated when plasma levels of chlorpromazine were high.

They were alleviated when, by reducing the daily dose, the plasma chlorpromazine level was brought closer to values observed in well controlled patients. Curry *et al.* (C18) concluded that unresponsiveness to chlorpromazine could result either from too low or too high a plasma chlorpromazine level. They believe that "a strong case can be presented for controlling (chlorpromazine) dosage on the basis of achieving a medium plasma level." So far their suggestion has not been tested experimentally.

4.10.2. *Hydrallazine*

The relation between the dosage, plasma concentration, and hypotensive action of hydrallazine has recently been examined (Z1). This drug, despite its many advantages as a hypotensive agent, fell from favor because of an unacceptably high incidence of severe side effects. Studies by Perry *et al.* (P8) have shown, however, that slow acetylators are more liable to develop the severe lupuslike syndrome associated with hydrallazine usage than fast acetylators.

Zacest and Koch-Weser (Z1) used a spectrophotometric method to measure plasma hydrallazine levels in patients. They found a "high degree of correlation between plasma concentration of hydrallazine and the magnitude of hypotensive action," on the one hand, and a poor correlation between plasma concentration and dosage on the other. The lack of correlation was due to the presence, within the population, of two metabolically distinct groups of individuals—one of which acetylated hydrallazine rapidly, the other slowly (cf. isoniazid). It seems possible, therefore, that the lupuslike syndrome which limits the clinical usefulness of hydrallazine is a direct consequence of high plasma hydrallazine levels and could be reduced, or prevented, by regular plasma monitoring.

4.10.3. *Theophylline*

Theophylline is one of the mainstays of therapy for acute and chronic obstructive airways disease. Until recently little was known about its pharmacokinetics and their clinical application, but there is now good evidence (J4) that both the therapeutic response and toxic side effects are related to the concentration of theophylline in plasma, rather than to its dosage.

Relatively little theophylline is excreted unchanged in the urine. Most is metabolized, at a rate that varies markedly from individual to individual, to give an average plasma half-life of 5.2 ± 1.5 hours (J4). The maximum therapeutic response, as determined by the measurement of specific airway resistance, is observed when the steady-state plasma theophylline level, measured 6 hours after the last dose, is over 10 μg/ml

(J1, J4, T6). Little benefit can be demonstrated at lower plasma levels. In one study adjustment of dosage to maintain "steady-state" plasma theophylline levels within a recommended optimum therapeutic range of 10–20 μg/ml led to improved therapeutic control (J4). To achieve this a more than 8-fold difference in the daily dose was necessary (J4), due to variations between one subject and another in the rate of metabolic degradation of theophylline.

Side effects include unpleasant gastrointestinal symptoms, nausea, and vomiting and are very common in patients with a "steady-state" plasma theophylline level above 20 μg/ml. They are not observed at plasma theophylline levels under 13 μg/ml, regardless of the daily dose. It seems unlikely, therefore, that these troublesome toxic side effects are due to local tissue damage produced by theophylline in the gut, but rather that they are a systemic response to a high plasma theophylline level and, therefore, preventable by proper monitoring.

5. Conclusions

The growth in knowledge about the biochemical mechanisms involved in the absorption, distribution, metabolism, and excretion of drugs has cast serious doubts upon the validity of many time-honored practices in therapeutics. Recognition that the daily dose of a drug often bears little relation to the therapeutic effect in any individual patient has led to a search for better indicators of drug efficacy. Proposals that measurement of the concentration of drugs in blood might not only provide such an indication but enable drug treatment to be monitored and regulated to each individual patient's requirements are still based upon only a few examples. Even so, the complexity of the relationship between the concentration of drug in the blood, its concentration at the receptor site, and its biological effectiveness, coupled with claims of clinical usefulness that go beyond the experimental data, have led to disillusionment in some quarters. It is our firm belief, however, that as with all other aids to diagnosis and treatment, measurements of blood drug levels will increase both in number and scope, eventually to find their proper place as an indispensable tool in medicine. Clinical biochemists have an important role to play in this process by devising analytical procedures that are sufficiently sensitive, accurate, precise, and economically practicable to provide meaningful information rapidly enough to benefit individual patients. Clinical biochemists will need to collaborate with clinical pharmacologists and clinicians to extend the range of drugs which can and should be measured in blood and other tissues, and to establish the best conditions under which such measurements should be made, their clinical usefulness and limitations.

Acknowledgments

We gratefully acknowledge financial assistance from British Epilepsy Association, Abbey Life Assurance Company, Cilag-Chemie Foundation of Switzerland, Medical Research Council and South-West Metropolitan Regional Hospital Board.

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THE PROTEINS OF PLASMA LIPOPROTEINS: PROPERTIES AND SIGNIFICANCE[1]

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[1] The work by the AA cited in this review was supported by Grants from the Public Health Service (HL-08727), Illinois and Chicago Heart Association (C 71-6), and Atomic Energy Commission.

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[4] Operated by the University of Chicago for the U. S. Atomic Energy Commission.

1. Introduction

There has been a remarkable upsurge of interest in the area of research concerning plasma lipoproteins, and rapid advances have been made in the understanding of their structure and metabolism. Since numerous books (N1, S38, T3) and reviews (A3, G15, H2, O2, S7, S10, S16, S24) have already been published, some of them very recently, we have elected to avoid redundancy and to focus mainly on a discussion of the several polypeptides which have been isolated from circulating lipoproteins. Such a choice is motivated both by the structural and biological relevance of these polypeptides and by the rather explosive amount of published information on the subject. In this review, we have dealt primarily with studies on normal man. A few examples of pathological states were selected to indicate the usefulness of the study of genetic clinical variants in the definition of structure–function relationships of circulating lipoproteins. We recognize that any arbitrary selection of topics in a vast area of research like that of plasma lipoproteins has an *a priori* element of bias. We have attempted, nevertheless, to retain objectivity and place emphasis upon achievements and concepts that are likely to form a basis for future developments in the field.

2. Nomenclature for Plasma Lipoproteins

The most commonly adopted system of nomenclature is based on ultracentrifugal methods. It stems from the pioneer work of Gofman *et al.* [for review, see reference (N1)] and identifies plasma lipoproteins in terms of flotation rates in salt media of a given density. In more common usage, lipoproteins are referred to as very low- (VLDL), low- (LDL), high- (HDL), and very high- (VHDL) density lipoproteins, again as a reference of the salt medium used. These are clearly operational terms, yet the lipoprotein species so defined possess distinctive chemical, physical, and immunological properties. Lipoproteins separated according to density criteria can be fractionated further according to size, for example, making use of gel filtration techniques in media like Sephadex or agarose.

According to their electrophoretic mobility in supporting media, e.g., paper, agarose, or cellulose acetate, serum lipoproteins can be defined as pre-β, β-, and α_1-lipoproteins, grossly corresponding to VLDL, LDL, and

HDL, respectively. By starch block, some of the VLDL exhibit α_2-mobility, whereas by polyacrylamide gel electrophoresis the pre-β band migrates in post-β position, a phenomenon due to the sieving effect of the supporting medium.

Although the more commonly adopted nomenclature for plasma lipoproteins is based on ultracentrifugal or electrophoretic criteria, recent attempts have been made to replace "established" terminology with one based, not on the physical properties of the whole particle, but on the nature of one of its constituents, the protein moiety. According to this nomenclature (A3), there are essentially three families of lipoproteins in circulation: LP-A, LP-B, and LP-C; these exist either alone or in combination, and each has as a specific marker apoprotein A or B or C. According to Alaupovic (A3) and co-workers, any of the ultracentrifugally isolated lipoproteins would be a mixture of varying proportions of LP-A, LP-B, and LP-C, this based on the analysis of preparations obtained in the laboratory of the authors. At this time, the detailed properties of LP-A, LP-B, and LP-C have not been clearly defined, nor have the methods of their isolation sufficiently been tested. The concept, however, is of interest and deserves consideration, at least as a working hypothesis.

3. Comments on the Methods of Separation of Plasma Lipoproteins on a Preparative Scale

3.1. Ultracentrifugation

The preparative procedures currently in use have essentially been derived from early work by Gofman *et al.* [for review, see reference (N1)]. By such procedures, lipoproteins are separated from serum components by flotation, taking advantage of the significant differences in density between lipoproteins and other serum proteins. For an authoritative review on the subject, the reader is referred to a recent article by Lindgren *et al.* (L3) in which basic principles and methodologies are critically analyzed. Unfortunately, there is no one universally accepted method for isolating "pure" lipoproteins in the ultracentrifuge. Even a cursory analysis of the published methods indicate significant variations from laboratory to laboratory in terms of types of rotor employed, temperature and length of the ultracentrifuge runs, density cuts, type of salts, etc. The choice of a given method is probably a reflection of the investigator's bias and his laboratory conveniences; thus, the inadequacy of such a method can be assessed only by analysis of the products obtained. In this context, it is becoming increasingly evident that characterization of particles ought to include an analysis of their polypeptide composition

according to methods outlined in the following sections. As recently stressed by Lindgren *et al.* (L3), solvent densities must always be rigorously checked by either pycnometry or refractometry and the salts employed must be suitably recrystallized and stored in order to preserve their anhydrous conditions.

For the convenience of the reader, we have outlined the method of sequential flotation employed in our laboratory for separating chylomicrons VLDL, LDL, HDL_2, HDL_3, VHDL, and $d > 1.25$ bottom (Table 1). This method, the result of years of experience, has been highly reproducible in terms of the normal human population examined in this laboratory. Such a method may not necessarily apply to dyslipoproteinemic states, where modifications may be necessary, depending on the type of abnormality under consideration. It should also be stressed that any lipoprotein isolated is in need of purification; this may be achieved by ultracentrifugation based on the assumption that "contaminants" are in loose association with the main complex. Whenever this purification is not achieved, other methods may be used as outlined below. For a discussion of the application of density gradient ultracentrifugation to the study of plasma lipoproteins, the reader is referred to a recent review (L3).

3.2. Chromatography

Chromatographic procedures have been applied increasingly in the fractionation and purification of plasma lipoproteins (B11, L3, W2). Agarose media have proved to be particularly valuable because of their sieving properties for particles in the size range of plasma lipoproteins, including the low- and very low-density classes (S1).

3.3. Electrophoresis

Besides starch block, electrophoresis has not seen wide use in the separation of plasma lipoproteins on a preparative scale. Since the applications of electrophoresis to the study of plasma lipoproteins have been dealt with in recent reviews (H2, L3, M4), they will not be discussed here.

3.4. Precipitation Techniques

Primarily through the work of Oncley *et al.* [for reviews, see references (C4) and (B11)], relatively simple procedures have been devised for the specific precipitation of the low- or the high-density lipoproteins by the use of polyanions in the presence of divalent cations. It is necessary, however, to purify the preparations thus obtained, but the procedures thus far described do not permit subfractionation of the major lipopro-

TABLE 1

METHOD OF SEPARATING CHYLOMICRONS AND THE VARIOUS LIPOPROTEIN CLASSES BY SEQUENTIAL FLOTATION IN THE ULTRACENTRIFUGE (ALL RUN AT 15°C)

Initial steps[a]		Purification steps
Serum (contains 0.1 g EDTA/100 ml)		
1. CHYL	*d* 1.006 10,000*g* 10 min 30.2 rotor	Refloated × 2 at *d* 1.006 under same ultracentrifugal conditions
2. VLDL	*d* 1.006 140,000*g* 20 hr 30.2 rotor	Refloated × 2 at *d* 1.006 under same ultracentrifugal conditions
3. LDL	*d* 1.063 140,000*g* 24 hr 30.2 rotor	Density adjusted to *d* 1.019 (centrifugation as in step 3). Top = LDL_1 (*d* 1.006 − 1.019). Undernatant adjusted to *d* 1.063 to obtain LDL_2 (*d* 1.019–1.063). Other density cuts performed if required
4. HDL_2	*d* 1.125 150,000*g* 24 hr 40.3 rotor	HDL_2 contains LDL and albumin. LDL is eliminated by centrifugation as in step 3. LDL floats, HDL_2 sinks. The latter is then refloated at *d* 1.21.
5. HDL_3	*d* 1.21 150,000*g* 24 hr 40.3 rotor	It contains albumin, *no* LDL. The preparation is refloated × 3 at *d* 1.21 as in step 5
6. $VHDL_1$	*d* 1.25 150,000*g* 24 hr 40.3 rotor	$VHDL_1$ is heavily contaminated by albumin. At least three centrifugation steps at *d* 1.21 necessary to remove contaminant. However, albumin is difficult to remove
$d > 1.25$		Contains very high-density lipoproteins and albumin

[a] CHYL = chylomicron; VLDL = very low-, LDL = low-, HDL = high-, and VHDL = very high-density lipoprotein.

tein classes or of their subcomponents (i.e., VLDL subfractions, LDL, and LDL_2, HDL_2, HDL_3, VHDL, etc.).

3.5. Immunological Procedures

(A3, H2, S16) In general, immunological procedures have been employed for assessment of the purity of the various lipoprotein preparations. At this time, however, the availability of specific antisera may permit the application of immunological procedures in preparative work, particularly by the technique of affinity chromatography.

3.6. Outline of Methods for Assessing the Purity of Plasma Lipoproteins

These methods are summarized in Table 2. Physical, chemical, and immunological procedures are not exclusive of each other, but rather ought to be employed together so that an unequivocal assessment of the "purity" of the particles under consideration can be obtained.

4. Summary of Properties of Plasma Lipoproteins

Table 3 lists the protein–lipid distribution and the principal physical properties of the major lipoprotein classes separated by ultracentrifugation. The chemical nature of the lipid moiety (Table 4) has been investigated extensively, and has recently been authoritatively reviewed (N1). Phospholipids (predominantly as lecithin), cholesterol (predominantly as ester), and glycerides (mainly as triglyceride) are the major lipid species of serum lipoproteins occurring in different proportions, depending upon the lipoprotein class. Essentially all lipids are bound to protein by noncovalent linkages, a fact which permits their ready extraction by organic solvents or displacement by detergents. The physical properties

TABLE 2
Methods Employed in the Assessment of the "Purity" of Plasma Lipoproteins

Physical	Chemical	Immunological
Analytical ultracentrifuge	Lipid analysis	Use of antisera specific against HDL or LDL in its various polypeptides
Electrophoresis: agarose, polyacrylamide gel, cellulose acetate	Protein composition	
Gel filtration	Carbohydrate composition	
Optical methods (UV, ORD, CD)[a]		

[a] UV = ultraviolet irradiation; ORD = optical rotatory dispersion; CD = circular dichroism.

TABLE 3
MAIN PHYSICAL PROPERTIES OF HUMAN SERUM HIGH-DENSITY LIPOPROTEINS

Parameters	Chylomicrons	VLDL	LDL	HDL_2	HDL_3	$VHDL_1$
Flotation rate,[a] $S_{f(1.063)}$	>400	20–400	0–20	—	—	—
Flotation rate, $S_{f(1.21)}$	—	—	—	3.6–9	0–3.5	—
Solution density of isolation	<1.006	<1.006	1.019–1.063	1.063–1.125	1.125–1.21	1.21–1.25
Average hydrated density, g/ml	0.93	0.97	1.03	1.094	1.145	1.155
Molecular weight	$>0.4 \times 10^9$	$5–10 \times 10^6$	$2.2–2.7 \times 10^6$	3.6×10^5	1.75×10^5	1.51×10^5
Diameter, Å (as sphere)	>700	250–700	180–260	70–100	40–70	—
Electrophoretic mobility (paper or agarose)	Origin	Pre-β	β		α-Lipoproteins	
Lipid:protein ratio	98:2	92:8	79:21	60:40	45:55	38:62

[a] S_f = Svedberg unit, 10^{-13} cm/sec/dyn/g. The subscript denotes the solution density at which flotation studies were carried out. [See Scanu and Kruski (S13).]

TABLE 4
HUMAN SERUM LIPOPROTEINS PERCENTAGE PROTEIN:LIPID DISTRIBUTION[a]

Lipoprotein class[b]	Protein	Lipid			
		Unesterified cholesterol	Cholesterol esters	Phospholipids	Glycerides
Chylomicrons	2	0.8	7.7	7.5	88
VLDL	10	7.0	12.0	18.0	53
LDL	21	8.0	35.0	22.0	9
HDL_2	41	5.4	16.2	29.5	4.5
HDL_3	55	2.9	11.7	22.5	4.1
$VHDL_1$	62.4	0.3	3.2	28.0	4.6

[a] The data are taken from recent reviews (N2, S12).
[b] VLDL = very low-, LDL = low-, HDL = high-, and VHDL = very high-density lipoprotein.

of lipoprotein lipids have been studied by X-ray techniques, electron microscopy and spectroscopic methods (fluorescence, electron, and nuclear magnetic resonance). The results of such studies, which have provided some insight into the nature of protein–protein, protein–lipid, and lipid–lipid interactions in serum lipoproteins, have been reviewed recently (S8, S9, S12, S16).

5. The Apolipoproteins

5.1. Methods of Isolation: Delipidation Procedures

The purpose of the techniques described here is to obtain a protein, completely free of lipids and in its "native" state. Neither of these goals is readily achieved. Delipidation is usually incomplete; the delipidated protein ordinarily contains 1–3% lipid by weight. In terms of conformation, the delipidated apoprotein exhibits some spectral differences from its parent complex. Such spectral changes between lipidated and delipidated apoproteins, however, have been proved to be reversible when the apoproteins are appropriately reexposed to lipids (S8, S9).

Since the early delipidation procedure was applied initially to the whole serum (S15) and later to the isolated serum lipoproteins (S18), many other methods of delipidation have been reported [see reference (S16) for review], employing mixtures of organic solvents (ethanol–ethyl ether; chloroform–methanol, acetone, etc.) or detergents (sodium dodecyl or decyl sulfate, Triton X-100, Nonidet, etc.). Techniques for delipidation have not been standardized, nor is there a comprehensive comparative assessment of the various proposed methods presently avail-

able. In general, it has been necessary to carry out delipidation techniques in the cold, using freshly distilled solvents and operating under conditions potentially capable of preventing lipid peroxidation and protein denaturation. The apoprotein powders obtained have proved to be stable for many months in the cold, this in sharp contrast with the relative instability of the intact lipoprotein complexes.

In one procedure (S30), the ratios of the extracting solvents have been adjusted to prevent protein precipitation and to thus carry out the whole delipidation procedure in a liquid–liquid system. The maintenance of lipoprotein protein "in solution" offers some advantages, especially in the case of the LDL apoprotein (apo LDL), whose poor solubility in aqueous media is well recognized. However, their prolonged storage may lead to problems, such as aging or bacterial contamination, that are not encountered with either dried or lyophilized materials. Furthermore, the latter material dissolves readily in water as in the case of the VLDL and HDL polypeptides. In terms of apo LDL, solubilization is possible in the presence of denaturing agents like urea or guanidine hydrochloride, or anionic or nonionic detergents (see below).

5.2. Solubility Properties

The delipidated serum lipoprotein proteins exhibit solubility differences in aqueous media. The polypeptides of HDL and the "D" polypeptides of VLDL are readily soluble in aqueous media, particularly in slightly alkaline low-ionic strength buffers (S28, S30). In contrast, the LDL protein does not dissolve in such buffers and, like many other "water-insoluble" proteins, requires denaturing agents, detergents, or suitable chemical modification. The many techniques for the solubilization of apo LDL have been reviewed recently (G15). A thorough assessment of such techniques is not possible since not all the solubilized products have been characterized. The choice of the method presently depends on the investigator's preference and experimental needs.

Recently, it has been shown that fraction V of apo HDL, closely resembling the "D" polypeptides of apo VLDL, is moderately soluble in aqueous solutions of ethanol, a fact to be taken into consideration in the delipidation of serum lipoproteins with extracting mixtures containing this organic solvent. The relevance to recovery problems was pointed out previously (S11).

5.3. General Principles of Fractionation

Early studies had led to the recognition that even the water-soluble apoproteins exist in solution as discrete molecular aggregates (S16, S24). The nature of the aggregating units was clarified only recently in studies

where fractionation procedures were carried out in the presence of urea, guanidine hydrochloride or acidic buffers. Under these conditions, production of the monomeric forms of the apo HDL and apo VLDL polypeptides is induced. These monomeric units have been shown to differ in size and charge, thus permitting their fractionation by gel filtration techniques (R7, S20) and ion-exchange column chromatography (E1, S30, S31), preferably in combination (E1, S19). Preparative polyacrylamide gel electrophoresis has also been employed, but the fractionated products have not been thoroughly characterized (C1). The use of highly concentrated urea solutions in the fractionation of serum lipoprotein polypeptides may pose technical hazards since cyanate-induced carbamylation may occur, causing an irreversible chemical modification of the proteins. The detailed conditions for avoiding such complications have been described; they rely mainly on the use of freshly prepared solutions of freshly recrystallized urea at low temperature with minimum exposure time between protein and urea (E1, S19).

In the following sections we give an outline of specific methods that have been employed successfully in the fractionation and purification of the polypeptides of apo HDL and apo VLDL. We also provide some comments on apo LDL, the exact nature of which is still not unambiguously resolved.

5.4. Specific Methods of Fractionation and Analysis

5.4.1. *Apo HDL*

The work which clearly established the polypeptide heterogeneity of human serum apo HDL was initially conducted in the laboratories of Shore and Shore (S31), Scanu *et al.* (S20), and Rudman *et al.* (R7). Since the time of the early reports, refinements of methodology have been published both by Shore and Shore (S31) and Scanu *et al.* (E1, S17). From this work, it now appears well established that apo HDL consists of three major polypeptide classes, named operationally, in terms of their elution from Sephadex columns (Fig. 1), III, IV, and V (see Table 5 for alternative nomenclature). When each of the Sephadex fractions is subjected to further fractionation by DEAE column chromatography, pure fractions are obtained as assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), 8 *M* urea-PAGE, isoelectric focusing and immunoprecipitin reaction against monospecific antisera raised against each of the lipid-free polypeptide. According to the experience gained in our laboratory, it is necessary to combine gel filtration and ion-exchange techniques to obtain pure products. According to Shore and Shore (S30, S31), DEAE chromatography alone may serve the pur-

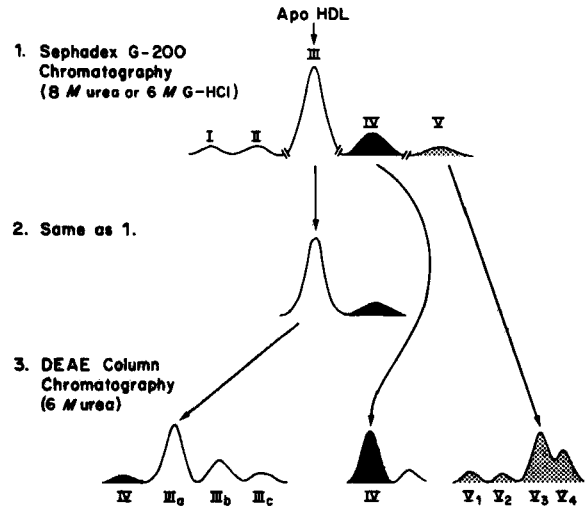

FIG. 1. Fraction of human serum high-density lipoprotein apoprotein (apo HDL), scheme 1. As in scheme 2, the method makes use of the combination of gel filtration and ion-exchange chromatography. Fraction IV is obtained in its dimer form.

pose. In Fig. 1, we have schematically represented one of the methods of fractionating apo HDL developed in this laboratory and now in routine use for preparative scale of each apo HDL polypeptide. Such a method makes it possible to obtain IV polypeptide in its native form.

An alternative method of fraction (see Fig. 2) has been developed which takes advantage of the observation that cleavage of the single disulfide bond induces the dimer → monomer transition of fraction IV (B6, S17, S19). If the fractionation procedure is carried out in the presence of a reducing agent, such as β-mercapthoethanol, the reduced IV can be reoxidized into the dimer form by oxygen. The choice of the method

TABLE 5
NOMENCLATURE OF THE POLYPEPTIDES OF HUMAN SERUM APO HDL

Sephadex method (S20)	Alaupovic (A3)	Lux *et al.* (B5, L4)	Rudman *et al.* (R7)
I–II	[a]	[a]	[a]
III	A-I	Gln-I[b]	II
IV	A-II	Gln-II[b,c]	III
V	C	—	—

[a] No equivalent described.
[b] Named after its COOH-terminal residue.
[c] Formerly, R-Thr (S30, S31).

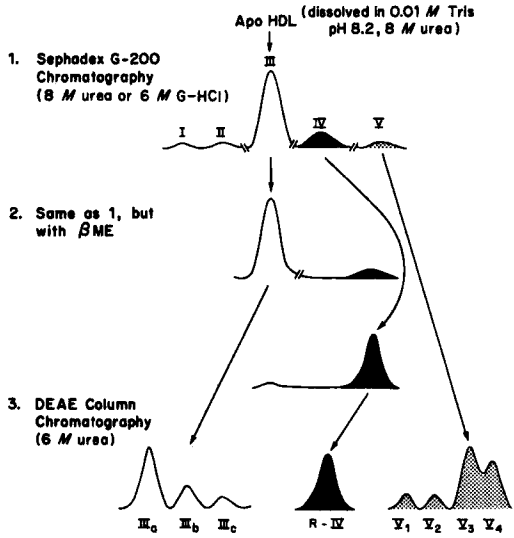

FIG. 2. Fractionation of human serum high-density lipoprotein apoprotein (apo HDL), scheme 2. Such a procedure takes advantage of the dimer → monomer conversion of fraction IV induced by the cleavage of the single disulfide bridge. R-IV = reduced fraction IV; βME = β-mercaptoethanol. Peaks: □ = fraction III; ■ = fraction IV; ▨ = fraction V.

depends upon the type of product needed. However, in either instance, both gel filtration and ion-exchange chromatography are needed for ensuring products that appear to be homogeneous by polyacrylamide gel electrophoresis in SDS or 8 *M* urea or by immunological means. The purity of each product can be further assessed by the technique of both analytical and preparative isoelectric focusing also in the presence of 6 *M* urea. The results have indicated that the technique affords a separation comparable to that obtained by DEAE chromatography. In consequence, this laboratory has elected to adopt the latter technique routinely and to reserve isoelectric focusing only for special cases.

Besides fractions III, IV, and V, two other fractions, I and II have been described in preparations of human serum apo HDL_2 and HDL_3. Although they are generally considered to be aggregates of the other fractions discussed above, these high-molecular-weight components have not yet been thoroughly studied. Such work is currently under way in this laboratory.

5.4.2. *Apo VLDL*

Primarily because of the work of Brown *et al.* (B8, B9, B10), it is now possible to fractionate the VLDL polypeptides by a combination of gel

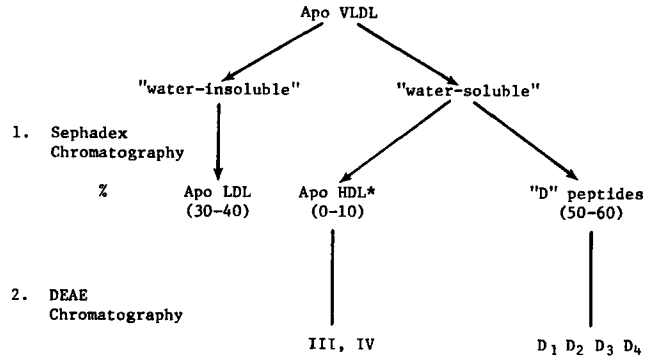

FIG. 3. Protein components of human serum very high-density lipoprotein (VLDL) apoprotein (Apo). *According to the experience gained in this laboratory, this component is negligible. As shown by Brown *et al.* (B9, B10), it becomes relevant in VLDL of patients with types IV and V hyperlipoproteinemia.

filtration and ion-exchange chromatography. Apo VLDL consists of "water-soluble" and "water-insoluble" components (Fig. 3). The water-insoluble components have been shown to have the same chemical and immunological properties as the LDL protein, and account for about 40% of the whole apo VLDL. The major part (about 50%) of the "water-soluble" proteins, comprises the D peptides, which are separable from the other apo VLDL components by gel chromatography and which can be further divided into fractions D_1, D_2, D_3, and D_4. This fractionation can also be achieved by the technique of isoelectric focusing (A5). The VLDL obtained from sera of patients with types IV and V hyperlipoproteinemia has been shown to contain (1–10% total protein by weight) an additional "water-soluble" component, which consists of polypeptides III and IV of apo HDL. In normal VLDL these fractions are usually negligible.

VLDL, obtained by ultracentrifugal flotation at $d < 1.006$ g/ml, can be subfractionated according to particle size by either density gradient ultracentrifugation or gel filtration in an agarose column. In such subfractions the ratio of apo LDL to D polypeptides has been shown to vary, the latter being relatively more abundant in the lighter and larger fractions (E4).

5.4.3 *Apo LDL*

In spite of some evidence presented in the literature (K2, S31), there are no unequivocal data available supporting the chemical heterogeneity of the protein of LDL. The preparations of LDL employed could have been contaminated by some VLDL or HDL components only uncovered

by delipidation. Alternatively, charge heterogeneity may have resulted from chemical modification artifactually produced during the process of apo LDL isolation and solubilization. In this context it should again be stressed that apo LDL does not dissolve in aqueous buffers unless high alkaline pH values are reached or under denaturing agents or surfactants used alone or in combination (G15, S8). This may at least partially justify the present uncertainties concerning the properties of apo LDL (see below).

5.5. General Comments on Nomenclature

Currently, there is no universally accepted nomenclature of the polypeptides isolated thus far from plasma lipoproteins. After the initial studies that firmly established the heterogeneity of the VLDL and HDL polypeptides, there was a tendency among some investigators to classify each of the isolated polypeptides by its COOH-terminal amino acid residue (S30, S31). For instance, the two major polypeptides of apo HDL were called R-Thr and R-Gln, based on the COOH-terminal analysis by the authors. Similarly, the low-molecular weight peptides of VLDL were called R-Val, R-Gln, and R-Ala with subscripts to indicate polymorphic forms (B8). The early reservations (S16) moved against this system of nomenclature were strengthened by later reports showing that the polypeptide "R-Thr" had instead glutamine in the COOH terminal position (E1, K6, L4) and that "R-Val" was in fact apo VLDL-serine (H4, M1). Furthermore, polymorphic variants of a given polypeptide involving the C-terminus have been recognized (E1), also some polypeptides with the same COOH-terminal amino acid residue have been shown to differ markedly in amino acid composition or other chemical and physical properties (S31). In view of the above, two tendencies currently exist: one nomenclature refers to the three major classes of polypeptides as A, B, C, the apoproteins of the A, B, and C lipoprotein families, respectively (A3). In such a nomenclature the various polypeptide subfractions are identified by a subscript, i.e., A_1, A_2 . . . or C_1, C_2 . . . etc., regardless of their mode of fractionation. The other nomenclature classifies the various polypeptides in operational terms. Thus, apo VLDL, apo LDL, and apo HDL refer to the ultracentrifuge-prepared lipoproteins: VLDL, LDL, and HDL, respectively (S8, S24). Each of the subfractions is then defined according to its order of elution from chromatography columns. Thus fractions III and IV, are those apo HDL components eluting in position III and IV from Sephadex columns (S20); or D_1, D_2, D_3, or D_4 are the apo VLDL polypeptides eluting in that order from ion-exchange columns (B8, B9). The value of an operational classification is that it is noncommittal and can be safely employed until all structural properties

of each polypeptide are well defined. At that time, a suitable nomenclature for serum lipoprotein polypeptides is likely to be adopted that permits understanding both by workers and nonworkers in the field.

5.6. Properties

5.6.1. *Apo HDL, Fraction III (or Apo LP Gln-I or A-I)* (see Table 6)

It now appears to be accepted that this apolipoprotein represents about 70% of apo HDL by weight. In its pure form it has been shown to have a molecular weight (MW) of about 27,000 (E1, L4) with NH_2-terminal aspartic and COOH-terminal glutamine (E1, K6, L4). The presence of COOH-terminal threonine has been reported by Shore and Shore (S30). Variants of fraction III which have been separated by DEAE column chromatography are probably related to differences in amide content (E1, L4, S31). Characteristically, fraction III contains no cysteine or cystine. Forms of III have been described with no isoleucine or 1 mole of this amino acid per mole of protein (E1, S30). A partial amino acid sequence has been reported (S32): NH_2-Asp-Glu-Pro-Pro-Gln-Ser-Pro-Trp-Asp-Arg-Val-Lys-Asp-Leu-Ala-Thr-Val-Tyr-Val-Asp-Val-Leu-Arg-Asp-Ser-Gly-Arg-Asp-Tyr-Val-Ser-Gln-Phe-Gln-Gly-Arg-Ala-Leu-Gly-Gly. . . . It has been noted that this fragment may represent the nonhelical portion of fraction III since it contains all the proline residues of the molecule. In recent studies, III has been subjected to fragmentation by cyanogen bromide (B1). Four fragments were obtained, as anticipated on the basis of the three methionine residues in the molecule. A significant observation was that the major fragment, which represented the COOH-terminal portion of the polypeptide, was the one having the highest α-helical content. Based on the information already ac-

TABLE 6
Properties of Human Serum Apo HDL Fraction III (Apo Gln-I or A-I)

Characteristic amino acid composition	Absence of cystine, cysteine, and isoleucine; variants containing 1 mole of isoleucine per mole of protein have been reported (E1, S33)
NH_2-terminal	Aspartic
COOH-terminal	Glutamine (E1, K6, L4); threonine has also been reported (S30)
Molecular weight	27,000 (E1, L4); a value of about 15,000 has been reported (S33)
α-Helical content	About 70% (L5, S20)
Polymorphic forms (at least three components)	Separable by DEAE column chromatography. Their relationship not established (E1, L4, S33).

TABLE 7
PROPERTIES OF HUMAN SERUM APO HDL FRACTION IV
(APO GLN-II OR A-II)

Characteristic amino acid composition	Absence of tryptophan, histidine and arginine
NH_2-terminal	Pyrrolidone carboxylic acid (B5, S19)
COOH-terminal	Glutamine (B5, K6, S19, S31); a variant with threonine has been reported (S19)
Molecular weight	
Dimer	≃17,000
Monomer	≃8500 (B5, S17, S19)
α-Helical content	About 40% (L5); decreased after reduction and restored by reoxidation (S6)
Polymorphic forms	Controversial

quired, the complete amino acid sequence of III should shortly be forthcoming.

5.6.2. *Apo HDL Fraction IV (or Apo LP Gln-II or A-II)* (see Table 7)

Contrary to early belief (S32), it is now established that, in its native form, fraction IV represents a dimer having its two identical or nearly identical monomers linked together by a single disulfide bridge (B5, S17, S19). The complete amino acid sequence of this polypeptide has recently been published (B5) and is represented in Fig. 4. Each monomer has been

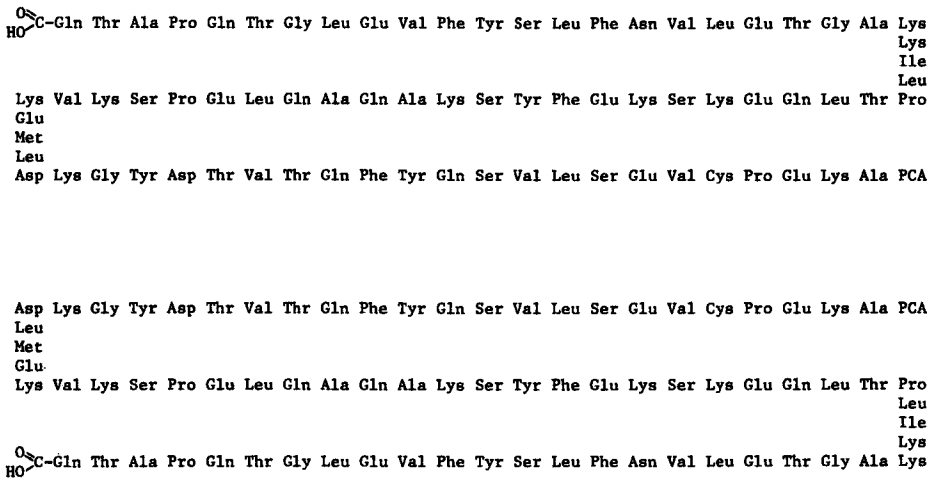

FIG. 4. Amino acid sequence of human serum high-density lipoprotein apoprotein (apo HDL), fraction IV (dimer). Data from Brewer *et al.* (B5).

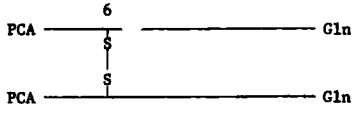

FIG. 5. Schematic representation of fraction IV (Apo Lp-Gln-II). The disulfide bridge is in position 6 from the NH_2-terminus. PCA = pyrrolidone carboxylic acid; Gln = glutamine.

shown to have: a molecular weight of about 8500, pyrrolidone carboxylic acid in the NH_2-terminal position and glutamine at its COOH-terminal (B5, S19). The disulfide bridge was found to be in position 6 from the blocked NH_2-terminus (Figs. 4 and 5). Variants of IV have been described (S19). In one case the COOH-terminal residue was threonine instead of glutamine (S19). Other variants are likely to be encountered as structural studies are extended to additional subjects. The structural properties of IV led some to suspect the existence of a precursor form of IV. This precursor has not been identified, however.

5.6.3. *Apo HDL, Fraction V (or C or D)*

Although it has been shown that at least some of these polypeptides derive from exchange with the VLDL protein (E4, S22), a stringent chemical corroboration of these findings has not been published. Studies from these laboratories have now provided such a documentation at least in terms of polypeptides D_1, D_2, D_3, and D_4 in apo VLDL (for properties, see below). Other minor polypeptides are also members of V, but their properties are still in the process of being defined (L2a).

5.6.4. *Apo LDL (Apo B)*

The information available in this polypeptide or these polyeptides is far from definitive. Controversy exists on size and molecular weight of the apo LDL monomers, their number, and chemical properties (Table 8). Also, the question of polypeptide heterogeneity has not been resolved; this is not surprising in view of the difficulty of protein solubilization discussed earlier. At the time of this writing, apo LDL is the mostly poorly characterized protein of all serum lipoproteins. This apoprotein is

TABLE 8
PROPERTIES OF LOW DENSITY LIPOPROTEIN APOPROTEIN

Molecular weight	27,000 (P2) to 245,000 (S39)
Number of subunits	2 (P2) to 20 (S39)
NH_2-terminal	Glutamic (S16, S24)
COOH-terminal	Serine (S16, S17)
Carbohydrate content	3–4% (S16)
Conformation	Mixture = α-helix, random coil and β-structure (G15, S8)

found both in the LDL and VLDL classes and chemically and immunologically indistinguishable from each other.

5.6.5. *Apo VLDL, D_1, or C_1*

This peptide is also referred to as apo Lp-Ser based on its COOH-terminal amino acid residue (H4, M1), initially believed to be valine (B8, B9). Its NH_2-terminal residue is threonine with the amino acid composition listed in Table 9. The sequence of this peptide announced recently (S34) has the noted feature (Fig. 6), also shared by apo LP-Gln II (B5) and apo LP-Ala (B6) of having a number of basic adjacent to acidic residues.

5.6.6. *Apo VLDL, D_2, or C_2*

Also referred to as apo LP-Gln for its COOH-terminal residue, this has been shown to have threonine as its NH_2-terminal residue. Its amino acid composition (Table 9) shows characteristics that are distinct from the

TABLE 9
AMINO ACID COMPOSITION AND NH_2- AND COOH-TERMINALS OF THE PRINCIPAL D PEPTIDES OF VLDL[a,b]

Amino acid	D_1	D_2	D_3	D_4
Asp	81.0	64.0	85	85
Thr	42.0	101.0	58	58
Ser	101.0	99.0	117	118
Pro	13.0	42.1	26	26
Gln	147.0	104.0	120	118
Gly	20.0	28.1	45	46
Ala	51.0	75.2	118	117
$Cys_{1/2}$	0	0	0	0
Val	30.0	45.0	68	70
Met	11.0	21.0	19	20
Ile	43.1	10.0	0	0
Leu	96.0	92.0	57	58
Tyr	0	53.0	21	20
Phe	44.1	24.0	44	46
Lys	147.0	70.0	71	70
His	0	0	11	12
Arg	43.0	13.0	23	24
Trp	17.0	—	26	28
NH_2-Terminus	Threonine	Threonine	Serine	Serine
COOH-Terminus	Serine	Glutamic	Alanine	Alanine

[a] These peptides are also found in apo HDL fraction V. D_1 and D_2 contain no sialic acid. D_3 and D_4 contain 1 and 2 moles of sialic acid per mole of protein, respectively.
[b] Values are expressed as moles per 100,000 g of protein.

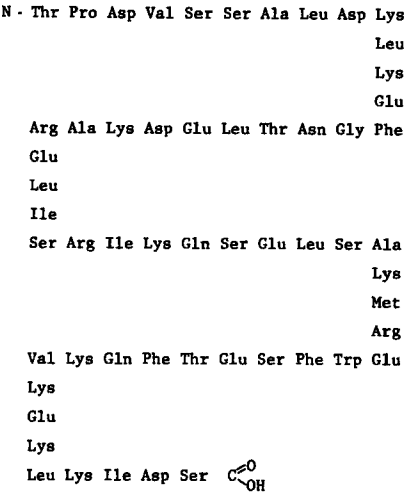

FIG. 6. Amino acid sequence of serum very high-density lipoprotein D_1 or C_1 (apo Lp-Ser). Data from Shulman *et al.* (S35).

other peptides of this group including immunological properties. The sequence of this peptide is yet unknown.

5.6.7. *Apo VLDL, $D_{3,4}$, or $C_{3,4}$*

These two polypeptides have been shown to have identical amino acid composition (Table 9) but to differ from each other in sialic acid content. D_3 and D_4 have 1 and 2 moles of sialic acid per mole of protein, respectively (A5, A6, B9, B10, E5). A third form without sialic acid has been isolated by preparative isoelectric focusing (A5). Both D_3 and D_4 have the same NH_2-terminal (serine) and COOH-terminal (alanine) amino acid residue and a molecular weight of about 10,000. The complete amino acid sequence has recently been announced (B6) and is reported in Fig. 7. These studies show that the polysaccharide having sialic acid as its terminal sugar, is linked to threonine 74 of the polypeptide chain.

6. General Comments on the Properties of Serum Lipoprotein Polypeptides

The development of technology for the isolation, fractionation, and purification of the principal polypeptides of VLDL, LDL, and HDL is undoubtedly a major achievement in the area of lipoprotein research. The availability of such polypeptides in pure form has permitted studies on their characterization and the definition of their chemical, physical, and immunological properties. The primary structure of many of these poly-

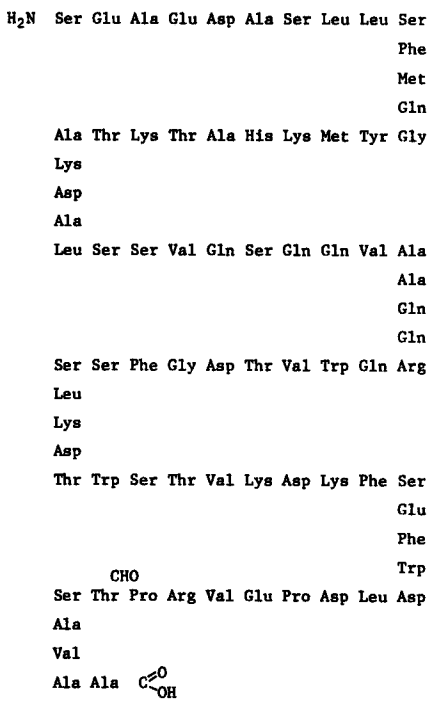

FIG. 7. Amino acid sequence of serum very low-density lipoprotein D_3 or C_3 (apo Lp-Ala). Data from Brewer *et al.* (B6).

peptides is either known or about to be known, a fact which permits not only more detailed work on their structure, but also a systematic analysis of their function. It is now becoming increasingly evident that some or all of these polypeptides cannot be considered just as carriers of lipids, but at least based on *in vitro* experiments (see following sections), that they enter into important biochemical processes related to lipid metabolism. The basic role of lipoprotein polypeptides in lipid metabolism will become apparent from the experiments reviewed in the following two sections dealing with both *in vitro* work and the investigation of some inherited disorders of lipoprotein metabolism.

7. Functional Properties (Table 10)

7.1. Lipoprotein Lipase

Lipoprotein lipase, an enzyme responsible for the hydrolysis of the triglyceride moiety of lipoproteins, has been shown to be activated by serum high-density lipoproteins (B4, K5, S5, S14), by very low density

lipoproteins (B4, G1), or simply by apo HDL in the presence of phospholipids (S4, S5). In experiments with partially purified lipoprotein lipase preparations from human postheparin plasma (F3) or rat epidymal fat tissue (C2), the cofactor activity of apo HDL was shown to reside in its minor component fraction V, which contains at least three polypeptide chains of molecular weight about 10,000 (S20). Other investigators (L1) have demonstrated that of the VLDL polypeptides only apo Glu and apo Ala activate crude lipoprotein lipase preparations from rat testicular fat pads, this in general agreement with the data obtained with an enzyme isolated from cow's milk (H3). It was recently determined, however, that apo Ala of VLDL acts preferentially as an inhibitor of crude lipoprotein lipase preparations from bovine milk (B7) and that its previously observed (L1) activation could be explained in terms of a very small degree of contamination with the apo Glu peptide (B7). In general, it appears that the results in the *in vitro* activity of lipoprotein lipase are influenced by the source and purity of the enzyme as well as of the lipoprotein cofactors. In this context, Ganesan *et al.* (G2), using different sources and degrees of purity of lipoprotein lipase, have stated on the basis of their findings that lipoprotein lipase activity has two patterns of apoprotein specificity: one activated by apo Ser, the other by apo Glu, both from VLDL. According to these authors, human postheparin plasma lipoprotein lipase in type III hyperlipoproteinemia would have a significant difference in the two enzyme activities (G3) as compared to normal.

Current available information does not permit definitive conclusions on the nature, specificity, and mechanism of action of the protein cofactor(s) of lipoprotein lipase. It is very difficult to correlate the observations described above (summarized in Table 10) since the enzyme preparations used were not pure or well characterized, and were derived from various sources. For instance, two species of lipoprotein lipase have been reported to exist in rat adipose tissue (G4), and major differences between enzymes of liver and adipose tissue have been noted (G16). Also, the nature of the apoprotein preparations employed as protein cofactor(s) of lipoprotein lipase has not been clearly specified in all the studies; contaminated materials may account for the spurious results observed. At present, it is not known how apoproteins such as apo Glu, apo Ala, and apo Ser could exhibit their activator or inhibitor activity on lipoprotein lipase. If these different apoproteins indeed prove to be cofactors for lipoprotein lipase, the nature of the lipid-protein specificity must be established and thus the role played by carbohydrates, since some of these apoproteins are glycoproteins.

In addition to a serum protein cofactor, calcium has been shown to be necessary for attainment of maximal reaction rates of partially purified

TABLE 10
ACTIVITY OF LIPOPROTEIN PROTEINS ON SOME ENZYMES OF LIPID METABOLISM

	Purification of enzyme			Activation by protein cofactor		
Enzyme	Source	Purification (-fold)	Reference	Protein cofactor	Enzyme source	Reference
Lipoprotein lipase	Human and rat postheparin plasma	10,000–15,000	Fielding (F1)	HDL	Chicken adipose tissue	Scanu (S4, S5)
	Rat postheparin plasma	200–250	Posner and Morales (P3)	Fraction V of HDL	Human postheparin plasma	Fielding *et al.* (F3)
					Rat epidymal fat tissue	Chung and Scanu (C2)
	Bovine skim milk	5000–7000	Egelrud and Olivecrona (E2)	Apo Glu and apo Ala of VLDL	Rat testicular fat pads	LaRosa *et al.* (L1)
					Bovine milk	Havel *et al.* (H3)
	Rat adipose tissue (2 forms)	Not specified	Garfinkel and Schotz (G4)	Apo Glu of VLDL	Bovine milk	Brown and Baginsky (B7)
				Apo "Val" (source unspecified)	Human postheparin plasma	Ganesan and Bradford (G1)
				Apo "Val" and apo Glu (source unspecified)	Human, rat, and dog postheparin plasma	Ganesan *et al.* (G2)

				Apo Glu and apo Ala (source unspecified)	Human and rat adipose tissue; human and bovine milk	Ganesan *et al.* (G2)
				Apo Ser and apo Glu of VLDL	Human postheparin plasma of type III patients	Ganesan *et al.* (G3)
				Tris-soluble proteins of VLDL	Rat epidymal fat tissue	Chung and Scanu (C2)
LCAT[a]	Human plasma	500	Ho and Nichols (H5)	HDL	Human plasma	Glomset *et al.* (G14); Akanuma and Glomset (A1)
	Human plasma	1200	Glomset (G12)			
	Human plasma	2500–3000	Fielding and Fielding (F2)	Fraction III of HDL	Human plasma	Fielding *et al.* (F4)
Enzymes of cholesterol biosynthesis	Rat liver	150	Dempsey (D1)	Rat pro-SCP	Rat liver	Ritter and Dempsey (R2, R3)
				Human pro-SCP	Rat liver	Ritter *et al.* (R6); Dempsey *et al.* (D2)
				Apo HDL	Rat liver	Ritter and Dempsey (R3)
				Fraction IV of HDL	Rat liver	Dempsey *et al.* (D2); Ritter and Dempsey (R4)
				Adrenal SCP	Rat liver	Kan *et al.* (K1)
				Rat SCP	Rat liver	Scallen *et al.* (S2)

[a] Lecithin: cholesterol acyltransferase.

lipoprotein lipase from rat postheparin plasma (P3). The nature of the cofactor role of this metal has not been clearly established. Clearly, more work remains to be done in this important area on one of the key enzyme(s) in lipid metabolism.

7.2. Lecithin:Cholesterol Acyltransferase (LCAT)

Since a recent review (G12) has appeared describing the reaction mechanism, properties, and role of LCAT, an acyltransferase that transfers fatty acids from lecithin to cholesterol of plasma lipoproteins, this section will be concerned mainly with the observed relationship between the activity of this enzyme and HDL (A1, C3, G14). Some current information on the subject is summarized in Table 10. The requirement for HDL protein in the esterification of cholesterol by LCAT was shown to be associated with only one of the several HDL apoproteins, namely fraction III or apo Gln I (F4). On the other hand, it was shown that the other major apoprotein of HDL, fraction IV or apo Gln II, either significantly reduces the activity induced by fraction III (F4) or acts as a slight activator of such an enzyme (G5). It has also been demonstrated that differences in lipid composition between the subclasses of HDL (N2, S36) influences LCAT activity (F4). Nichols and Gong (N3) recently demonstrated that LCAT reacts more favorably with lipid–protein aggregates which contain lecithin and cholesterol in a ratio of 3:1 than with aggregates containing the same lipids in a ratio of 1:1. These findings were corroborated in another laboratory, where maximal LCAT activity was found at a lecithin to cholesterol ratio of 4:1 in the presence of fraction III of HDL (F5). The products of the LCAT reaction, in particular, cholesteryl ester, also have an important effect on the activity of the enzyme utilizing fraction III of HDL as cofactor (F5). The reason why HDL is a preferred substrate for LCAT is not known with certainty. It has been suggested that lecithin, cholesterol, and apoprotein make up specific sites on the lipoprotein surface that have a special affinity for LCAT (G12). Some evidence in support of this hypothesis has been provided by studies of the preferential binding of LCAT to HDL (A2).

Relatively little is known about the physical and chemical properties of LCAT. This transesterifying enzyme has not yet been obtained in pure form: even the most highly purified preparations of the enzyme are contaminated with other proteins. Consequently, a thorough assessment of the findings discussed above must wait until LCAT is sufficiently pure.

7.3. Enzymes of Cholesterol Biosynthesis

Liver microsomal enzymes of cholesterol biosynthesis have been shown to be stimulated 4-fold or more by an activator isolated from the

105,000*g* supernatant fraction of liver homogenates (R2) and adrenal homogenates (K1). The activator has been purified 720-fold (R5) and identified as a heat-stable protein which binds squalene and the water-insoluble precursors of cholesterol noncovalently (R1, R2, R3). The latter finding led to the hypothesis that this protein functions as a vehicle for all water-insoluble precursors of cholesterol and becomes part of the active site of each microsomal enzyme in cholesterol biosynthesis (R2, R3). Accordingly, this protein has been named squalene and sterol carrier protein (SCP) (oligomer); pro-SCP (16,000 daltons) is the protomer form (R2, R3). Certain aspects of these findings have been confirmed in other laboratories (R1, S2). Pro-SCP has been isolated from both rat and human liver homogenates and shown to function identically both in the stimulation of partially purified microsomal enzymes of cholesterol biosynthesis and in the control of pathways of cholesterol formation (D2, R4, R6).

An interesting finding was that apo HDL functions as well as pro-SCP in cholesterol biosynthesis, suggesting that SCP may be a component of the high density lipoprotein fraction of serum (R3). Of the various apoproteins of HDL, only fraction IV has been found to stimulate the partially purified microsomal enzymes of cholesterol formation, not in an identical, but in a manner similar to SCP (D2, R4). As compared to the chemical properties of fraction IV summarized in 5.6.2, human pro-SCP contains 6–7 half-cystine residues; furthermore, reduction and carboxymethylation of SCP does not produce the disulfide cleavage observed with fraction IV (R5a). It has been suggested that, although fraction IV and human pro-SCP are not identical, they may contain the same sequence of amino acids which confer the activation potential of these proteins on the microsomal enzymes of cholesterol biosynthesis (R4). Further investigation may resolve this intriguing hypothesis and the suggested intracellular role of apo HDL in cholesterol biosynthesis. In related studies, it has been shown that apo HDL has affinity for squalene; this property may have regulatory significance in cholesterol formation (O1).

The isolation of an SCP protein from rat liver homogenates has also been reported (S2). This protein has been found to be heat-labile, to be detectable only in the liver, and to have a molecular weight of approximately 50,000 daltons by gel filtration (S2) and 28,000 daltons by sedimentation equilibrium (S3). Although the functional properties of the heat-labile SCP (S1) are similar to the heat-stable SCP (R2, R3), these proteins appear to be different. According to Scallen *et al.* (S3), their SCP preparation resembles chemically serum LDL; this based on the similarity in amino acid composition between these two proteins. In the

absence of a stronger chemical evidence or immunochemical data, such a hypothesis should presently be regarded as an interesting working hypothesis.

Both the heat-stable SCP protein of Ritter and Dempsey (R2) and the heat-labile SCP protein of Scallen *et al.* (S2) bind other lipids (e.g., phospholipids and fatty acids) in addition to water-insoluble cholesterol and its precursors (R2, R3, R5, S2). In view of this apparent lack of specificity, Ritter and Dempsey (R5) have suggested that the carrier protein may be more generally called *lipid carrier protein* (LCP), although its binding to squalene and sterol carrier protein may more directly reflect its functional role in cholesterol biosynthesis. Obviously, more work is needed to clearly define both structural role and functional properties of this protein or proteins.

8. Relevant Lipoproteins from Patients with Dyslipoproteinemia (Table 11)

8.1. Lipoproteins of Cholestasis

The characteristic elevation of plasma unesterified cholesterol and phospholipid concentrations in subjects with biliary obstruction is due to the presence of an abnormal low density lipoprotein, LP-X, which does not react with antibodies to normal LDL (M5, R8, S26, S40, S41). The protein-lipid composition of LP-X is unique and distinguished by a low content in protein (H1, P1, S26) and a very high content of phospholipid and unesterified cholesterol (H1, S26). The protein moiety of LP-X consists of a combination of albumin and a specific apolipoprotein, apo X; the variable concentrations reported may reflect the method of isolation used (P1, S26). Electron microscopy shows that the native LP-X is a spherical particle with a diameter ranging from 300 to 700 Å, having a strong tendency to aggregate and undergo remarkable structural changes; that is, to aggregate from a globular shape into little rolls or stacks of disk-shaped structures which flow together to form myelinlike figures (H1, S25). Immunochemical and delipidation data have been interpreted to indicate that apo X is located on the surface and albumin in the core of the particles (A4). The localization of phospholipids on the surface was suggested by phospholipase A_2 treatment of LP-X (S25) and was also supported by X-ray diffraction analysis (H1). According to the latter authors, the abnormal particles are surrounded by a continuous lipid bilayer of phospholipids and cholesterol, with both fatty acid chains and cholesterol occupying the center of such units (H1). Apo X, the characteristic nonalbumin protein moiety of either intact or partially delipidated LP-X, has been said to be immunochemically and

TABLE 11
LIPOPROTEINS OF PATHOLOGICAL RELEVANCE FROM PATIENTS WITH DYSLIPOPROTEINEMIA

Diagnosis	Class of lipoprotein affected			Nature of abnormality
	VLDL	LDL	HDL	
Cholestasis	−	+	−	(1) LP-X, an albumin–lipoprotein complex, is a spherical particle (300–700 Å diameter) (A4, H1, P1, S25, S26) (2) Apo X, nonalbumin protein, is an apoprotein different from apo LDL but may be identical to apo VLDL (A4, P1, S27)
Familial LCAT deficiency	+			(1) VLDL are abnormal in electrophoretic mobility, migrating as β-lipoproteins (G12)
		+		(2) LDL are immunochemically identical and composition similar to the cholestatic lipoprotein, LP-X (M1, M3, T2)
			+	(3) HDL, abnormal in size, composition, and appearance, consists of a major large molecular weight subfraction, migrating as α_2-globulin and a minor smaller molecular weight subfraction, migrating in the prealbumin region (F6, G13, N5)
a-β-Lipoproteinemia	+	+	+	(1) Abnormal "LDL" by morphology and chemical composition (2) Abnormal HDL in subclass distribution and polypeptide composition (S27)
Tangier disease	−	−	+	(1) HDL_T is the only detectable high density lipoprotein in Tangier homozygotes; HDL and HDL_T are detectable in Tangier heterozygotes (L6) (2) HDL_T is immunochemically different from HDL (L2, L6) (3) Major HDL apoproteins are present in HDL_T, but apo Gln-I disproportionately decreased with respect to apo Gln-II (L6)

(*continued*)

TABLE 11 (*Continued*)

Diagnosis	Class of lipoprotein affected			Nature of abnormality
	VLDL	LDL	HDL	
Lp(a) variant	−	−	+	(1) Lp(a)-lipoprotein is immunochemically identical with anti-LDL and anti-VLDL but not with anti-HDL, develops pre-β mobility during electrophoresis, has a greater sialic acid content and lower total lipid than LDL (S28, S36, U1) (2) Apo Lp(a)-lipoprotein has a different amino acid composition than HDL and LDL (S36)

electrophoretically different from apo HDL, apo LDL, albumin, α-globulins and other serum proteins (A4, S27). Recently, other investigators have demonstrated that a more highly purified preparation of apo-X reacts immunochemically with anti-HDL (P1). The physical, chemical, and immunological data by Seidel *et al.* (S27) and Alaupovic *et al.* (A4) suggest that apo-X and the "C" or "D" peptides of VLDL may be identical apolipoproteins. This possible identity has been based on the following evidence: (a) similar electrophoretic patterns in different supporting media such as agar, agarose, and polyacrylamide gels; (b) similar phospholipid:protein ratios of LP-X and VLDL delipidated under identical conditions; (c) identical N-terminal (threonine and serine) and C-terminal (alanine, "valine," and glutamic acid) amino acids; and (d) identical immunoprecipitin lines with anti-C and anti-LP-X (A4, S27). A more complete physicochemical characterization of the polypeptides of apo X and apo VLDL must be accomplished, however, before apo X and apo C can be considered identical.

The presence of LP-X in the plasma of patients with liver disease has been considered as a sensitive indicator of biliary obstruction and, thus, useful in the differential diagnosis of diseases of the liver (S29, W1). However, the recent demonstration (see Section 8.2) that particles resembling LP-X occur also in the plasma of patients with LCAT deficiency poses serious reservations regarding the specificity of the proposed test.

8.2. Lipoproteins of LCAT Deficiency

Familial LCAT deficiency, an inborn error of metabolism that affects the levels of plasma cholesteryl esters, was recently discovered in Scandinavia (G7, G8, G9, G10, G11, N4). Patients with this disease have

corneal opacity, anemia, and proteinuria, elevated levels of plasma unesterified cholesterol and lecithin, and concomitantly reduced levels of cholesteryl esters and lysolecithin. Although the primary defect in the disease has not been conclusively identified, many of these pathological features seem to depend on the absence of plasma LCAT activity (G12, G13).

Each of the major lipoprotein classes is abnormal by several criteria (F6, G13, N5). The electrophoretic mobility of the patients' VLDL is that of a β-lipoprotein rather than a pre-β-lipoprotein (G12). Such an abnormality has been attributed to reduced levels of one or more of the VLDL peptides (G12).

Another striking abnormality involves the HDL of these patients. Originally HDL were thought to be absent from the patients' plasma because α-lipoproteins were absent in cellulose-acetate electrophoresis. However, they were later demonstrated by preparative ultracentrifugation (G13) and shown to be abnormal in size, composition, and appearance upon electron microscopy (F6, N5). The abnormal HDL can be fractionated in agarose columns into a major, large molecular weight subfraction and a minor one with a smaller molecular weight (F6, G13, M2, N5, T1). On agarose electrophoresis, the large molecular weight subfraction migrates as an α_2-globulin, whereas the component of smaller molecular weight migrates in the prealbumin region (N5). Both subfractions react with normal anti-HDL (N5). The smaller molecular weight subfraction compared to the larger component serves as a more effective substrate than the larger component for LCAT activity (G13).

One of the most striking lipoprotein abnormalities of familial LCAT deficiency is the presence in the LDL fraction of abnormally large particles, containing variable but unusually great proportions of unesterified cholesterol and lecithin (F6, G14, N5). Recently, an abnormal LDL lipoprotein, identical to cholestatic lipoprotein, LP-X (see Section 8.1) was demonstrated in plasma from patients with familial LCAT deficiency (M1, T2). Identity of the abnormal LDL lipoprotein and LP-X was shown by electron microscopy, composition, and immunological techniques (T2). The amount of LP-X in plasma of patients with obstructive jaundice ranged from 40 to 1200 mg/100 ml (M3) whereas plasma from patients with familial LCAT deficiency contained 49 to 152 mg/100 ml (T2).

It remains to be demonstrated whether all of the abnormalities of patients lipoproteins can be explained on the basis of LCAT deficiency or whether other secondary factors play a role in this disease.

8.3. Lipoproteins of a-β-Lipoproteinemia (ABL)

A-β-Lipoproteinemia is a rare, inherited disorder characterized mainly by the absence of a normal circulating LDL, retinitis pigmentosa, in-

volvement of the central nervous system, malabsorption of fats and an abnormal morphology of the red blood cells. The clinical and biochemical features of this disease need not be discussed in detail here, since very recent authoritative reviews on the subject have appeared (F7, K3). The absence of normal LDL is a most striking finding in ABL, but abnormalities in the other plasma lipoproteins have also been reported; these have been defined in detail only recently (S21). According to these studies, which were conducted on five different cases of ABL, there are two classes of particles in circulation. One class ("LDL") occurs in very minute amounts, has the flotational properties of LDL, and the polypeptide makeup of HDL, but structural properties (electron microscopy and circular dichroism) resembling neither LDL nor HDL. The second class comprises primarily HDL_2-type particles with a significant decrease in cholesterol esterification and, compared to the normal particle, a higher content of sphingomyelin and less lecithin. The HDL particles in ABL were also found to have some polypeptides usually not encountered in normal HDL together with a problem of glycosylation of one of the small molecular weight peptides ($D_{3,4}$ or R-$Ala_{1,2}$). It thus appears that ABL is a disorder affecting all plasma lipoproteins. The relationship among the various observed biochemical abnormalities remains unknown at present.

8.4. Lipoproteins of Tangier Disease

Tangier disease, a rare autosomal recessive disorder, is characterized by the near absence of plasma high-density lipoproteins and the storage of cholesteryl esters in foam cells in many tissues (F7). Prominent clinical features include splenomegaly, enlarged, orange-colored tonsils, and a relapsing sensory-motor neuropathy which may be quite disabling (F7, K4).

The so-called HDL_T, is the only lipoprotein present in the high-density lipoprotein class of Tangier homozygote at a concentration of 0.5–4.5% of normal (L6). From experiments employing immunoelectrophoresis, it appears that the obligatory heterozygote for Tangier disease also has circulating HDL_T although it has not yet been possible to confirm this by isolation of the abnormal lipoprotein or to obtain meaningful estimates of the relative amounts of HDL and HDL_T in the plasma of heterozygotes (L6). HDL_T appears to be a unique marker for Tangier disease insofar as can be determined from examination of plasma from over 300 persons with normal lipoprotein concentrations of other types of dyslipoproteinemia (L6).

HDL_T is different from normal HDL as indicated by immunochemical studies, including mixing experiments conducted *in vivo* and *in vitro* (L2,

L6). HDL_T contains cholesterol, cholesteryl esters, and phospholipids in roughly the same proportions as normal HDL (L6). The triglyceride content may be slightly higher (K4, L6). However, the data presently available on the lipid composition of HDL_T must be regarded as only approximate, since they are not corrected for any possible contamination of "sinking prebeta lipoprotein" (Lp(a) antigen) (S36) (see also Section 8.5). The contribution of such a contaminant to the lipid composition of the high density fraction, while normally small, could be significant in Tangier's disease owing to the 50- to 100-fold reduction in the amount of HDL. The lipid composition and physical properties of purified HDL_T must be examined before this matter can be clarified.

Both of the major HDL apoproteins, apo Gln-I and apo Gln-II, are present in apo HDL_T; but apo Gln-I is disproportionately decreased with respect to apo Gln-II, the ratio of their concentrations being 1:12 in apo HDL_T as compared with 3:1 in apo HDL. Several minor apoprotein components which comprise 5–15% of apo HDL are detected in approximately normal proportions in apo HDL_T. Tangier apo Gln-I and apo Gln-II appear to be immunochemically and electrophoretically identical to their normal counterparts. The amino acid compositions of the normal and Tangier apo Gln-II have been reported to be very similar. On the other hand, normal and Tangier apo Gln-I may be slightly dissimilar: their minor differences in the amino acid composition has been attributed to the possible presence of small protein contaminants in Tangier apo Gln I. However, additional studies will be required for a precise definition of the structural relationship between normal and HDL_T polypeptides. The results presently available have been taken to support the hypothesis that the hereditary defect in Tangier disease is a mutation in an allele-regulating synthesis rather than in the structure of apo Gln I (L6).

8.5. Lp(a) Variant

The Lp(a)-lipoprotein represents an inherited antigenic polymorphic system of the β-lipoproteins; it is found in 30–35% of the population (B2, B3). Lp(a+) and Lp(a−) can be distinguished by immunodiffusion with heteroprecipitins (B2) and by polyacrylamide gel electrophoresis (G6). Recent quantitative studies using radial diffusion showed that the amount of Lp(a)-lipoprotein varies from individual to individual (E3) and that the lipoprotein carrying the Lp(a) determinant [Lp(a+)] is found in the plasma fraction having a density range of 1.06–1.12 g/ml (S23, S37, W3), although with properties dissimilar from those of normal HDL.

Lp(a)-lipoprotein contains less total lipid than normal LDL although

the lipid composition is similar to that of LDL (S28). Its mobility (pre-β) in agarose electrophoresis also differs from that of HDL or LDL; this pre-β mobility of Lp(a)-lipoprotein may be the result of its sialic acid content, which is five times greater than that of LDL (S36). Electron microscopy shows Lp(a)-lipoprotein to have a spherical structure similar to LDL; and electrofocusing gives an isoelectric point of 4.9 for Lp(a)-lipoprotein and 5.5 for LDL. It reacts immunochemically with anti-LDL, anti-Lp(a)-lipoprotein (U1), and anti-VLDL serum (S28), but not with anti-HDL or anti-albumin serum (U1). The amino acid composition of apo Lp(a)-lipoprotein differs from that of apo HDL and apo LDL (S36).

After total delipidation of the Lp(a)-lipoprotein, only apo VLDL is soluble in 0.9% NaCl and detectable by immunochemical means (S28). It therefore seems unlikely that the specific antigenic determinant of the Lp(a)-lipoprotein is located on or is part of the apo VLDL protein moiety. At present, the nature of the antigenic determinant of Lp(a)-lipoprotein is not known.

9. General Considerations on the Role of Apolipoproteins in Lipoprotein Structure

The work on the isolation and characterization of the various lipoprotein apoproteins has clearly shown that these proteins are distinct not only from each other but also from the other proteins of circulating plasma. Thus, they represent a special class of polypeptides which circulate in association with lipids to form lipoprotein species having rather distinct physical, chemical, and immunological properties. The question why there are so many lipoprotein polypeptides has not yet been resolved; but the increasing interest in studies on the definition of their structure-function relationship are likely to provide an answer to this problem in a not too distant future. Based on current structural information (S8, S9), it would appear appropriate, regardless of their function (see Section 7) to make a distinction between "structural" or "intrinsic" polypeptides, those strictly determining or participating in any given lipoprotein structure, and the "extrinsic" polypeptides, those common to more than one lipoprotein species and originating from the now well-established exchange process existing among them. According to this concept, polypeptides III (or A-I) and IV (or A-II) would be the intrinsic components of HDL; and the B polypeptides, the intrinsic components of LDL. Moreover, fraction V (comprising the D or C polypeptides) would be "intrinsic" for VLDL and "extrinsic" for HDL. In spite of the evidence supporting it, this concept does not yet rest on a firm experimental basis. Much additional information should come from studies on

the lipid-binding capacity of each delipidated apolipoprotein or mixtures thereof, particularly in terms of understanding the nature and mode of interaction between proteins and lipids in the native lipoprotein complex. Many such studies are now under way in several laboratories. The reports of preliminary results are rather encouraging although they clearly point to the difficulties inherent in this type of experimentation.

10. Clinical Significance of Apolipoproteins

Until relatively recently, plasma lipoproteins were referred to as classes either in ultracentrifugal or electrophoretic terms. Antibodies against each of these major lipoprotein classes had been prepared and utilized in lipoprotein identification as well as quantification. Given the four major lipoprotein particles: chylomicrons, VLDL (or pre-β), LDL (or β-), and HDL (or α-), hyperlipemic states have been classified in terms of elevation of one or more of these lipoprotein classes. Hypolipemias have seemingly been ascribed to either decreased concentrations or absence of low- or high-density lipoproteins (see Section 8). The recent studies which have led to the isolation and characterization of their principal polypeptides have opened new approaches to the study of dyslipoproteinemias and provided a sound chemical basis for their classification and definition in molecular terms. In Tangier disease, for example, the abnormality appears to involve not the whole HDL apoprotein, but rather one of its two major polypeptide chains (fraction III or apo Gln-I). Further, in a-β-lipoproteinemia, besides the striking absence of normal circulating LDL, there are also HDL with unusual polypeptide distribution and composition. The current trend is to quantify the various polypeptides in whole plasma. For this purpose, the immunological approach with special emphasis on radioimmuno assay techniques appears particularly fruitful. The technical problems involved are not simple, since liproprotein polypeptides do not circulate free but are bound to lipids. The problem is not insurmountable, however, and developments in this area are to be expected with a fair degree of optimism. At this time, monospecific antibodies against each of the major lipid-free apoproteins can be obtained. This is clearly a major development which has obvious implications from clinical medicine and for lipid metabolism in general.

11. Concluding Remarks

Research on plasma lipoproteins has entered a highly productive period during which major advances have been made from both the conceptual and technological standpoints. The information gained on lipoprotein apoproteins has particular relevance, especially in terms of pri-

mary structure and function. As the work in this area continues, it is anticipated that besides the structural work outlined in this review, the tertiary structure of each of these polypeptides will also be clarified, and this in turn will provide a better basis for approaching the problem of the structure of each lipoprotein in its native state. These polypeptides, once obtained in lipid-free form, retain their lipid-binding properties and it is in fact possible, in the case of HDL, to restore a complex with properties very similar to those of the native particle. It is important from the functional standpoint to realize that some of the plasma lipoprotein polypeptides seem to have, besides their well-recognized carrier-function, a regulatory role in the activity of key enzymes in lipid metabolism (see Section 7). All these findings are of obvious relevance to clinical medicine, and should provide a strong physicochemical basis for the understanding of primary or secondary dyslipoproteinemias or of any other disorder associated with a derangement of the lipoprotein metabolism. Plasma lipoproteins have represented, and will continue to represent, a very useful research tool for the basic scientist and the clinical investigator alike. This area of research has again confirmed that basic and applied work can successfully interdigitate to provide answers to problems that have both physicochemical and clinical relevance.

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IMMUNOGLOBULINS IN POPULATIONS OF SUBTROPICAL AND TROPICAL COUNTRIES

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1. Introduction

Over the past two decades several reviews on the immunoglobulins in populations in the temperate regions have been written. In many of these, the structure, function, and synthesis of the various immunoglobulins have been dealt with; consequently in the present article mention will be made only of those aspects of the physicochemical properties of the immunoglobulins that have not been fully covered in previous articles and those that are particularly relevant to diseases in populations of subtropical and tropical climates.

Malnutrition is responsible for well over half the number of deaths in children in tropical populations. Those who survive the ravages of it must continue to suffer throughout their lives from some aspects of the subnormal immune responses that invariably accompany malnutrition. As yet we know little about the long-term effects of malnutrition on the immune system; however, with the availability of modern immunological techniques one can indeed look forward to answers to these problems.

Protein-calorie malnutrition has a devastating effect on the thymolymphatic system, and very early in the course of malnutrition in infants, the thymus usually shows a pronounced atrophy, followed by that of the spleen and then the rest of the lymph nodes. How these are related to (a) the defect of the cell-mediated immunity which we frequently observe in subjects with parasitic infection and (b) the autoimmune disorders of the heart, the kidneys, and the spleen in subtropical and tropical populations are not immediately apparent, but they are useful areas for further investigations.

The immunological disorders of Indian childhood cirrhosis and of tropical splenomegaly syndrome, where there are disturbances of the immunoglobulin synthesis as well as defects of cellular immune responses, may be the results of a combination of infections and perhaps certain types of unusual autoimmune phenomena—or even to vitamin deficiencies.

The association between malaria and Burkitt's lymphoma, and between splenomegaly and chronic lymphocytic leukemia are perhaps the results of the long-term effects of an overworked thymolymphatic system.

Many parasitic diseases are associated not only with an impairment of humoral antibody synthesis, but also with an impaired cellular response, and one wonders whether these may have originated from an earlier nutritional effect on immune mechanisms (S2).

Because of the prevalence of parasitic diseases in populations of subtropical and tropical countries and their contributions to the high serum immunoglobulin levels in most of them, a note of caution should be sounded concerning the interpretation of serum immunoglobulin results which may not be clear-cut. In such instances, one must be aware that a single isolated estimation of the serum immunoglobulin concentrations in an individual patient frequently does not provide a useful diagnostic or prognostic aid. Therefore, it should be emphasized that serial estimations provide far more useful information than a single isolated measurement. In addition, the comparison of the serum immunoglobulin concentrations in populations living in subtropical and tropical countries with those living in temperate countries must be interpreted with the knowledge that the normals in these two sets of populations are subjected to completely different environmental factors, which must influence their immunoglobulin concentrations accordingly.

2. Physicochemical Properties

2.1. IgG

This immunoglobulin has a molecular weight of 160,000 with a sedimentation coefficient of 7 S. It accounts for 70–80% of the total γ-globu-

lins and is freely distributed between the intravascular (40%) and extravascular (50%) body fluid spaces. The carbohydrate content is about 3.1%, and it is the only one of the five known immunoglobulins to cross the placenta. Unlike the case in temperate regions, higher mean serum IgG levels are found in the African newborn than in corresponding maternal sera (M5, M36).

There are four subclasses of IgG: IgG_1, 70%; IgG_2, 16%; IgG_3, 10%, and IgG_4, 4%. IgG_4 is capable of inhibiting IgE-mediated passive cutaneous anaphylaxis (PCA) reaction in baboons when injected in relatively high concentrations.

The following Gm alleles are associated with the various heavy-chain subclasses of IgG: IgG_1 subclass is associated with the Gm 1, 2, 3, 4, 7, 8, 9, 17, 18, 20, and 22; IgG_2 is associated with Gm 23, 8, 9, and 18; and IgG_3 is associated with Gm 5, 6, 10, 11, 12, 13, 14, 15, 16, 19, and 21.

The tabulation shows Gm alleles found in the different races (S17).[1]

Race	Alleles
Negroid	$Gm^{1,5,13,14}$, $Gm^{1,5,14}$, $Gm^{1,5,6}$
Caucasoid	Gm^{1}, $Gm^{1,2}$, $Gm^{3,5,13,14}$
Mongoloid	Gm^{1}, $Gm^{1,2}$, $Gm^{1,13}$, $Gm^{1,3,5,13,14}$
Bushman	Gm^{1}, $Gm^{1,5}$, $Gm^{1,13}$, $Gm^{1,5,13,14}$
Pygmy	$Gm^{1,5,13,14}$, $Gm^{1,5,6}$,
Melanesian	Gm^{1}, $Gm^{1,2}$, $Gm^{1,3,5,13,14}$, $Gm^{1,5,13,14}$

IgG_1 and IgG_3 seem to cross the placenta readily whereas IgG_2 and IgG_4 cross in lower concentrations (W2). These IgG subclasses which cross the placenta readily may have a faster catabolic rate than the other IgG subclasses. IgG_3 has a fractional catabolic rate (FCR) of 17% and $T_{1/2}$ of 8 days whereas $T_{1/2}$ for total IgG is 22 days with an FCR of 7% (S14). This physicochemical property of IgG subclasses is no doubt related to the lower IgG values found in African cord blood than in their respective maternal blood.

In the normal European, the mean IgG synthesis varies from about 23 to 36 mg/kg/day whereas in the African the corresponding values are 50 to 169 mg/kg/day and, since only about 3% of the total body content of IgG is catabolized per day, this may, in part, explain some of the high levels of serum IgG reported in subtropical and tropical populations.

IgG is the characteristic antibody of all internal secretions including blood and cerebrospinal fluid (CSF). Normal urine contains about 5–10 mg/day. In secondary immune responses like malaria and helminth infection, very large quantities of IgG are produced especially to soluble antigens and toxins. High values are found in many parasitic infections.

[1] Reproduced by courtesy of the University of Chicago Press.

2.2. IgA

IgA accounts for 10–15% of serum Igs. Serum IgA is mainly 7 S type whereas the 10 S and 11 S IgA are found in the external secretions. About 60% of the total body IgA is synthesized in the submucosal plasma cells of the lamina propria of the gut (see Fig. 1).

Dimers and polymers of IgA are found in the various external secretions such as gastrointestinal, colostrum, seminal, and vaginal secretions, saliva, tears, nasal, bile, urine, and tracheobronchial secretions. Parotid saliva has an IgG:IgA ratio of about 0.01 compared to 4.5 for serum (T5). Four types of polypeptide chains have been identified in S IgA: the light chain, MW 22,500; the heavy chain, MW 55,000; the secretory component (SC), MW 60,000 and the joining (J) chain, MW 20,000.

The joining chain (J in Figs. 1 and 2) is attached to IgA in the submucosa, forming the 10 S dimer which, by the process of pinocytosis, enters the mucosa and there becomes attached to the secretory component (SC), giving rise to the 11 S dimer. Secretory component prevents hydrolysis of S IgA in the lumen of the gut. The 7 S IgA monomer does not become attached to the J chain but enters the circulation via the venous-lymphatic circulation. This physicochemical property of IgA probably accounts for its relatively increased values in the serum of some children with kwashiorkor.

In the gastrointestinal tract there are about 20 IgA per IgG secreting cells, and it is thought that Peyer's patches seed the rest of the gut with IgA-secreting cells which probably originate from the thymus. About 30 mg/kg body weight/day of IgA is synthesized per day, and about 40%

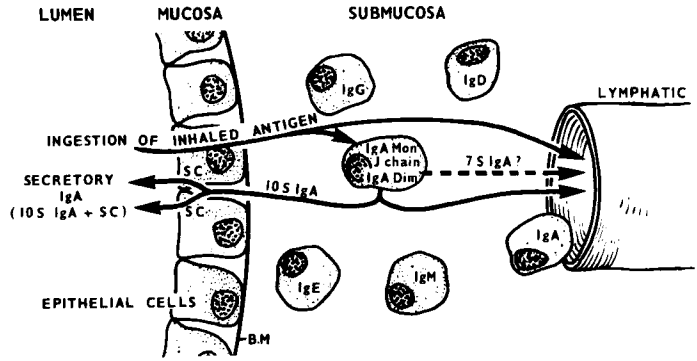


FIG. 1. Schematic diagram SC, secretory cell; BM, basement membrane; Mon, monomer; Dim, dimer representing the synthesis of the various forms of IgA by gut mucosal cells. From Tomasi (T5). Reproduced by courtesy of the publishers of the *New England Journal of Medicine*.

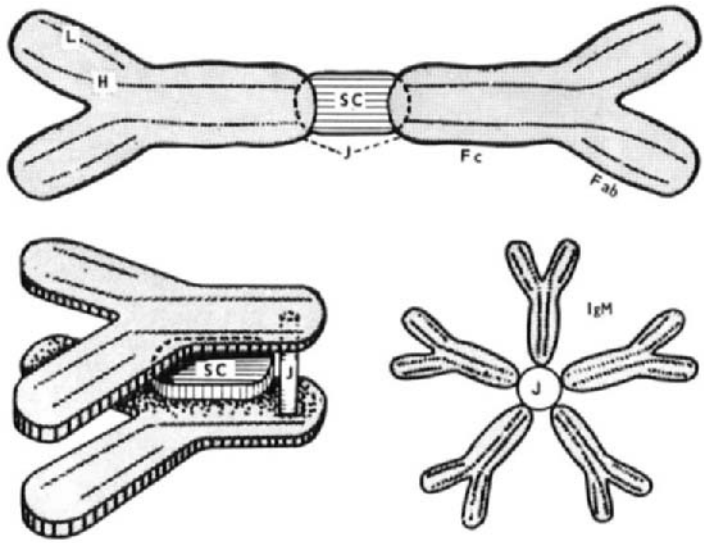


FIG. 2. Diagram representing the comparative structures of secretory IgA and IgM. L, light chain; H, heavy chain; J, joining chain; SC, secretory component. Reproduced by courtesy of the *New England Journal of Medicine*.

is present in the intravascular spaces. It is of interest that about four times as much IgA as IgG is catabolized per day, and its half-life of 6 days is about one-fourth that of IgG.

Unlike IgG, IgA does not cross the placenta, but like IgG, both the 10 S and the 11 S dimers of IgA are known to fix complement and seem to have the ability to block IgE reaction. This property, as well as its phagocytic role, adds to its efficient antibody role in the mucosal surfaces of the body. Normal serum concentration in tropical populations are similar to, or slightly higher than, those of persons living in the temperate regions. However, it should be noted that normal Caucasians do not attain adult levels of IgA until the fifteenth year whereas in some tropical children the adult level of IgA may be reached by the tenth year of age (W7) (see Fig. 2).

2.3. IgM

Molecular weight ranges from 850,000 to 1,000,000 with a sedimentation rate of 19 S, although minor components of 29 S and 38 S exist. Eighty percent of the total body IgM is found in the intravascular space and does not cross the placenta.

Ultrastructural studies revealed that IgM exists as circular pentamers or hexameric forms, which were predicted from early physicochemical studies. The carbohydrate content is about 8% (M34).

The polyvalency of IgM causes it to bind more firmly to an antigen than either a univalent or bivalent antibody. In this respect, a single molecule of IgM can cause lysis of a cell. Furthermore, the joining chain (J) has been detected in polymeric IgM and IgA, but not in the other immunoglobulins. About 10% of the IgM in external secretions has the secretory component attached. One wonders whether these characteristics of the IgM are responsible for its take-over role in the gut, when there is a defective synthesis of IgA as in celiac or in Crohn's disease or in ulcerative colitis.

IgM is usually the first line of defense in the systemic circulation whereas IgA is the first line of defense in the external mucosal surfaces of the body.

IgM may be regarded as the most primitive of the immunoglobulins. It is the first antibody produced in response to an antigen in the primary immune response. In human gestation it is the first Ig to be produced in the fetus in response to infection, e.g., syphilis, malaria, toxoplasmosis, and rubella: in some of the lower vertebrates it is the only immunoglobulin as yet detected.

Large quantities of IgM are produced in response to particulate antigens like malaria, trypanosomiasis, helminths, and immune complexes and IgM malarial antibodies have been found associated with thrombocytopenia in patients with malaria infection (B4).

The macroglobulins are known to activate the first component of complement, and whereas a single molecule of IgM can fix complement it requires two or more molecules of IgG or IgG_2 or IgG_3 to do so. IgG_4 does not fix complement.

IgM is synthesized at 5 mg/kg/day, and its catabolic rate is about two to three times that of IgG.

2.4. IgE

The discovery that this skin-sensitizing antibody IgE was identical to the anti-ragweed antibodies (J3) confirmed the earlier painstaking work of Ishizaka and Ishizaka (I1). It has a sedimentation coefficient of approximately 8 S with a relatively high carbohydrate content of 11.7% and a molecular weight of 196,000. IgE is not known to cross the human placenta, nor is it known to fix complement. Studies with this immunoglobulin have been limited by its extremely low concentration in normal human serum although much higher values are found in Africans. Evidence is now accumulating that there is a highly significant correlation between chronic respiratory disease and IgE in the serum (P1).

IgE binds to tissue receptor sites through its F_c region, leaving the F_{ab} region free to react with the allergen. The half-life of IgE is 2 days

in comparison to 23 days for IgG, with a daily turnover of a very small fraction 2000 times less than that of IgG. Levels of serum were between 16 and 20 times higher than in Swedish children and, in a group of African children with rarified infection with *Ascaris lumbricoides*, the serum IgE levels were 28 times higher (J4).

It would thus appear that parasitic infections, especially ascariasis, stimulate the production of IgE. Filipinos with roundworm infestation had markedly elevated levels of IgE in their sera (R5), and raised IgE levels were associated with *Toxocara canis* (H6).

Cord blood has a mean IgE concentration of about 3800 mg/100 ml, implying that, since IgE does not cross the placenta, the fetus has the capacity to synthesize its own IgE.

Most patients with a variety of advanced untreated neoplasms had low or undetectable IgE levels in the serum. Subnormal levels of IgE were also found in patients presenting with a variety of neoplasms in the early stages (J2). Although it is yet too early to appreciate the significance, this relationship between IgE and neoplasm seems rather interesting.

2.5. IgD

Seventy-five percent of this Ig occurs in the intravascular space and, like IgE, it has a very short half-life: $T_{1/2} = 3$ days. A very small amount—0.4 mg/days kg/day is synthesized—and these factors account for its very low level in the body. The function of IgD is not clearly understood.

The IgD profile in a normal Nigerian population was similar to that of the British and American populations (T7). Interestingly, in more than half the patients with African trypanosomes, IgD was absent from their sera (M35). Three out of six diabetic patients had insulin antibodies of the IgD class (D3), and IgD levels were five to six times higher in Ethiopian children than in Swedish children of the same age (J4). Patients with penicillin-induced hemolytic anemia had IgD receptors on their erythrocyte surfaces and sera from some subjects allergic to penicillin G were shown to have IgD antibodies specific for the benzylpenicilloyl-antigenic determinant of this antibiotic (C19).

Patients with protein-calorie malnutrition, especially children with marasmus and chest infections, had very high levels of serum IgD (R7). Antigen binding activity of IgD to diphtheria-toxoid and to bovine γ-globulins in some human sera have been reported (G4, H3).

Twenty-five patients with systemic lupus erythematosis had high IgD antinuclear factor, and mean higher serum IgD concentrations were found in two groups of Vietnamese populations who live in an area endemic for malaria.

TABLE 1
SERUM LEVELS OF IMMUNOGLOBULINS (MG/100 ML) IN VARIOUS POPULATIONS

Reference	Country	Race	Number of subjects	IgG	IgA	IgM
L1	Senegal	Black	125	(2400)	(330)	(170)
		White	100	(2100)	(260)	(74)
M7	Nigeria	Black	25	(1521)	(237)	(87) dry
				(3657)	(237)	(318) rainy season
M35	Congo	Black	112	1135–5220	70–400	32–478
				(2151)	(189)	(111)
M11	Jamaican	Black	150	664–1972	230–302	44–160
				(1318)	(266)	(102)
	Nigerian	Black	65	3097–3937	157–317	55–177
				(3657)	(237)	(121)
C13	U.S.A.	Black	54	—	118–1065	64–380
					(394)	(191)
L4	U.S.A.	White	112	(112)	(157)	(120)
		Black	108	(1408)	(175)	(133)
H4	U.S.A.	Black	6	1500–2500	(113)	(128)
		White	23	—	(112)	(75)
T7	Nigeria	Black	93	101–340	21–200	21–210[a]
		White	95	41–140	21–200	11–20
A11	Venezuela	Mixed	36	1197–2222	135–450	35–210
				(1647)	(285)	(74)
W7	New Guinea	Non-Watut aborigine	30	870–2560	68–564	68–575
				(1549)	(215)	(238)
		Watut aborigine	12	1370–2640	86–300	87–1650
				(1919)	(146)	(478)
L3	Argentine	Mixed	17	(1310)	(270)	(160)
M30	Brazil	Mixed	11	(1685)	(206)	(238)
A5	Mexico, Mexico City	Mixed	112	400–2500	70–450	16–150
				(914)	(223)	(48)
	Acapulco	Mixed	38	700–2520	108–450	49–159
				(1456)	(209)	(92)
S10	South Africa	Bantu	79	(3220)	(186)	(240)
		Pygmy	91	(2780)	(220)	(300)

[a] Values are expressed as percentages of a standard serum.

3. Normal Values of Serum Immunoglobulin Levels in Subtropical and Tropical Populations

There is general agreement that the serum γ-globulin levels in persons living in the tropics are higher than in persons living in the temperate regions (E2, E4, E5, H7, M37, R3, S16).

And, although many reasons—for example, ethnic origin—have been advanced to account for the higher levels of Ig's in tropical populations,

TABLE 1A
CONCENTRATION OF IMMUNOGLOBULINS IN SELECTED MALE BLOOD-DONOR POPULATIONS AGED 20–29 YEARS[a]

Town and country	Number of individuals examined	Geometric means of estimates of concentrations of immunoglobulins (IU/ml with 95% confidence limits)		
		IgG	IgA	IgM
Algiers, Algeria	100	143 (97–213)	164 (84–317)	190 (84–429)
Perth, Australia	94[b]	143 (94–219)	127 (56–286)	191 (86–425)
Santiago, Chile	100	156 (83–292)	163 (73–365)	158 (109–228)
Birmingham, England	51	123 (73–207)	115 (46–289)	133 (47–372)
Offenbach, Germany	45	124 (86–178)	108 (48–244)	133 (59–298)
Osaka, Japan	98	146 (102–210)	129 (70–237)	144 (68–308)
Mexico City, Mexico	100	127 (82–196)	97 (29–327)	63 (12–333)
Utrecht, Netherlands	100	116 (65–206)	94 (40–223)	127 (48–334)
Ibadan, Nigeria	100	287 (146–567)	80 (31–207)	211 (34–1413)
Uppsala, Sweden	94	126 (90–177)	126 (57–282)	135 (52–345)
Lausanne, Switzerland	100	135 (87–208)	136 (56–334)	176 (81–380)

[a] From Rowe (R6). Reproduced by courtesy of the publishers of *The Lancet.*
[b] 95 for IgA.

the one that is most compelling is the presence of endemic parasitic infections in tropical populations.

With the introduction of specific immunochemical assay methods for the quantitation of the individual immunoglobulins, more detailed results have become available. Tables 1 and 1A show the wide range of normal values for the serum immunoglobulin concentrations for different populations.

3.1. EFFECTS OF SEX, RACE, AND ENVIRONMENT

In an extensive study in Central Africa (M35) the concentration of IgA, IgM, and IgD immunoglobulins did not seem to play an important role in the hypergamma globulinemia of normal Bantus, and the concentrations of these three immunoglobulins in the serum were fairly close to that found in white populations. IgG concentration, on the other hand, varied according to sex, age, and the region inhabited, and there were no racial differences in the mechanism by which the IgG passed the placental barrier. The increased γ-globulins observed in the Pygmies and Bantus have been ascribed to their environmental conditions (S10).

3.2. SEASONAL EFFECTS

Seasonal variations have been reported for the higher serum Ig's in Nigerians, with mean higher values in the rainy season than in the dry

season. Adult values for IgG and IgM were two to three times higher in the rainy season than in the dry season, while levels of IgA and IgM in the fetal cord blood of infants tended to increase in the wet season (M7). In young Gambian children only the IgM showed material seasonal changes, values in the rainy season being higher than in the dry season, owing to current or recent infectious disease challenge (M22).

No marked effect of season was observed on mean values for IgD and IgA, but IgM values in groups aged less than 20 years were higher in the rainy season than in the dry season. Mean IgG values were constantly higher at the 1% level in some groups during the dry season (M23).

3.3. Comparative Values between Races

In a comparative study between British male medical students in their early twenties from Birmingham, England, and Nigerian males living in the neighborhood of Ibadan, the distribution of serum IgG in the Nigerian populations was the most striking of all four Ig's measured. The difference between the British and Nigerian values, for the serum IgG especially, was such that there was barely any overlap and, although the British group showed a Gaussian distribution and a compact range of values, the Nigerian group was non-Gaussian and broadly distributed. The IgM distribution was undoubtedly elevated in the Nigerian population, some individuals having exceptionally high values. Both IgA and IgD distribution were similar to that found in other temperate regions (T7). It is clear that the environment, more than the genetic factors, was responsible for the marked differences of the immunoglobulin concentration between the two groups of populations.

West Africans resident in Britain had average γ-globulin levels for those in Britain less than two years of 2.2 g/100 ml as compared with 2.0 g/100 ml for those resident two to four years and 1.6 g/100 ml for those resident five to eight years (S3). Specific host factors in which the immune mechanism are implicated was believed to account for the higher levels of γ-globulin in American Negroes and Puerto Ricans than in white races (S9).

In Australia, adult Whites, aged 20–45 years, in Sydney had significantly lower IgG and IgM than age-matched New Guinea Watuts (W7), and in addition the pattern of increased serum levels of IgG, IgA, and IgM in normal New Guinea natives resembled that observed in reactive hyper-gamma globulinemia in indigenes from other tropical areas including Nigeria (M8, T7), Senegal (M32), and Gambia (R6). The highest levels of IgM occurred in New Guinea subjects with splenomegaly with a range of values from 150 to 300 mg/100 ml.

Of the five Ig's investigated in Ethiopian preschool children, the levels

of IgG and IgD were significantly higher than those found in Swedish children of the same age. The mean levels of IgD were five to six times higher than in the Swedish children. It was striking that the concentration of IgE was between 16 and 20 times higher than in Swedish children, and in a group of children with verified infections with *Ascaris lumbricoides* the level was 28 times higher (J4).

In an extensive study of 819 apparently healthy American subjects, age range 1 year to 92 years, the authors noted that socioeconomic rather than genetic differences could account for the differences of immunoglobulin values which they have attributed to race (B10). Thus, white males had 37.9% less IgG ($P < 0.0001$) and 18.7% less IgA ($P = 0.0105$) than black males, whereas serum IgM concentration in black and white males were not significantly different. White females had 23.5% less IgG ($P < 0.0001$), 14.9% less IgA ($P = 0.029$), and 38.8% more IgM ($P = 0.004$) than black females. Their results confirm that there are significant differences in serum immunoglobulin levels related to the sex of the individuals as well as to the socioeconomic factors. Bantu females were observed to possess higher serum IgM levels than their male counterparts (S10).

3.4. Effects of Altitude and Climate

Residents of Mexico City, which has a temperate climate and is located at an altitude of 2240 meters above sea level, had significantly lower serum IgG and IgM than residents of Acapulco, which lies on the Pacific coast of Mexico and has a tropical climate. There was no significant difference between mean IgA levels in the two groups. Those subjects who had Negro racial features did not have any significant differences of immunoglobulin levels from the non-Negro group and, although malaria had been eradicated from the Acapulco area a few years previously, intestinal parasites which are common there might well account for the higher IgG and IgM in that population. The diminished immunoglobulin production in persons at high altitude may be part of a compensating mechanism to avoid excessive blood viscosity when erythrocytosis tends to occur because of high altitude (A5).

3.5. The Development of Ig's in Subtropical and Tropical Populations

Much of the early work on the development of the proteins in tropical populations, utilizing mainly electrophoretic technique, have been summarized (E6). Using both the quantitative electrophoretic and qualitative immunoelectrophoretic methods to study the developmental patterns of

the serum proteins in a comprehensive study, it was observed that both African umbilical cord blood and maternal blood contained significantly higher concentrations of γ-globulins than comparative European bloods. Whereas the mean concentration of γ-globulins in the umbilical cord bloods of European babies was significantly higher than the concentration in the blood of their respective mothers, African mothers on the other hand, with γ-globulin concentration below 1.6 g/100 ml, generally had babies whose γ-globulin levels were higher than that of their mothers while those with a γ-globulin concentration over 1.6 g/100 ml had babies whose γ-globulin concentration was below that of their mothers.

These observations were confirmed when the more specific quantitative immunodiffusion method for immunoglobulin determination was applied to the problem. Thus, in cord-blood serum and maternal serum from 38 mother and child pairs belonging to the Central African Negro population, with the exception of four, all such pairs showed higher IgG concentrations in the mother than in the infant, in contrast to the lower serum IgG in white mothers than in their respective infants' cord sera (M10). The concentration of IgG in fetal circulation was thought to depend partly upon selective transfer across the placental barrier and partly upon the presence of antigen in the fetal environment. Maternal fetal IgG ratio was also lower in serum of the Jamaican than in serum of the Nigerian, and, since the groups were racially similar, it was suggested that the presence of endemic malaria in Nigeria may be accountable for the pattern of transfer between mother and fetus in Africa (M11) (see Table 2).

The plasma concentrations of four Ig's were measured in several different ages in a large Gambian community, and the results are compared with those of British adults. By about the fifth year of age Gambian children attained adult Gambian levels of IgG, but levels similar to those found in British adults were attained by Gambian children at about one year of age. IgM was detected in all Gambian plasma samples, including one obtained 24 hours after birth. During the first four or five months of life, serum IgM levels in Gambian children rose rapidly to about 20% of the standard. However, between five months and six years there was no progressive increase, but beyond seven years of age, mean levels of IgM rose progressively throughout the remainder of the age span. One Gambian child, 24 hours after birth, had no detectable IgA in the plasma, but a progressive increase was found with increasing age (R6). Both IgM and IgA were detectable in trace quantities in cord blood of both Bantu neonate and Nigerian neonate (M9). Although in Egyptians IgA and IgM were not detectable in infant blood at birth, IgG was present at a higher level than in maternal blood (G1).

TABLE 2
MATERNAL–FETAL IMMUNOGLOBULINS IN SUBTROPICAL AND TROPICAL POPULATIONS[a]

Reference	Country	Race	Number of subjects	IgG	IgA	Igm
M36	Congo Republic	Black	38 Mothers	1966–2638 (1986)	—	—
		Bantu	38 Cord blood	1311–2402 (1731)	—	—
M9	Nigeria	Black	45 Mothers	2150–3250 (2700)	156–312 (244)	56–128 (92)
			45 Cord blood	1726–3266 (2408)	Trace in most	11–20 (16)
M11	Jamaica	Black	38 Mothers	444–1604 (1029)	226–254 (240)	181–235 (208)
			38 Cord blood	605–2021 (1313)	Nil in most	Trace in most
G1	Egypt	Egyptian	21 Mothers	(1990)	(180)	(110)
			18 Cord blood	(1831)	Nil	Nil

[a] Dash means not done. Mean values are given in parentheses.

3.6. EFFECTS OF PREGNANCY

In pregnant Gambian women serum IgG concentration appeared to be lower than in nonpregnant women (R6). Furthermore, in Gambians between 10 and 50 years of age, mean levels of IgM in males ranged between 56% and 83% of the female means, suggesting that the female sex hormone may be a factor in the control of IgM synthesis. Heterophile antibodies and serial immunoglobulins estimation were performed on 87 pregnant Nigerian women protected throughout gestation with antimalarial drugs (M15). The results are shown in Fig. 3.

Mean serum levels of IgG and IgA remained fairly constant throughout the earlier part of gestation although there was a tendency for the serum IgG level to decrease around term. On the other hand, serum IgM levels tended to increase throughout gestation and, in some pregnant women who had high levels of serum IgM, there was also a corresponding high titer of heterophile antibodies.

A pronounced fluctuation of serum immunoglobulin concentrations in maternal blood occurs around term, and in some women the serum IgG concentration may decrease by as much as 20% or more at the time of delivery and between week 34 and week 36 of gestation (M15).

4. The Immunoglobulins and Nutritional Status

It has been known for a long time that protein-calorie malnutrition (PCM) is always accompanied by a decrease in the levels of the serum

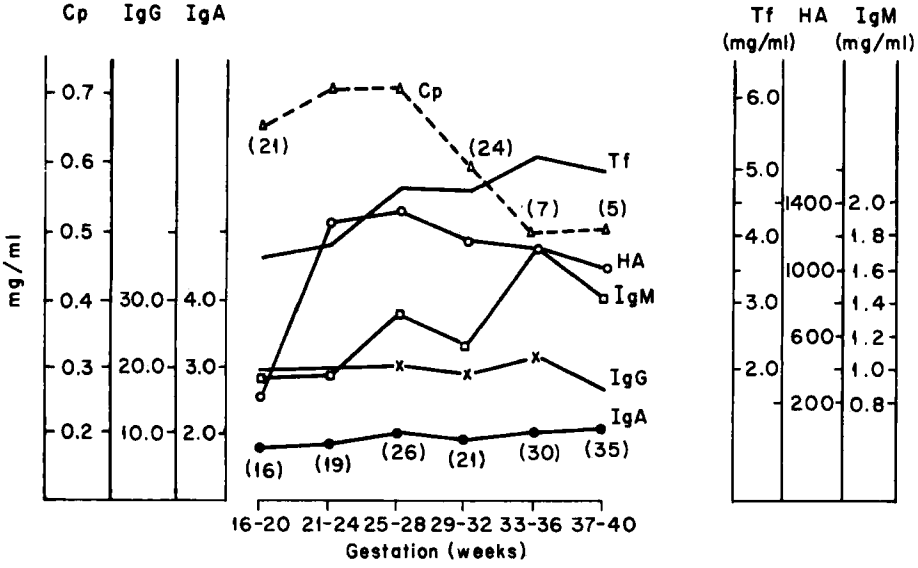


FIG. 3. Pattern of heterophile antibodies (HA reciprocal units of dilution), immunoglobulins (IgA, IgG, and IgM), transferrin (Tf), and ceruloplasmin (Cp) throughout gestation in malaria-protected pregnant women. The numbers of investigations at each period of gestation are indicated in parentheses at the bottom of the graph; where this differs, as in the case of ceruloplasmin, the numbers are so indicated. Note the increase of serum IgM and transferrin until just before term, when there is also a decrease of the IgG. Ceruloplasmin and heterophile antibodies show a decrease in mean concentration from 25 to 28 weeks of gestation.

albumin (G5). However, the situation is not so clear-cut with regard to the serum immunoglobulins. By means of semiquantitative free-micro-electrophoresis of Antweiler on 13 cases of malignant malnutrition and 9 cases of nutritional edema, the serum γ-globulins were observed to be normal or higher than normal (A7). There have been several subsequent reports, based on various electrophoretic techniques, on quantitation of the serum proteins in children with PCM. These results show lack of agreement—most likely because of the differences of electrophoretic techniques and because of the varied nutritional status of the children examined. However, in a larger series of 146 consecutive children with kwashiorkor, aged 6–80 months, there was a significant fall in the mean absolute concentrations of total protein, albumin, α_2-globulin and β-globulin, but the changes in mean serum γ-globulin concentrations were not statistically significant (E3).

4.1. Serum Ig Levels in Young Infants with Kwashiorkor

In Egyptian children with kwashiorkor whose clinical disease became apparent before 7 months of age there was a regular long-delayed and

deficient development of serum IgG, IgA, and IgM; however, older children who presented with their disease at 18–48 months had raised serum IgG, lower than normal IgM, and variable concentrations of IgA (A9), implying that severe kwashiorkor starting very early in infancy may be associated with profound depression of total serum immunoglobulin levels.

The immunoglobulin values were reported to be significantly depressed in Ugandan children with kwashiorkor when compared to five controls. Seven out of twenty of these children with kwashiorkor had serum IgG values above 1400 mg/100 ml; six had values between 700 and 1000 mg/100 ml, and two had values of 1240 mg/100 ml (B8). As judged from the ages of the children with kwashiorkor, the eleven who had serum IgG values ranging from 700 to 1200 mg/100 ml may be considered slightly reduced, while the rest appeared to be in the normal range. In one child who was followed up, there was a marked rise in serum IgM; this could have resulted from an acute infection and/or from increase in the IgM with increasing age of the child and/or to hemoconcentration, since most children with PCM lose their edema soon after treatment has commenced.

4.2. Serum Ig Levels and Infection in Kwashiorkor

Eleven Cape Colored children suffering from kwashiorkor and a variety of infections, such as conjuctivitis, otorrhea, respiratory tract infections, gastroenteritis, and candidiasis, and 11 well-fed children suffering from similar infections showed no differences between serum IgG and IgM, most of the results being high compared with those for normal children. The IgA values of the kwashiorkor cases were much higher than those of the controls and normal children (K1). Similar results were obtained in 24 South African children with PCM and suffering from a variety of infections. Synthesis of IgG, IgA, and IgM were not quantitatively impaired, but the convalescent mean levels of serum IgG of 1486 mg/100 ml were higher than the admission levels of 1106 mg/100 ml. Serum IgM behaved similarly to serum IgG, with admission level of 93 mg/100 ml and convalescent level of 143 mg/100 ml although serum IgA levels at these occasions decreased from 154 mg/100 ml to 116/100 ml (W4). These results are summarized in Table 3.

Using semiquantitative immunoelectrophoresis in 21 Egyptian children with kwashiorkor without evidence of infections, serum IgG precipitin arcs were more or less unchanged and were similar to the normal pattern. Thirteen of the children with severe kwashiorkor had serum IgM precipitin arcs that were shorter and less dense than the normal arcs. The majority of the rest had normal serum IgM lines. Eighteen of the chil-

TABLE 3
SERUM IMMUNOGLOBULIN VALUES (MG/100 ML) IN CHILDREN WITH PROTEIN-CALORIE MALNUTRITION[a]

Reference	Country	Number of subjects: Kwashiorkor patients	Number of subjects: Controls	Age (months)	IgG	IgA	IgM
B8	Uganda	20		7–66 (28.7)	700–1820 (1200)	70–360 (164)	60–300 (101)
			5	12–48 (27.6)	1480–2100 (1740)	52–132 (90)	66–240 (124)
K4	Mexico	39	—	—	(971)	(158)	(126)
		—	32	—	(1092)	(196)	(89)
K1	S. Africa	11	—	20.8	(1335)	(152)	(1952)
		—	11	(17.5)	(1212)	(71)	(150)
W4	S. Africa	22	—	7–34 (20)	492–2479 (1106)	77–308 (154)	27–324 (93)
		—	12	7–31 (19)	661–3302 (1475)	28–357 (99)	34–430 (120)
M14	Nigeria	47 (moderate)	—	12–72 (30)	1010–3314 (2312)	69–293 (181)	46–206 (126)
		37 severe	—	12–72 (30)	1038–3144 (2091)	108–266 (187)	40–164 (102)
Z4	Senegal	26	—	12–25 (20.7)	(1178)	(216)	(118)
		—	52		(1512)	(146)	(126)

[a] Values in parentheses are mean values.

dren with severe dermatosis and kwashiorkor had serum IgA precipitin arcs longer, denser, and more curved than the corresponding normal pattern (E8).

The higher than normal serum IgA in many children with protein calorie malnutrition may be related to increased synthesis of IgA by the intestinal lamina propria in response to increased antigenic stimuli from bacteria and virus. This is probably supported by the observation that children with kwashiorkor were found to maintain their polio antibodies during malnutrition, and their immune mechanism seemed to be quite capable of inhibiting poliovirus infection, indicating that the intestinal receptor cell for poliovirus operates normally in kwashiorkor (B8). It is now known that polio antibodies are mainly associated with IgA.

4.3. REFEEDING AND THE SERUM IG'S IN KWASHIORKOR

During an extended refeeding period there were no significant differences between the serum immunoglobulin values in two large groups of

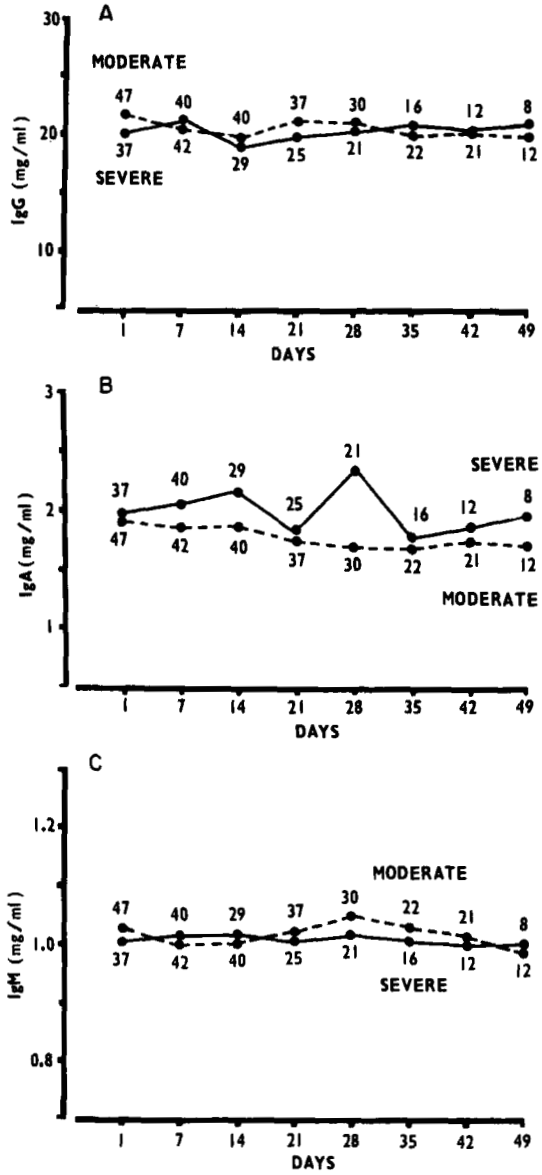


FIG. 4. Immunoglobulin patterns in two groups of children with kwashiorkor during an extended feeding period. (A) IgG; (B) IgA; (C) IgM. The figures at each point represent the number of patients investigated.

Nigerian children with both moderate and severe kwashiorkor, although there was a tendency toward higher serum IgA values in some children with the severe forms of the disease (M14) (Fig. 4A–C).

In 26 infants with kwashiorkor aged 12–25 months the serum IgG was reported to be low in the acute phase of kwashiorkor but increased during recovery. No change was observed in the serum IgM, but the IgA was significantly higher in the acute phase of kwashiorkor and throughout the recovery period. The interesting finding that the high admission level of serum IgD in the children with malnutrition decreased slightly after treatment (Z4) warrants further examination.

It is not unlikely that this increase of the serum immunoglobulin levels following refeeding in some malnourished children could well be due to hemoconcentration caused by reduction of the edematous body fluid as well as to the normal development of serum immunoglobulin concentrations with age, which is known to increase most rapidly in normal children between the ages of 3 and 9 months. The distribution of the immunoglobulins throughout the body fluid compartment—IgG readily distributed between the intra- and extravascular compartments and IgM exclusively in the intravascular space—are likely to be quantitatively affected by the redistribution of body fluids during convalescence from PCM.

Figure 5 shows the distribution of the immunoglobulins in children with PCM, on admission in comparison to age-matched controls. Here it can be seen that by far the majority of the children with PCM had immunoglobulin levels similar to those of their controls although it should be mentioned that about 10% of all children with PCM may have abnormally reduced or raised concentrations of one or the other, or all three of the major serum immunoglobulins (IgG, IgA, or IgM). Most of those children with low immunoglobulin levels did not always have the severe form of PCM and were without apparent infection. In addition, on refeeding, the reduced serum immunoglobulin levels returned to normal concentrations in some of the children, but there remained the occasional child with PCM whose depressed immunoglobulins did not return to normal levels on treatment, but remained low throughout—probably resulting in increased infection and finally death (M12).

4.4. Serum Ig's in Children with Marasmus

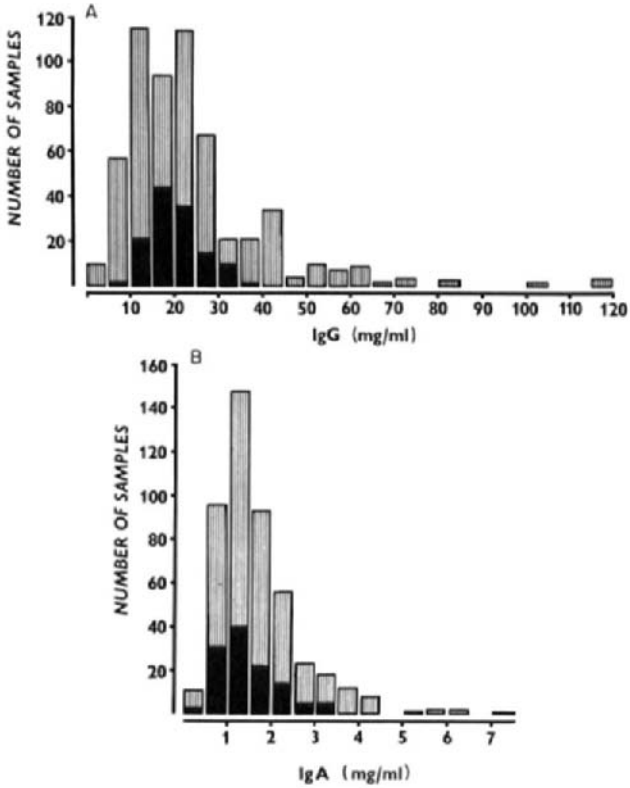
Results from two different groups of children reveal that the serum immunoglobulin levels in children with marasmus are not significantly different from those with kwashiorkor. Sixteen marasmic infants free from all signs of apparent kwashiorkor but all having gastrointestinal disturbances or respiratory infections had immunoglobulin levels as fol-

lows: in the 3–6 month age group the mean serum levels of IgM, IgA, and IgG in marasmic infants were significantly higher than the respective mean levels for healthy infants, whereas in the 7–12 month age group the levels of serum IgG and IgA were similar in the marasmic and healthy infants, but the marasmic ones had higher IgM levels (N1).

However, in 17 marasmic infants aged 12–36 months and free from infections, serum IgG immunoprecipitin arcs were similar to those of the normals; serum IgM precipitin arcs were longer, thicker, and more curved in the marasmic children than in the controls, and the majority had normal serum IgA precipitin arcs (E8).

4.5. Serum Ig's at Death in Children with Kwashiorkor

It is of interest that 8 cases of kwashiorkor, antemortem and postmortem, showed immunoglobulin levels not below normal levels, even in those with total germinal center depletion, although IgA was raised in 4 cases (S4).



Figs. 5A and B. See caption on facing page.

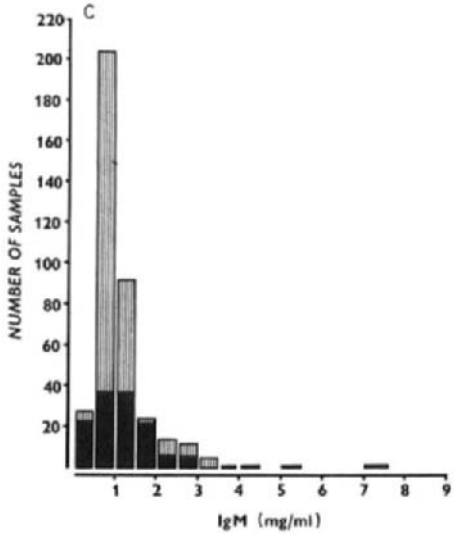


FIG. 5. Distribution of serum immunoglobulins in children with kwashiorkor (▥) and age-matched controls (■). (A) IgG; (B) IgA; (C) IgM.

4.6. SYNTHESIS OF IG'S IN MALNUTRITION

Immunoglobulin synthesis in malnutrition is clearly related to—among other factors, such as age—extent of infection of the patient. Thus the γ-globulin in 7 children with kwashiorkor maintained on a restricted protein intake of 0.5–1.29 g of protein per kilogram per day were relatively unaffected. But although the albumin synthesis rate during kwashiorkor was only about a third of the normal, the rate of γ-globulin production in children whose illness was complicated by infection was considerably increased. The synthesis rate in children with kwashiorkor and with infection was three times higher than in uninfected malnourished children and was comparable to that of adults exposed to hyperendemic malarial infection, and it has been suggested that the increased production of γ-globulin in severe protein-depleted subjects may seriously reduce the rate of synthesis of other biologically important proteins, such as albumin and transferrin (C17).

It is likely that the lack of agreement of results concerning the immunoglobulin levels in children with PCM stem from the fact that the values found in children in the subtropical and tropical populations are often compared with values from the temperate regions, where it is now abundantly clear that the serum immunoglobulin patterns in the white race differs significantly from patterns in the black race. Furthermore, the nutritional status of each group of children must vary from country

to country, as does the immunochemical technique. Immunoglobulin levels in kwashiorkor children have also been compared with levels in children who are much older.

4.7. Specific Antibody Production in Malnutrition

Although the synthesis of the immunoglobulins in the majority of children with malnutrition, age above 7 months, is not impaired, there is now mounting evidence that the ability to produce specific antibody to some well known antigens may be subnormal (M14).

4.8. Antibody Production to Typhoid and Diphtheria in Malnutrition

In rabbits with undernutrition due to a shortage of calories there was a depressed agglutinin production against typhoid vaccine (C3, R4). A similar incapacity to produce antibodies to typhoid vaccine was reported in children with severe protein malnutrition (B11). During treatment of 5 children, mean age 24 months, with chronic primary malnutrition, it was observed that their response to a single standard dose of 100 units of purified diphtheroid toxoid was small and slow. Furthermore there was no correlation between specific antibody response and serum protein levels (O2).

A moderate degree of restricted diet in rats had little, if any, effect on the secondary antitoxin production, but in later work prolonged and severe undernutrition had a definitely deleterious effect on secondary antitoxin production (H2).

4.9. Antibody Production to Yellow Fever, Smallpox, Polio, and Other Viruses in Malnutrition

The antibody response to yellow fever vaccine was impaired in protein-deficient children with kwashiorkor compared to the well-nourished controls. Polio antibody production was normal in the malnourished children, all of whom also responded in the normal fashion to smallpox vaccination. They had no evidence of disseminated vaccinia (B8). In Guatemala, on the other hand, smallpox vaccination of children who had fully recovered from severe protein-calorie malnutrition led to a drop in their nitrogen retention with the added complication of disseminated vaccinia (V3).

Underfed mice had depressed antibody response to Western equine encephalomyelitis vaccine (R10). Prisoners of war on protein-deficient diets had significantly lower antibody to both fowl red cell and tobacco mosaic virus (G2). The percentage of undernourished subjects reacting

at the higher dilutions of serum was definitely smaller than that of the controls.

On the other hand, there are reports stating that severely ill nutritionally depleted patients were found to be capable of producing antibody as well as or better than the healthy controls. Furthermore, antibody production was found to continue up to the time of death from wasting disease (B1).

4.10. The Thymus in Malnutrition

Simon, in 1845, described the thymus as an early critical barometer of nutrition (S11) and Hammar (H1) was so impressed by the characteristic lesions produced in the thymus by malnutrition (J1) that he applied the term "accidental involution" to describe the atrophied changes produced in the thymus by malnutrition. Of all the thymolymphatic organs the thymus first showed the most pronounced involution in the malnourished individuals, followed by the spleen (A8) and then the rest of the lymphatic organs (J5) (Table 4).

Children dying from malnutrition have been first noted in 1937 to have had a profound depletion of the thymus gland (V2). This has now been confirmed in several parts of the world (T6, W5).

4.11. Effects of High Levels of Corticosteroids on the Thymolymphatic System in Malnutrition

The atrophied changes of the thymolymphatic system is thought to be in part due to the high levels of plasma glucocorticoids in malnutrition (M12, S4, S12). In accordance with this view is the observation that protein-deficient rats had a very marked increased uptake of 1,2-$^3H_2$-

TABLE 4
Loss in Organ Weight of the Thymolymphatic System Following Malnutrition[a]

Organ	Percentage loss of weight	
	Dogs	Rabbits
Total body	27.1	23.5
Spleen	73.5	62.8
Thymus	68.1	87.9
Mesenteric lymph node	56.9	52.5
Cervical and popliteal lymph nodes	41.3	38.8
Appendix	—	43.2

[a] Jolly (J5).

corticosterone into their thymus and spleen as compared to their well fed controls and a high concentration of free-circulating plasma cortisol was associated with depressed cell-mediated immunity in malnutrition. High doses of corticosteroids can increase IgG catabolism and impair its synthesis and this factor may account for the occasional low level of serum IgG observed in a few of the patients with malnutrition.

The massive atrophied change which takes place first in the thymus followed by the spleen could well explain the marked depressed function of thymus-dependent lymphocytes (T cells)—phytohemagglutinin-responsive cells—in peripheral blood of subjects with protein calorie malnutrition (J6, M18, S6, S12).

4.12. Skin Transplant Reaction in Malnutrition

Protein-deficient mice, although responding normally to phytohemagglutinin, were still able to reject skin autografts more rapidly than normally nourished controls which showed striking depression of antibody synthesis to sheep red blood cells (J7). Similarly malnourished rats which had marked suppression of plaque-forming cells and rosette-forming cells showed no difference in skin transplant rejection from their well fed controls (M18).

4.13. Conclusion

Synthesis of immunoglobulins is not impaired in most patients with malnutrition. The majority of children above 7 months of age have normal serum immunoglobulin values. About 10% have elevated or low values, which are now not always related to the severity of the nutritional defect, but also to whether infection is present or not. Production of specific antibody to many antigens, as well as cell-mediated immunity, are depressed, however, in malnutrition.

5. The Immunoglobulins in Organ-Specific Diseases

5.1. Heart Diseases

From the results of the variety of studies undertaken, it now appears certain that no one single immunological factor is responsible for the various forms of heart diseases which are seen in populations in subtropical and tropical countries. Thus, in patients with endomyocardial fibrosis (EMF), rheumatic heart disease, or idiopathic cardiomegaly, heart antibodies were present in 42% and thyroid antibodies in 15%; serum cryoglobulins were present in 74% of the patients but was more frequent in those patients who had EMF than in any of the other groups (V1). The suggestion was therefore made that patients with EMF were

associated with an autoimmune phenomenon. Subsequently other individuals, without evidence of any heart disease, who came from areas of lower malarial endemicity to live in areas of high malarial endemicity were found with similar immunological disorders of high titers of malarial antibody and the presence of high levels of IgM and circulating autoantibodies to heart, thyroid, and gastric parietal cells. The point was therefore made that such an immunological background may condition the susceptibility of these individuals to certain disorders like EMF (S8). Nevertheless, patients with EMF were reported to show a 64% positive skin test to cattle filarial antigen. However, when a microfilarial antigen prepared from human blood was used, it was observed that only 7 of 31 patients with a variety of heart disease gave a positive immunoprecipitin reaction against the antigen (C5).

Table 5 summarizes the serum immunoglobulin levels in patients from Nigeria with organic heart disease. Two patients with EMF had secondary macroglobulinemia; there was a general tendency, however, toward higher IgM values in this group than in any other group of patients with heart disease. In addition, patients with rheumatic heart disease had higher mean IgA levels than did patients of the other groups. It should be borne in mind, however, that the widespread prevalence of protozoal, bacterial, and helminth infections in these populations may obscure significant differences in the serum immunoglobulin levels in those patients with various forms of cardiac diseases (C5).

TABLE 5
SUMMARY OF IMMUNOGLOBULIN CONCENTRATION IN PATIENTS WITH ORGANIC HEART DISEASES[a]

Serum immunoglobulin type[b] (mg/ml)	Endomyocardial fibrosis	Rheumatic heart disease	Hypertensive heart failure	The rest
IgG				
Mean	22.65	22.90	23.76	23.10
SE	1.82	2.89	2.11	2.29
No.	(28)	(10)	(20)	(25)
IgA				
Mean	2.67	5.04	2.45	2.80
SE	0.2	1.45	0.38	0.43
No.	(28)	(8)	(18)	(24)
IgM				
Mean	3.83	2.79	2.30	2.35
SE	1.24	0.38	0.36	0.38
No.	(28)	(10)	(17)	(24)

[a] From Carlisle *et al.* (C5). By courtesy of the publishers of *British Heart Journal.*
[b] SE, standard error; No., number of patients.

5.2. Liver Disease

The immunoglobulins in the serum of patients with other diseases implicating the liver have been dealt with in other sections, and Hobbs has recently extensively reviewed this subject (H5).

This section is confined to the disturbance of the serum immunoglobulins in an unusual type of liver disease: Indian childhood cirrhosis or infantile cirrhosis is one of the major causes of mortality in Indian children and the disease is uniformly fatal. Its etiology is unknown, but recent studies strongly suggest that there is a disturbed immunological process, probably involving both the humoral and cellular aspects of the immune response.

A marked increase of all three major serum immunoglobulins, in particular the serum IgM, was observed in 30 children aged between 8 and 30 months with Indian childhood cirrhosis (C6). The finding of a low total and altered B_1C component of complement suggested that complement was probably being utilized in the antigen–antibody reaction, which may be related to the extraordinary liver cell destruction in these unfortunate children (R2). The detection of hepatitis-associated antigens along with elevated serum immunoglobulin levels, as well as high titers of serum hemagglutinating antibodies, may support some associated viral etiology (R1).

5.3. The Kidneys

The association between *Plasmodium malariae* infection and the nephrotic syndrome in children has now been established (S13), and malarial antigen–antibody complexes of IgG, IgA, and IgM, and B_1C have been detected by immunofluorescent deposits along the basement membrane of the glomerular capillaries. With respect to the proteinuria, the majority of Nigerian children with the nephrotic syndrome had poorer selectivity of IgG and, usually, B_1C, than most European nephrotic children. A few children with the nephrotic syndrome who responded well to steroid chemotherapy have very low values for the clearance ratio of IgG:albumin. Renal biopsy studies confirmed that granular deposits of immunoglobulins as well as B_1C complement were deposited in the basement membranes (A6). The utilization of complement by malarial antigen–antibody complexes being responsible for the kidney lesion in the nephrotic syndrome seems an attractive concept.

In an extensive study (A4) of 88 Nigerian children with the nephrotic syndrome, 80% were observed with a nonselectivity proteinuria, and steroid therapy was ineffective in most of these children. Unlike European and American children with the nephrotic syndrome, less than half of the Nigerian children who had highly selective type proteinuria showed

a good clinical response to steroids. On the other hand, the majority of Indian children with the nephrotic syndrome responded fully to steroid treatment. In these the differential protein clearances of albumin, IgG, transferrin, B_1C, and α_2-macroglobulin confirmed the highly selective nature of their disease. Those nephrotic children who did not respond had a poorly selective proteinuria (C7). Ugandan patients who had immunofluorescent evidence of IgG, IgM, IgA, and B_1C deposited along the glomerular basement membrane had glomerular nephritis resembling that of poststreptococcal rather than that of quartan malaria (W14). This observation strengthens the argument that immune complexes from any source whatever, be it malaria or helminths or bacteria, may well be responsible for the nephritis seen in populations in subtropical and tropical countries.

5.4. Diseases of the Spleen

The enlargement of the spleen in subtropical and tropical populations has attracted much attention, particularly so in Africa and Australia.

Charmot, in 1959 (C8), first described the syndrome "splénomégalie en Afrique noir." Subsequently several publications on splenomegaly in Africans appeared from Charmot and his colleagues, linking the association of various parasites with the etiology of splenomegaly [for reviews, see references (C9, C10, C11, D2)]. In 1963 Charmot *et al.* wrote: "The principal cause of macroglobulinemia in Africa are trypanosomiasis and then leprosy, but alongside them, there is a small number of adults with chronic splenomegaly. A possible explanation of this is malaria plus some immunological disturbance." The impressive contribution to the study of tropical splenomegaly made by Charmot, starting as early as 1957, in which he studied in great detail both the clinical and laboratory aspects of the disease aroused the attention of fellow workers in the field, and resulted in the disease being then known as "la maladie de Charmot." Its geographical distribution at that time included Madagascar, Algeria, the Congo, Majunga, and Tananarive, but now it is known to be much more widespread.

In a letter addressed to the author in 1965, Charmot wrote: "I am above all interested in the γ^1M accompanying certain adult chronic splenomegalies. I think it is a matter of a disorder in the immune response to malarial infection."

In Uganda, Marsden *et al.* (M31) and in Zambia, Lowenthal *et al.* (L5) reemphasized the association between tropical splenomegaly and malarial infection, and, with the introduction of specific immunochemical assay methods, interest in the association between tropical splenomegaly and malaria was reawakened.

Wells (W7) in a series of excellent articles, noted that although the

serum immunoglobulins were elevated in all groups of New Guinea indigenes, the highest levels of serum IgM occurred in those with splenomegaly, the serum IgM having a wide range of values from 150 to 3000 mg/100 ml. These observations supported the earlier ones made by Charmot that splenomegaly and macroglobulinemia may represent an unusual immunological response to repeated malarial infections in susceptible individuals. In this population, clinical splenomegaly tended to be associated with greater hepatic sinusoidal infiltration and high malarial fluorescent antibody titers (M31). Furthermore, higher levels of rheumatoid factor, thyroglobulin antibodies, euglobulins, and cryoglobulins were detected in patients with splenomegaly than in control subjects (W8).

In Nigeria, Sagoe (S1) confirmed the markedly elevated levels of the serum IgM in patients with tropical splenomegaly, noting that the responses to prolonged chemotherapy with proguanil was found only in cases of tropical splenomegaly syndrome who had high serum IgM levels and normal lymphocyte transformation to phytohemagglutinin. Nonresponders to proguanil had low serum IgM levels, and their lymphocytes failed to transform with phytohemagglutinin and later developed a malignant lymphoma or chronic lymphatic leukemia.

Populations of Rwanda migrants in Uganda who came from areas of low malarial endemicity to live in areas of high malarial endemicity had considerably heavier spleens and an immunological syndrome consisting of elevated titers of malarial antibody, very high levels of serum IgM, and increased circulating autoantibodies to heart, thyroid, and gastric parietal cells than the local population (S8).

In New Guinea the mesoendemic malarious area of Watut, childhood splenomegaly was often associated with significantly higher levels of all three immunoglobulins whereas adults with splenomegaly showed markedly higher levels of serum IgM but significantly lower IgA concentration than did subjects with impalpable spleens. Malaria was the only apparent cause of the splenomegaly in the population (C22), and the transition from simple malarious splenomegaly to tropical splenomegaly syndrome occurred between the ages of 6 and 20 years which coincided with the changing pattern of serum imunoglobulin levels. The higher serum IgM concentrations noted in females than in males was also associated with a higher incidence of splenomegaly in females (C23).

The mortality in New Guinea from tropical splenomegaly may be as high as 57% in those with the severe form of the disease. However, splenectomy reduced the high levels of serum immunoglobulins as well as the mortality rate (C22, C24).

The significance of the reduced levels of serum complement and the

high levels of serum IgM and their association with immune complexes, as well as the lymphocyte response to a larger range of specific antigens in patients with tropical splenomegaly (Z3), necessitates further investigation.

In experimental work, the splenomegalic response of malarious mice fed on a low protein diet was less pronounced than that of those on a higher protein diet, indicating that the tropical splenomegaly in malarious mice may not be related to their nutritional status (S18) although it would appear that the situation may be a more complex one in humans who have been assaulted with parasitic infections over a longer period.

6. The Immunoglobulins in Parasitic Diseases

6.1. Malaria

6.1.1. *Serum γ-Globulin and Immunoglobulins in Malaria*

The characteristic hypergammaglobulinemia of tropical and subtropical populations have been chiefly ascribed to malarial infection; up until the 1950's quantitation of the γ-globulin in the serum of patients was wholly by the electrophoretic method, which does not separate the γ-globulin into its different immunoglobulin components (D5, T2).

Thus, in East Africa, the serum protein patterns of three groups of Africans who were exposed to different rates of malaria transmission suggested that there might be som causal relationship between malarial incidence and the serum γ-globulin levels (H8). Gambian children protected for three years from malaria by treatment with the antimalarial drug chloroquin had significantly lower γ-globulin than the unprotected children (M19, M20, M24). Comparison of the serum γ-globulin of malaria-protected children whose hemoglobin genotype was AA with unprotected children of the same hemoglobin genotype revealed the effect of malarial prophylaxis on the serum γ-globulin concentration. There was no significant difference in γ-globulin concentration in both groups of children up to the age of 12 months. However, significant differences in γ-globulin concentration were observed in older children up to the age of 26 months—the protected children having significantly lower concentrations of γ-globulin, indicating that the immune response to malaria was a most important factor in inducing the hypergammaglobulinemia in tropical populations. Furthermore, the observations that the mean γ-globulin for these unprotected children with hemoglobin genotype AS was higher than the value for the unprotected children, suggested that the sickle cell hemoglobin gene may somehow augment the hypergammaglobulinemia in

children unprotected from malaria. Also, when quantitation of the three main immunoglobulin classes by specific immunochemical methods was applied to the problem, it was indeed interesting to find that American Negroes with sickle cell disease had significantly higher mean serum IgG levels of 1682 ± 398 mg/100 ml and IgA 416 ± 178 mg/100 ml ml than healthy American Negroes who had mean serum IgG of 1250 ± 329 mg/100 ml and mean serum IgA of 162 ± 66 mg/100 ml. Both groups of Negroes had similar levels of serum IgM (C1).

6.1.2. *Experimentally Induced Malaria*

In human volunteers, when infected with human and simian malaria, there was a direct correlation between rise of malarial fluorescent antibody production and the increased serum γ-globulin levels. When an immunoelectrophoretic technique was used for the study of the serum immunoglobulins in malarial infection, there was a consistent increase in the B2M macroglobulins (IgM) in 10 hospitalized human volunteers who were infected with *Plasmodium vivax* and *Plasmodium cynomolgi.* The impressive rise in the IgM antibodies was later followed by an increase in the IgG after repeated malaria stimulation—indicating that the high IgM was due to the primary response whereas the subsequent rise of the IgG globulin was due to the secondary immune response. Fractionation of the sera from the malaria-infected volunteers by gel filtration in Sephadex G-200 columns revealed that the malarial antibodies consisted of both 19 S macroglobulins and 7 S γ-globulin antibodies (A1). Later work has shown that antibodies to malarial parasite infection may also be found in the serum IgA fraction (T3). However, populations living in an endemic malarial area are subjected to repeated malarial infection and therefore exhibit an immune reaction characteristic of the secondary immune response (W10) and when their sera were fractionated by gel filtration on Sephadex G-200, most of the malarial antibody activity was detected in the 7 S fractions.

In carefully controlled experiments in 12 human volunteers infected with *Plasmodium vivax* and 5 with *Plasmodium cynomolgi,* serial estimates of the serum immunoglobulins were made before, during, and after the primary malarial attack and in one case after relapse, which revealed that large amounts of IgM globulin were produced in all subjects. Twenty-six days after the infection with *P. vivax* or *P. cynomolgi* the serum IgM concentration reached its maximum of 560 mg/100 ml from a preinfection value of 113 mg/100 ml. On day 91 after infection, the serum IgM level approached its original value and malarial fluorescent antibody titer (MFAT) followed the same pattern of the serum IgM, although the MFAT remained at relatively higher levels for 252 days

after parasitemia and longer than did the serum IgM. Both the serum IgG and IgA increased by 42% and 55%, respectively, after malarial infection in human volunteers (T4).

It is now clear that fluorescent antibody to malaria persists for years after the patent malaria infestation has long disappeared, and malaria antibodies were still detectable in some West Africans after they had been in Britain for up to seven years (K5, V4).

In a longitudinal study of experimentally induced malarial infection in rodents, the serum IgM made a relatively higher contribution early in infection, but both IgG and IgM malarial antibody persisted throughout the period of study for 2 months (C20).

6.1.3. *Malaria and Immunoglobulins in Infants*

In areas where malaria is hyper- or holoendemic, newborn infants had high titers of malarial antibody which decreased during the first 6 months of life and then showed a gradual rise throughout childhood until adult levels were attained. The pattern of the malarial fluorescent antibody titer in children in a malaria endemic area, reflected the development of the serum IgG more closely than that of any other immunoglobulins, and the early fall in the titer of the malaria fluorescent antibody coincided with that of the loss of maternal IgG from the children's circulation, implying that malarial antibody is quite capable of traversing the human placenta (M13) (Table 6, Fig. 6).

Studies on a much larger population of Gambian children confirmed

TABLE 6

AGE OF SUBJECTS AND NUMBER OF SERA INVESTIGATED[a]

Mean age range	IgG	IgM	IgA	MFAT
Cord blood	32	32	32	32
1–2 weeks	11	11	11	0
2–4 weeks	36	34	35	1
4–8 weeks	55	52	52	9
2–3 months	45	33	34	12
3–4 months	67	41	42	14
4–6 months	103	72	70	14
6–12 months	144	120	123	43
1–2 years	40	37	36	0
2–5 years	90	89	70	0
5–12 years	80	79	57	0
12–20 years	19	19	19	0
Above 20 years	51	51	51	30

[a] From McFarlane *et al.* (M6). By courtesy of the publishers of *Tropical and Geographical Medicine.*

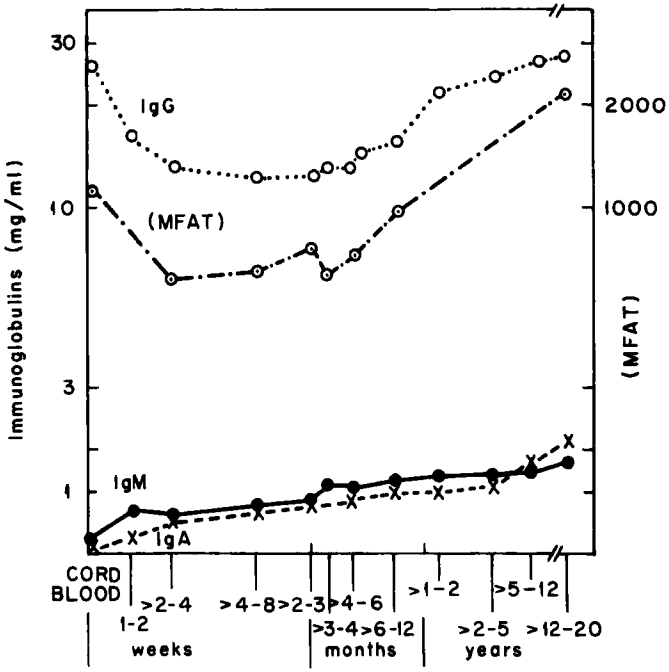


FIG. 6. Pattern of development of IgG, IgM, IgA (mg/ml) and malarial antibodies (reciprocal of dilution units) in Nigerians, plotted on semilog paper. MFAT, malarial fluorescent antibody titer (M6). By courtesy of the publishers of *Tropical and Geographical Medicine*.

that malarial parasitemia was associated with an increased mean serum IgG level in age groups up to 20 years, but with increased serum IgM levels only in the first two years of life. Indeed, Gambian plasma which contained precipitating antibodies to malarial antigens showed higher mean levels of IgG and IgM in most of the age groups tested. No consistent relationship was observed between malaria and mean levels of serum IgA or IgD (M23). These authors confirmed that although most of the immunoglobulin levels in the same individual were relatively stable in adults over the study period, the serum IgM levels were highest during the wet season.

6.1.4. *Malaria and Pregnancy*

In pregnancy the frequency of malarial parasitemia and the density of infection are higher in the pregnant women than in either the same individuals before pregnancy or in a nonpregnant control group of the same age (G3). Higher malaria fluorescent antibody titers were detected in parasitized pregnant women than nonparasitized pregnant women in

both Gambian and Nigerian populations (M23, W11). These observations are compatible with the tendency to reduced levels of IgM and IgG at term, observed in several tropical populations, and with the decreased γ-globulin synthesized by a malarial, unprotected, 3-month-pregnant woman when compared to that synthesized by nonpregnant subjects (C16).

6.1.5. *Synthesis of Immunoglobulins in Malarious Subjects*

The synthesis of IgG in African subjects unprotected from malaria was approximately twice (169 mg/kg/day) that of Africans protected from malaria and seven times that of Caucasians (23 mg/kg/day), implying that malaria must account for a good proportion of the high immunoglobulin levels found in persons in a malarious area (C18). In fact absorption experiments have shown that about 3–10% of the immune human IgG was specific malarial antibody (C25).

Passive transfer experiments in subjects infected with malarial parasites have indicated that protective malarial antibodies exist in immune sera. It would appear from the work in human volunteers and in field surveys that such malarial antibodies in fact belong to both IgG, IgA, and IgM. A powerful antiparasitic effect was noted when adult Gambian γ-globulin was given to persons suffering from malaria parasitemia (C18). Nigerian adult and cord blood γ-globulin also had an antiparasitic effect in children with *Plasmodium falciparium* infection (E7).

6.1.6. *Separation of Malaria Antigens*

Apart from antibodies detected by (a) the schizont-infected red cell agglutination test, (b) the agglutination of sporozoites, (c) complement fixation, (d) passive hemagglutination and by the direct and indirect immunofluorescent methods [for review, see reference (V4)], malarial antibodies have also been detected by malarial antigens prepared from heavily infected human placenta, infected human brain, and short-term *in vivo* cultures of cells from heavily parasitized subjects (W11) (see Tables 7 and 8).

Thus, using a modification of the Spira and Zuckerman technique (S15), two malarial antigens—T1 and T2—were purified from malaria trophozoites recovered from the blood of heavily infected children. Two further antigens—P1 and P2—have been prepared from pre-schizonts and schizonts recovered from densely infected placental blood. Gel diffusion analysis showed that these antigens reacted with malarial antibody from immune sera which migrated in the γ-globulin region (M21). Two groups of antigens, α and β, recovered from the aqueous extracts of heavily *P. falciparum* parasitized maternal blood were referred to as sur-

face antigens (T8). Gel diffusion tests of malarial antigen prepared from a heavily parasitized human brain after autopsy gave strong precipitin lines with immune sera which had elevated levels of all three immunoglobulins (W9). It now appears that malarial antigens can be classified according to their heat stability. However, using several combinations of carefully controlled immunochemical techniques, higher titers of antibodies were detected with malaria schizonts as the source of antigen than with the trophozoites, and in addition, malaria antibody was detected with antisera to IgG and IgM and at low levels to IgA, but not with antisera to IgD or to IgE (T3).

TABLE 7

A. MALARIAL ANTIBODY TITERS AND IMMUNOGLOBULIN CONCENTRATION IN SOME NIGERIANS[a]

Study No.	Agar gel diffusion test	Serum immunoglobulins (mg/ml)			Malarial fluorescent antibody titer	Parasitemia
		IgG	IgA	IgM		
1	+	25.60	2.50	1.12	640	—
2	+	20.00	1.70	0.84	1280	—
3	−	40.00	2.80	2.50	1280	—
6	+	42.00	3.30	4.50	1280	—
7	++	40.00	1.70	1.80	2560	—
8	−	25.00	3.90	2.80	5120	*P. falciparum* 200 mm³
9	+	49.00	2.85	0.80	5120	—
10	+	36.00	3.90	2.24	640	—
11	+	30.00	2.30	1.12	1250	—
12	++	24.00	4.01	2.80	5120	*P. falciparum* 980 mm³
13	−	30.00	2.30	1.60	2560	—
14	−	72.00	3.50	3.36	5120	—
15	−	22.00	2.80	0.80	160	—
16	−	27.00	3.90	4.50	5120	—
17	+	40.00	2.30	3.00	2560	—
18	+	68.00	4.40	0.80	640	—
19	+	22.00	1.00	11.05	5120	*P. falciparum* 20 mm³
20	−	29.00	1.80	1.70	2560	—
21	+	48.00	5.10	3.60	1280	*P. falciparum* 1420 mm³
22	+	35.50	3.00	7.70	1280	*P. falciparum* 60 mm³
23	−	20.00	4.00	4.60	5120	*P. malariae* 540 mm³
24	+	28.00	1.25	1.85	2560	Not determined
25	++	31.00	2.75	2.00	5120	Not determined
26 (cord serum)	++	25.00	Nil	0.17	2560	Not determined
Mean values		34.54 ±2.80	2.806 ±0.55	2.802 ±0.55	2752 ±380	

TABLE 7 (*Continued*)

B. Analysis of Immunoglobulins and Malarial Antibody in Terms of Malarial Infection[a]

Blood film	No. of patients with a positive precipitin line	Malarial antibody titer	IgG (mg/ml)	IgM (mg/ml)	IgA (mg/ml)
Malaria, +	5/6[b]	3,840 ± 812	29.1 ± 4.5	5.4 ± 1.3	3.50 ± 0.56
Malaria, −	10/17	2,150 ± 387	39.2 ± 3.7	2.4 ± 0.47	2.77 ± 0.063
Difference between groups		$P = 0.05$	$P = 0.1$	$P = 0.1$	$P = 0.1$

[a] Modified from McFarlane and Voller (M8). By courtesy of the publishers of *Clinical and Experimental Immunology.*

[b] Five out of six patients gave a positive precipitin test. Malarial antigen was extracted from human brain for gel diffusion tests.

TABLE 8

Discrete Malarial Antigens Classified according to Wilson *et al.* (W13)

Property	Labile (L) antigens	Resistant (R) antigens	Stable (S) antigens
Susceptibility to heat	Destroyed at 56°C; unstable during storage at 4°C but stable for 2 years at −70°C	Destroyed at 100°C	Survive boiling for 5 minutes
Subclasses	Four La and three Lb	2	20
Occurrence	Antibodies to La antigens present at all ages in Gambian sera; cross the placenta readily Antibodies to Lb antigens are rare	Extremely rare	Antibodies to S antigens present in 60% of children aged 6–36 months does not appear to cross the placenta
Antibody type	Mainly IgG	? IgA	Both IgG and IgM; ? IgG2 and IgG4
Approximate molecular weight	La = 250,000	150,000	60,000
	Lb = 32,000	150,000	60,000
S 20 values	?	?	4.4

6.1.7. *Malaria Antigen Variations*

A survey was made of the various techniques that are at present available for the detection of malarial antibodies. It was noted that, when the direct agglutination method of schizont-infected red cells during the early stages of malaria infection was used, the antibody produced would specifically agglutinate parasites of that early stage. This result implied that antigenic variation of the malaria parasite occurred, which frequently led to variations of the specific malaria antibodies produced at each stage of the infection process (V4). This factor may account for some of the variable results obtained, and suggestions have been put forward that the period of years required for malaria-infected subjects to develop their maximum protective immunity must constitute the time required to produce antibodies to each malaria antigen variant (B7).

6.1.8. *Role of T and B Lymphocytes in Immunity in Malaria Infection*

It now appears certain that destruction of malaria parasites *in vivo* depends in part, if not wholly, upon the presence of humoral antibodies, and this must explain the success achieved with the passive transfer experiments. The exact role of the T and B lymphocytes in immunity in malaria infection in man still needs clarification. In rats the ability to resist *P. berghei* infection seems to be thymus dependent, and higher parasitemias were encountered in the thymectomized rats, which also developed severe anemia (B6).

6.1.9. *Malaria Infection and Autoimmunity*

It was observed at the beginning of this century by Hammar (H1) in Germany, and earlier by Simon (S11) in London, that stress from any cause, particularly from malnutrition or infection, may cause a profound diminution in size of the thymus gland; later work has shown that this atrophy of the thymus is related to impairment of cell-mediated immunity (see Section 4). Several workers have reported a suppression of the immune responses to various antigens in persons who have chronic infection with malaria. The infrequent occurrence of autoimmunity, like systemic lupus erythematosis in Nigerians as compared with Jamaicans and American Negroes, has been ascribed to the suppressant effect of malaria infections on the immunological system (G7). Furthermore, hybrid NZB × NZW mice infected with *Plasmodium berghei* at the age of one month had a significantly lower mortality from renal disease than did the control mice—implying that malaria infection has a protective role on the autoimmune disease of mice, although it was not unlikely that the malarial

parasite might have become contaminated with a virus that could also have accounted for the immunosuppressive effects (G8). Burkitt suggested that Burkitts' lymphoma in man may owe its geographical distribution to the synergistic reactions between malaria and viral infections (B12), and the experimental model in mice in which concurrent infection with a murine plasmodium *P. berghei yoelii* increased the incidence of malignant lymphoma following the infection 6 months later with an oncogenic Maloney virus, support this hypothesis (W6).

6.1.10. *Complement and Malarial Infection*

Complement fixation test for the detection of blood-induced vivax malarial antibodies were utilized as long ago as 1938 and reviewed by Voller (V4) and although a rise in titer occurred some days after inoculation, there was little correlation between complement fixing antibody and the protective capacity of the sera. Thus the exact role of complement in the pathogenesis of malaria infection is not precisely known. However, in Nigerian children suffering from malarial nephritis, the soluble antigen-antibody complex seems to consist of the B_1C complement (S13). In addition renal biopsy studies done early in the course of the disease on children with nephrotic syndrome showed that, apart from IgG and IgM, the B_1C component of complement was also found as a granular deposit along the glomerular blood vessels (A6).

6.2. Leishmaniasis

In the Central and South American countries and in the West Indies, infection from *L. mexicana* produces a very long-lasting immunity in humans once they have been infected, and there is rarely more than one lesion on a patient (W12). On the other hand, in Africa, infection from *L. tropica* produces a diffuse cutaneous lesion which does not heal and is most disfiguring to the patient. There are no remarkable changes in the serum immunoglobulin levels in infected persons except perhaps for a slight elevation of the serum IgA. Hemagglutinating antibody titers are normal, and Brycesson (B9) has suggested that a specifically impaired cell-mediated immunity may account for the course of the infection with leishmaniasis in Africa.

6.3. Filariasis

The serum immunoglobulin changes in patients suffering from filariasis are characterized by a moderate increase of IgM and slight increase of IgG (M35). A patient who had microfilariae in a bone marrow biopsy also had massive macroglobulinemia complicating rheumatoid arthritis (M4).

Microfilarial antibodies have been detected in some Nigerian patients with heart disease (C5) (see Section 5.1).

6.4. Sleeping Sickness (African Trypanosomiasis)

This disease is caused by *Trypanosoma rhodesiense* and *T. gambiense*, which occur and multiply in all tissues including the connective tissue, the blood, and the lymphocytes. African sleeping sickness has been known since the 11th century and is such a devastating disease that it has been greatly responsible for large areas of Africa remaining unpopulated. Goodwin recently remarked that "the ancient dragon of Africa, the trypanosome, is firmly entrenched" (G6). There is no evidence of a long-lasting acquired immunity to trypanosome infection. This is most likely due to the marked capacity of the African trypanosome organisms to vary their antigenic structures. During an infection several variants of the organism are produced, and each new variant in form causes the production of its own antibodies, which in themselves stimulate the production of further new antigenic variants (M29).

Probably also contributing to the lack of an acquired immunity is the fact that the antibody produced to trypanosome infection is chiefly IgM, which is known to have a relatively short half-life.

As early as 1958 Bergot *et al.* (B5) first described the association between secondary macroglobulinemia in Africa and trypanosomiasis. This was confirmed by Dreyfus *et al.* in 1960 (D4) and later by Mattern *et al.* (M33), who adopted the immunoelectrophoretic technique to indicate the high IgM content in the sera of affected persons. In 1963, Charmot *et al.* observed (C12) that the principal cause of macroglobulinemia in Africans is trypanosomiasis.

A high IgM concentration in serum and cerebrospinal fluid (CSF) although not pathognomonic of infection with *T. gambiense* or *T. rhodesiense* is a very useful screening test. IgM estimation as screening test in African sleeping sickness has been successfully used, not only in Dakar but also in Botswana, the Congo, Tanzania, and Uganda. In trypanosomiasis, although the IgM is nearly all of the 19 S type, some 7 S IgM has also been detected. The serum IgG is not always elevated in trypanosomiasis and may only be slightly high than the normal.

Mattern *et al.* (M33) observed that there was a significant increase in the level of B_2M (IgM) in both the serum and CSF of patients infected with *Trypanosoma gambiense*. They observed that the increase of IgM in the serum may be 10 to 20-fold. Lamy (L1) found persons infected with trypanosomiasis showed that a mean serum IgM of 830 mg/100 ml may be reached. Masseyeff and Lamy (M32) also reported raised levels of serum IgM in patients infected with trypanosomiasis. The CSF of in-

fected patients is also known to contain IgM even when the serum protein content is normal. Several other authors have confirmed that serum IgM is distinctly elevated and is a useful aid in the diagnosis of human trypanosomiasis. IgG and IgA, although sometimes elevated, are not as conspicuous as the IgM. After 1–2 weeks, the sera of patients who are infected with *T. gambiense* or *T. rhodesiense* show a very prominent IgM precipitin line on immunoelectrophoresis. It should be remembered though, that other parasitic diseases also give rise to high IgM in the sera of infected persons although not as consistently as with trypanosome infection. However, the absence of an increased IgM precipitin line on serum immunoelectrophoresis, or the absence of raised IgM levels on radial immunodiffusion are very useful immunochemical results to rule out trypanosomiasis.

Houba and Allison (H9) have shown that immediately after treatment three East Africans with *T. rhodesiense* infection had markedly elevated heterophile antibodies in their sera and suggested that this may be due to increased antigenicity of the killed parasites. The heterophile antibody was associated with elevated IgM.

Conclusion. In African trypanosomiasis there is a very marked increase of the serum IgM—about 10–20 times the normal level. Estimation of the serum IgM can be regarded as a very useful test in the diagnosis of African sleeping sickness. The concentration of serum IgG is slightly raised and serum IgA is normal. In the tropics, trypanosomiasis may be regarded as one of the chief factors which contribute to secondary macroglobulinaemia.

6.5. Chagas' Disease (American Trypanosomiasis)

This disease is caused by *Trypanosoma cruzi*, which occurs, but does not multiply, in the blood of the host. Contrary to the findings in patients with African trypanosomiasis, the sera of patients with Chagas' disease, which is endemic in many South and Central American countries, have near-normal levels of immunoglobulin concentrations. Lelchuk *et al.* (L3) stated that individuals with acute American trypanosomiasis had mostly normal serum concentrations of the three major immunoglobulins. In the chronic stage of the disease, however, serum IgA and IgM concentrations were normal but IgG was significantly increased. Specific antitrypanosome antibodies of the IgM class were mainly found in the acute stage, but in the chronic stage the specific antibodies were mainly of the IgG and IgA classes. Tests for immunoconglutinin, rheumatoid factor, and antinuclear factor in the sera of patients with Chagas' disease were all normal.

Marsden *et al.* (M30) in Brazil also noted that in patients with chronic

Chagas' disease, both the serum immunoglobulin levels and complement titers were similar to those of other uninfected subjects living in the same area. The experimental findings in C_3H mice, that thymus-dependent cell mediated immune mechanisms are involved in controlling infection with *Trypanosoma cruzi* appear promising.

6.6. Ascarisis

Hypersensitivity to *Ascaris lumbricoides* is well known.

There is a generalized increase of all the immunoglobulins in helminth infections. In Nigeria I have observed high IgG levels and IgA in some patients with hookworm anemia. The IgM appeared to be normal in some of the sera tested, although there was one patient with values showing a 5-fold increase of serum IgM concentrations.

In preschool children in East Africa, Johansson *et al.* (J4) observed higher values of IgE in those with stool positive for ascaris ova than in those with stool negative for ascaris. The range of IgE values in those with positive ova in their stool ranged from 240 to 14,300 mg/ml in comparison to the range of values of 120 to 5200 mg/ml for those Ethiopian children with no ova in the stool. Serum IgA values were the same in both groups, but some IgG levels in the Ethiopian children were about twice as high as in Swedish children; the serum IgM levels were not strikingly different between the groups.

6.7. Hydatid Disease

Alterations in the pattern of the serum immunoglobulin concentration in patients suffering from hydatid disease (human echinococcosis) occur mainly in the IgG and to a lesser extent in the IgM. Both complement-fixing and hemagglutinating antibodies of human hydatid disease were also associated with the IgG and IgM (S7).

6.8. Schistosomiasis (Bilharziasis)

Schistosomiasis is found in various parts of the tropics, and infection is caused by the penetration of the larval cercaria into the skin of the host. A highly selective increase of serum IgG concentration in six of 24 patients has been observed (M35).

Increased serum levels of both IgG and IgM have been reported in patients with chronic schistosomiasis (A10). There seems to be a close relationship between the *S. mansoni* egg output per day and the level of the immunoglobulins in the sera of infected persons, and Bassily *et al.* (B2) reported that those patients who had uncomplicated *S. mansoni* infection (Group I) with egg output amounting to 36.3 thousands per

day had IgG of 2305 mg/100 ml; IgA, 225 mg/100 ml, and IgM, 190 mg/100 ml; however in those patients with *S. mansoni* infection with intestinal polyposis group II, and with egg output of 262.3 thousands per day, the corresponding figures were 2210, 254, and 207 mg/100 ml. The group III patients, those with schistosomal splenomegaly with cirrhotic liver and ascites and with 0.4 thousand egg output per day, had significantly lower levels of IgM. It is likely that the eggs, being particulate in nature, stimulate the production of some IgM in infected persons.

In Liberia, serum immunoglobulin determination carried out in a group of patients with 92% rate of infection from protozoan or helminth infections had mean serum levels (mg/100 ml) as follows: IgG, 2630; IgA, 199; IgM, 211; and IgD, 3.44. The elevated IgG and IgM were ascribed to the helminth infections (C4). Ninety-two percent of patients with schistosomiasis had a positive test for rheumatoid factor M-antiglobulins, and Lehman *et al.* (L2) observed that rheumatoid factor existed in high frequency and high titer among patients with either chronic or acute salmonella infection, and in high frequency and low titer among patients with schistosomiasis.

In an excellent review of the immunopathogenesis of schistosomiasis, Warren (W3) reported that the egg of the schistosome parasite was the prime factor responsible for the hepatosplenomegaly in the mouse; furthermore, that neonatal thymectomy and antilymphocyte serum had marked immunosuppression on the granuloma formation around schistosome eggs injected into the lungs of mice, implying that cell-mediated immunity must play some role in the protection of the host against schistosome infection.

6.9. Amebiasis (*Entamoeba histolytica*)

Because of the difficulties encountered in the clinical and laboratory diagnosis of amebiasis, only a relatively limited number of papers have appeared dealing with the immunoglobulins in amebiasis. Eleven patients with amebic hepatitis were reported by Michaux (M35) as having elevated mean serum IgG of 32.00 mg/100 ml; IgA of 326 mg/100 ml; and slightly elevated IgM levels of 168 mg/100 ml; serum IgD levels were normal. In a more detailed study on 43 patients with acute amebic colitis—11 with amebic liver abscesses and 17 symptomless patient (amebic cyst/trophozoite) carriers as well as controls—the serum immunoglobulin measurements showed more marked changes from the normal patterns. Table 9 summarizes the immunoglobulin results from the various patients with amebiasis. The highest serum immunoglobulin concentrations were found in those patients with liver involvement.

TABLE 9
SERUM IMMUNOGLOBULIN LEVELS IN AMEBIASIS[a]

Group of patients	No. of subjects	Level of IgG Range and mean values ±2 SD (mg/100 ml)	Level of IgA Range and mean values ±2 SD (mg/100 ml)	Level of IgM Range and mean values ±2 SD (mg/100 ml)
Acute amebic colitis				
Male	15	2578–5388 (3727) ($p < 0.001$)[b]	84–528 (210) ($p < 0.05$)	43–272 (108) ($p > 0.1$)
Female, nonpregnant	13	2132–7743 (4062) ($p < 0.001$)	44–763 (183) ($p > 0.1$)	68–240 (129) ($p > 0.1$)
Female, pregnant	15	1854–5106 (3077) ($p > 0.1$)	66–316 (145) ($p > 0.1$)	42–268 (107) ($p > 0.1$)
Amebic liver abscess	11	1931–9239 (4223) ($p < 0.001$)	97–351 (184) ($p > 0.1$)	55–378 (144) ($p < 0.01$)
Symptomless carriers	17	1570–4742 (2729)	66–349 (152)	69–145 (100)

[a] Modified from Abioye *et al.* (A3a).
[b] p for each value is compared with the corresponding value of the symptomless carriers.

6.9.1. *Amebic Liver Abscess*

Whereas the highest serum IgG and IgM concentrations were found in those patients with amebic liver abscesses, their IgA levels were within normal limits.

6.9.2. *Acute Amebic Colitis*

The 15 male patients and 13 nonpregnant female patients with acute amebic colitis had markedly elevated mean serum IgG concentrations in comparison to the normal mean and the slightly reduced levels in pregnant females with acute amebic colitis. Two nonpregnant female patients with acute amebic colitis had exceptionally high IgG values of 7700 mg/100 ml. Occasionally a patient was encountered with amebic colitis who had markedly reduced serum IgA, and one such patient had an IgA level of 12 mg/100 ml. It is interesting to note that pregnant females who had acute amebic colitis had the lowest values for all three immunoglobulins, and this may in part help to explain the unusually high mortality rate from amebiasis in pregnancy (A3).

Immunoglobulin follow-up studies on 14 patients with invasive ame-

biasis during treatment showed a consistent decrease of all three in over 90% of the patients, implying that the immunoglobulin estimation therefore appears to provide some indication of response to chemotherapy of patients with *E. histolytica* infection (see Table 10).

On the other hand, there was no such consistent and dramatic reduction of the immunoglobulin levels following treatment of the symptomless carriers (Table 11).

The mean serum IgA concentration was reduced by approximately 20% and the IgG and IgM by 8%, respectively, when serum from pa-

TABLE 10
SERUM IMMUNOGLOBULINS IN AMEBIASIS: FOLLOW-UP STUDY[a]

Patient No.	IgG (mg/100 ml)			IgA (mg/100 ml)			IgM (mg/100 ml)		
	Week: 0	1–2	5–6	0	1–2	5–6	0	1–2	5–6
	(1)[b]	(15)	(42)						
1	5300	4700	3100	235	165	200	120	110	100
	(1)	(12)	(40)						
2	4700	4000	3400	400	400	385	650	480	390
		(16)	(36)						
3	7100	4700	3000	400	380	340	550	450	350
		(14)	(34)						
4	7200	3400	2100	400	255	290	255	190	128
		(15)	(40)						
5	3200	2600	1900	961	85	90	300	410	178
		(15)	(40)						
6	4900	4400	3400	165	165	168	340	320	420
		(16)	(38)						
7	6600	4800	2000	400	165	178	600	508	410
		(13)	(41)						
8	6200	4700	4400	180	165	175	600	190	400
		(15)	(40)						
9	5400	4100	3400	440	230	250	190	110	120
		(15)	(40)						
10	7500	5000	3200	120	112	170	110	78	72
		(15)	(42)						
11	4700	4300	3680	400	290	190	340	310	300
		(14)	(35)						
12	6600	4600	4000	320	240	300	103	140	132
		(14)	(35)						
13	4400	3600	3000	90	110	125	110	110	102
	(4)	(15)	(40)						
14	3200	2300	2000	200	150	152	102	112	86

[a] Modified from Abioye *et al.* (A3a).

[b] Figures in parentheses indicate actual day of estimation of the Ig's.

TABLE 11
SERUM IMMUNOGLOBULIN LEVELS IN SYMPTOMLESS "CARRIERS"[a]

Patient No.	IgG (mg/100 ml)			IgA (mg/100 ml)			IgM (mg/100 ml)		
	Week: 0	1–2	5–6	0	1–2	5–6	0	1–2	5–6
		(15)[b]	(42)						
1	1700	1800	1650	72	88	76	70	128	180
		(17)	(40)						
2	2100	2000	1900	150	152	140	72	90	84
		(16)	(40)						
3	2100	1900	1950	90	110	102	72	75	88
		(17)	(38)						
4	1900	1900	1700	90	100	80	78	90	72
		(15)	(36)						
5	3200	2800	2400	165	162	104	105	130	90
		(17)	(40)						
6	3000	3200	3000	190	150	108	76	78	92
		(15)	(36)						
7	3100	3400	3300	150	154	148	111	108	126
		(16)	(40)						
8	1900	2000	2500	100	110	86	170	100	188
		(15)	(35)						
9	2800	2500	2300	85	90	90	128	130	120
		(14)	(35)						
10	2500	2600	2100	70	90	96	129	120	198

[a] Modified from Abioye *et al.* (A3a).
[b] Figures in parentheses indicate actual days.

tients infected with amebiasis as absorbed with an *E. histolytica* antigen prepared from the cultured organism (A2).

6.10. TRICHINOSIS

After infection with *Trichinella spiralis* there was an early relative increase of IgM-containing cells in the intestinal mucosa of rabbits, followed by a later increase of cells with IgG synthesizing ability, but the proportion of cells staining positively for IgA remained uniformly high in the intestine throughout the course of the infection with trichinella. Spleen sections, on the other hand, had higher numbers of IgM-producing cells than IgA-producing cells (C21).

7. The Immunoglobulins in Bacterial Diseases

7.1. SERUM IMMUNOGLOBULINS IN PULMONARY TUBERCULOSIS

There are reports that in infectious diseases, including pulmonary tuberculosis, increase of the serum IgG was characteristic, the IgA being

TABLE 12
AGE DISTRIBUTION OF TUBERCULOUS PATIENTS AND THE CONTROLS[a]

Age (years)	(A) All tuberculous patients	(A) Controls	(B) Sputum-positive patients	(B) Controls
0–5	11	20	0	0
6–9	10	11	0	1
10–19	26	19	10	19
20–29	46	20	24	21
30–39	54	14	30	14
40–49	33	13	17	13
50–59	12	1	4	1
60–69	8	1	1	1
70–79	1	1	0	1
	201	100	86	71

[a] From Malomo *et al.* (M27). By courtesy of the publishers of *Transactions of the Royal Society of Tropical Medicine and Hygiene.*

only slightly raised (M25); other reports (F1) suggested that, while IgG was elevated, both IgA and IgM were normal in the sera of Caucasian tuberculous patients.

In an extensive study undertaken in Nigeria of several patients suffering from pulmonary tuberculosis diagnosed by means of the radiological features of tuberculous bronchopneumonia on chest X-ray and detection of acid-fast bacilli in the patients' sputum by simple microscopy, serum immunoglobulin measurements were made (M27). The wide age distribution of the patients and controls are summarized in Table 12, and the immunoglobulin concentration results in Tables 13 and 14. The tuber-

TABLE 13
MEAN IMMUNOGLOBULIN LEVELS IN SERA OF ALL TUBERCULOUS PATIENTS AND CONTROLS[a]

	IgG (mg/ml)	IgA (mg/ml)	IgM (mg/ml)
Tuberculous patients (n = 201)	30.09	4.87	1.83
	±10.69	±2.65	±1.74
Controls (n = 100)	22.91	1.85	1.12
	±7.17	±0.78	±0.84
t	6.07	11.15	3.87
p	0.001	0.001	0.001

[a] From Malomo *et al.* (M27). By courtesy of the publishers of *Transactions of the Royal Society of Tropical Medicine and Hygiene.*

TABLE 14
MEAN IMMUNOGLOBULIN LEVELS IN SERA OF SPUTUM-POSITIVE PATIENTS AND CONTROLS OF SIMILAR AGE GROUPS[a]

	IgG	IgA	IgM
Sputum-positive patients (n = 86)	33.48	5.74	1.83
	±13.24	±2.94	±1.73
Controls (n = 71)	24.49	2.05	1.28
	±6.57	±0.78	±0.94
t	5.79	10.29	2.41
p	0.001	0.001	0.02, 0.01

[a] From Malomo *et al.* (M27). By courtesy of the publishers of *Transactions of the Royal Society of Tropical Medicine and Hygiene.*

culous patients were those who had a history of cough, weight loss, positive Mantoux test, physical signs of bronchopneumonia, and radiological evidence of tuberculous bronchopneumonia on chest X-ray. The patients who had these symptoms as well as positive acid-fast bacilli in their sputum are also shown in Table 14.

Eighty percent of the tuberculous subjects were between the ages of 10 and 49 years, 10% below 10 years, and 10% between 50 and 75 years. Acid-fast bacilli were detected in the sputums of 86 (42.8%) of the 201 patients on simple microscopy. The serum IgG and IgM levels were significantly higher in those patients who had acid-fast bacilli than in the controls. In the case of the serum IgM, however, the difference was just significant. Similarly, the mean values obtained for the serum IgG, IgA, and IgM for the tuberculous patients on the whole were also much higher than those of the controls (M27). Although all the serum immunoglobulins tested showed a distinct rise in tuberculosis, the rise of the serum IgA was the most significant. This was most likely because those tissues in the body which are known secretors of IgA, e.g., lungs, the intestinal mucosa, and salivary glands, are primarily affected in many tuberculous patients, probably causing an increase in the production rate of IgA.

7.2. LEPROSY

The patterns of the serum immunoglobulins in patients with leprosy are somewhat similar to those obtained in patients with tuberculosis, the predominant immunoglobulin in the serum being the IgA.

7.3. SYPHILIS AND YAWS

Treponemal antibodies and reagins are true immunoglobulins consisting mainly of IgM, but also of IgG. IgM appears early in the infection,

followed by IgG in the secondary phase, but after this IgM predominates and continues to do so throughout the later stages of the disease.

On purification on Sephadex columns of the antibodies in the sera of patients suffering from syphilis and yaws, the syphilis antibodies in the sera emerged from the DEAE-cellulose peak very soon after the void volume, and most of the activity was found in the first five fractions, indicating that the syphilis antibodies were of high molecular weight. The chromatograms invariably showed some syphilis antibody activity also in the IgG region. On the contrary, in those patients who were suffering from yaws, the chromatograms showed that most of the antibody activity emerged much later from the columns, indicating that the antibody to yaws were mainly of the IgG type (M2). Delhanty and Catterall (D1) stated that both IgM and IgG globulins were raised in primary, secondary, and latent syphilis. IgA was significantly elevated in the secondary stage. Higher IgM values were seen in the primary cases, but there was considerable overlap in values. IgM ranged from 0.5 mg/ml to 3.8 mg/ml, IgG from 10 mg/ml to 24 mg/ml, and IgA from 9.5 mg/ml to 5.5 mg/ml.

No association was found between Venereal Disease Research Laboratory (VDRL) titers and concentrations of immunoglobulins. In spite of the high serum IgM concentration observed in syphilitic sera, the VDRL titer of most syphilitic sera was not reduced by treatment with mercaptoethanol although most of the chronic biological false-positive reactors was reduced by this compound. The mitochondrial cardiolipin antibodies detected in the sera of early untreated syphilitics were of the IgG and IgM class (W15). After careful clinical grading of the different forms of syphilis, it was noted that in the primary stage of syphilis approximately two-thirds of the antitreponemal antibodies detected were in the IgM immunoglobulins: 71% in primary seronegative cases and 61% in primary seropositive cases. Most of the remaining antibodies were IgG, and a very few (4.6%) were IgA.

From the beginning of the secondary stage of syphilis, a marked fall was observed in the percentage of IgM immunoglobulins. In the latent stage of syphilis these antibodies comprised only slightly more than 40% of all fluorescent antibodies. At the same time, there was a marked rise in the antibodies in the IgG class, and this reached a maximum during the latent stage of syphilis when it comprised as much as 56% of all antibodies detected. In late symptomatic syphilis the serum IgM antibodies increased again to form 52% of all the antibodies detected (M28).

7.4. Gonorrhea

Serial estimates of immunofluorescent antibodies in sera obtained from 10 male volunteers who were infected with virulent strains of *Neisseria*

gonorrhoea revealed that 9 of 10 volunteers developed a 4-fold increase of IgG antibody reactivity to heat-labile surface antigens of the *N. gonorrhoeae*. Fewer patients showed increased IgM or IgA reactivities to heat-labile antigens. The heat-stable somatic antigens, prepared by heating the bacteria at 121°C, in contrast did not appear to stimulate any increased IgG or IgM activity, although 7 of 10 patients showed increased IgA antibody titers to the somatic antigens (C15). The immunogloubulin patterns in those patients with nonspecific urethritis were not significantly different from those of patients who had gonorrhea (S5).

In conclusion it can be said that syphilis antibodies ae mainly of the IgM class although smaller concentrations of IgG and perhaps IgA syphilis antibodies are also produced. In gonorrhea, on the other hand, the main antibody produced is of the IgG class.

7.5. Salmonella

Antibodies produced during the primary and secondary responses to the O antigens of *Salmonella typhosa* included the early synthesis of serum 19 S γ_1-globulins and the later synthesis of 7 S γ_2-globulins. On the other hand, the secondary responses to the H antigen of *Salmonella* comprised the early synthesis of large amounts of the 7 S γ_2-globulin antibody to the same level attained during the primary response (B3).

7.6. Cholera

In 1969 the Joint Study Group of the Indian Council of Medical Research—Government of West Bengal—WHO Cholera Research Project investigated the immunoglobulin levels in serum, intestinal juice, and feces from patients with cholera and noncholeraic diarrhea. They reported the following findings:

a. The mean level of IgA in the serum during convalescence from cholera was significantly higher than during the acute illness, strongly suggesting that during convalescence from cholera there was a rise in the synthesis of IgA.

b. The levels of IgG in serum did not change significantly during the course of choleraic or noncholeraic diarrheal illness or convalescence.

c. The levels of IgM in the serum were significantly higher in patients with cholera than in patients with the noncholeraic diarrhea or in normal Indians.

d. There were no statistically significant differences in serum IgE levels in the various Indian groups of controls and patients with diarrhea. However, Indian subjects had serum IgE levels 40 times higher than that of Europeans. Environmental, rather than genetic factors, were thought to be responsible for the immunoglobulin differences between the Indians and European subjects.

e. In the secretions of the small intestine, where the patterns of the immunoglobulin changes were similar to those exhibited by the serum, patients with cholera had the lowest levels of immunoglobulins, which were probably due to the abnormal pathology of the gut mucosa; on convalescence gut immunoglobulins, especially IgA and IgG, progressively increased in concentration.

f. IgA was the predominant immunoglobulin (76%) in the feces from patients with cholera; 46% in the feces from patients with noncholeraic diarrhea; 84–90% of the total immunoglobulin in feces from convalescent patients; and 73% in feces from normal Indians. There was an inverse relationship between the marked reduction of the IgA and the elevation of the IgG in the feces of the patients with noncholeraic diarrhea (W1).

This study, probably the first of its kind anywhere in the world, illustrates very clearly the relationship between local immunity in the gut and its relationship to bacterial infection. One looks forward to additional studies along these lines, which may help to solve some of the problems of parasitic infestation in tropical and subtropical populations.

8. The Immunoglobulins in Malignancies in Populations of Subtropical and Tropical Countries

8.1. Multiple Myelomatosis

8.1.1. *Diagnosis*

The triad for the diagnosis of multiple myelomatosis are radiological examination, bone marrow biopsy, and the immunoglobulin changes in both or either the serum and the urine. However, on several occasions when first performed, both the X-ray and bone marrow studies may not yield conclusive evidence to allow a firm diagnosis of myelomatosis to be made.

In these instances, careful examination of both the serum and urine by simultaneous electrophoresis invariably yields substantial evidence of the presence of a paraprotein in either or both the serum and urine of the suspect patient. When this situation arises, it is then best to repeat the radiological and bone marrow biopsy tests which would most likely confirm the presence or the absence of a malignant paraproteinemia.

It cannot be overemphasized that the absence of a monoclonal band of protein in the serum of a patient suspected of having multiple myelomatosis does not rule out the diagnosis. Quite frequently, the suspected patient may be excreting all the paraprotein in the form of a Bence Jones protein into the urine, and hence the simultaneous examination of both the serum and the urine by electrophoresis usually provides a rapid and excellent means of diagnosis.

Thus, in 1964, Clough and Reah (C14) observed that over a three-year period 15 new cases of multiple myeloma had been detected in their Biochemistry Department, entirely by careful examination of urine and serum proteins.

On examination of the urine and serum of numerous patients with suspected paraproteinemia in both Jamaicans and Africans between 1962 and 1966, it was concluded that whenever a low total serum γ-globulin level with a normal serum electrophoretic pattern were encountered in a suspected case of multiple myelomatosis, it was then essential to obtain also a specimen of urine from such a patient for further electrophoretic examination. Invariably simultaneous electrophoresis of such sera and urines proved to be diagnostic, even when the classical heat test for Bence Jones protein was negative. Consequently it was found that concurrent electrophoresis of serum and urine was the first means of detecting multiple myelomatosis in no less than 20% of the patients, which were subsequently confirmed either by bone marrow biopsy or X-ray examination or both (M3).

8.1.2. *Detection of Bence Jones Proteins*

Several authors have observed that the classical heat test is not always positive for Bence Jones protein when it is present in urine, and Osserman and Lawlor (O3) described three cases of multiple myelomatosis which gave a negative result for Bence Jones protein by the heat test, but a positive result by electrophoresis of the urine. Owen and Rider (O6) showed that the two main criteria on which the usual heat test for Bence Jones protein depend are unreliable and concluded that, as an aid to diagnosis, tests for the presence of heat-soluble Bence Jones protein as usually carried out tend to be of little value because of technical difficulties. Furthermore, biopsy of marrow in the sternum or other site, or of tumor if present, is generally considered to be the most reliable single means of diagnosing myelomatosis. However, the diffuse nature of the disease process often makes repeated examinations necessary before a typically abnormal specimen of marrow is obtained, and difficulty in interpreting a moderate increase in the percentage of plasma cells is not infrequent. Burtin (B13) stated that urinary electrophoresis provides a more reliable means of evaluation when it discloses the existence of a unique urinary protein with B-γ-globulin mobility.

8.1.3. *Concentration of the Urine*

Concentration of the urine of patients with suspected paraproteinemia for electrophoresis need not involve the purchase of any extra equipment, and highly satisfactory electrophoretograms on cellulose acetate mem-

brane can be achieved readily when 5–10 ml of urine is concentrated by simple dialysis against ordinary table sugar in a beaker. The urine in a dialysis tubing is simply placed above 10–20 g of the solid powered sucrose in a beaker; the dialysis tubing is then covered by about 20–30 g more sucrose. After about 2–3 hours, the urine is concentrated about 10-fold and can then be satisfactorily electrophoresed. The method has distinct advantages over other methods in that (a) it is inexpensive, (b) it can be set up by an inexperienced junior technician, (c) it requires no additional equipment, (d) large numbers of urines can be handled simultaneously, and (f) if the need arises the concentration process can be carried out overnight when larger volumes—up to 150 ml of urine—can be concentrated. If too much sucrose has penetrated the dialysis sac, this can be readily removed by inserting the dialyzing sac into 20 ml of isotonic saline for 10 minutes (M1). Several other methods are available for concentrating urines for electrophoresis: negative pressure dialysis, ultrafiltration, and lyophilization. However, since many of the smaller laboratories in the subtropics and tropics may not be able to afford the funds or the time, the simple method described above is to be highly recommended.

On electrophoresis of the urine, the electrophoretic pattern from a patient with multiple myelomatosis usually reveals either (a) a single band of homogeneous protein in the γ-globulin region or (b) a single band in the γ-globulin region along with a band in the albumin region or (c) several bands on the strip with the band in the γ-globulin region most prominent. Findings (a) or (b) invariably emerge when Bence Jones protein is excreted by a patient with multiple myelomatosis. If there is any doubt about interpreting the results, these can be readily confirmed by immunoelectrophoresis (see later).

Hypoproteinemia is not an uncommon finding in patients with multiple myelomatosis, although hyperproteinemia is the general rule. Twenty-five percent of a series of patients in Jamaica with multiple myelomatosis had low to normal concentrations of total γ-globulin levels, of these, 60% gave a perfectly normal serum electrophoretic pattern or a pattern with only minor nonspecific changes. In this group of patients, the laboratory diagnosis could have been readily missed if the urine was not simultaneously electrophoresed with the serum of the patient.

8.1.4. *Incidence of Myelomatosis*

Patients from subtropical and tropical populations coming to hospitals for treatment, do so in many cases long after their diseases have been well established. This is so because the majority of these patients are probably unable to walk the distance, the hospitals being too far away,

or because some of them prefer to consult the local herbalist. Many may not attend the doctor if their only complaint is a bone pain to which they might have grown accustomed. In short, the type of illness which usually compels a patient in the temperate regions to seek medical advice may not compel his counterpart in the tropics to do so. The result is that many such patients in subtropical and tropical populations die at home from their disease, which goes unrecorded, and therefore a particular disease may seem to occur infrequently in such populations. It is likely that this factor may be one of the important reasons why the incidence of multiple myelomatosis—a disease not easily recognized without the aid of laboratory services, particularly in Africa, has been reported as rare. The shorter life span of the average person in Africa may well be another factor which contributes to the rarity of multiple myelomatosis, which occurs in greater frequency in persons above the age of 50 years.

Reviewing the literature for Africa, Michaux recorded that, between 1959 and 1965, only 12 cases of multiple myelomatosis were recorded among the Bantu in Central Africa (M35). In blacks in South Africa the incidence of myelomatosis among males was 3.1 per million and among females 0.7 per million (O1), and in French West Africa the incidence was 1% of all hematosarcomas (C2). Although no mention was made of multiple myeloma among 223 tumors of the reticuloendothelial system among Nigerians (E1), the author has seen no fewer than six cases per year over a 6-year period with an authentic diagnosis of multiple myelomatosis in Nigerians.

A high incidence of multiple myelomatosis has been reported in Jamaican Negroes in three different studies (M3, M11, T1) and higher incidence in American Negroes than in American whites (M26). Further investigation into the racial incidence of multiple myelomatosis in tropical populations are awaited with interest, especially since more specific immunochemical methods are now available.

8.1.5. *Neuropathy and Multiple Myeloma*

Occasionally the nervous system becomes involved in myelomatosis, and a number of cases have been reported. The lesions in these patients include compression of neural structures by myeloma tissue or collapsed vertebra, herpes zoster, and amyloid infiltration of the peripheral nervous system. Although uncommon, a peripheral neuromyopathy in which there is no direct involvement of nerves or nerve roots has been reported, but the association of multiple myeloma with neuropathy and osteosclerotic lesion is rarer and even more unusual. Osuntokun *et al.* (O5) has reported such a patient in Africa whose outstanding clinical features were widespread neuromyopathy associated with multiple myeloma, and general-

ized osteoporosis with a focus of osteoblastic reaction in the lumbar spine. The patient also had tuberculosis adenitis. Immunochemical investigations of the serum and urinary proteins revealed IgG type K myeloma protein in the serum with k Bence Jones protein excretion in the urine.

8.1.6. *Postmortem Findings in Multiple Myelomatosis*

The commonest causes of death in the Jamaican patients with multiple myelomatosis were bronchopneumonia and other infectious complications, a finding which is compatible with the secondary antibody deficiency syndrome and impaired cellular immunity which occurs in patients with this disease. Bleeding manifestations and renal failure were not uncommon findings, and myeloma kidney was observed in 66% of the cases. Skeletal involvement was observed, but in many cases the typical lesions had to be searched for. Amyloidosis was present in as many as 21% of the patients (T1) and this may be associated with the high number of patients in Jamaica that are known to excrete Bence Jones protein in myeloma (M11).

8.1.7. *Measurement of Ig Concentration in Myelomatosis*

Benign paraproteinemia is a common finding among tropical populations, especially in those of the lower socioeconomic strata, and may very well obscure the true diagnostic merit of quantitating the serum immunoglobulin in tropical patients suspected of having myelomatosis. Thus the finding of a total serum IgG level above 3000 mg/100 ml in a white patient in the temperate regions is sufficient evidence to suspect the existence of paraproteinemia, but such a serum concentration of IgG is frequently found in tropical populations who live in areas where parasitic infestations are endemic. It is no wonder, therefore, that cases of myelomatosis may go undiagnosed among tropical populations. On immunoelectrophoresis, malignant paraproteinemia may be revealed by (a) an area of the precipitin line showing a double curvature or bowing, (b) an area of antigen excess in the precipitin lines which is manifested by a dramatic shortening of the precipitin line, (c) a massive thickened and elongated line, or (d) if the urine has also been subjected to immunoelectrophoresis against anti k or λ, the resultant precipitin line may reveal an abnormal curvature when compared with that of the normal control.

As mentioned in the Introduction, a single isolated determination of the three major serum immunoglobulins in a patient with multiple myelomatosis may only reveal that a particular immunoglobulin is markedly elevated. However, much more useful information is achieved if the serum immunoglobulin concentration can be determined at regular inter-

vals. In this respect, any sudden marked increase of the paraprotein would herald an escape phenomenon and treatment be modified accordingly.

The percentages of monoclonal paraproteinemia of the IgG or IgA or IgD classes seem to occur with equal frequency in subtropical and tropical populations, as they do in the temperate regions, but since high levels of serum IgE appear to be more widespread in populations of the subtropics and tropics, it is likely that the incidence of IgE myeloma may be more frequent among such populations than among those who live in the temperate regions, where raised levels of IgE do not occur so frequently.

8.1.8. *Diagnosis of Multiple Myelomatosis by Immunoelectrophoretic Methods*

The preparation of antiserum reagents may not be a feasible proposition in areas where animal house facilities are not adequate and skilled staff limited; consequently it may be much easier to purchase commercially prepared antiserum since in the long run it may be more economical —especially in areas where the use of antiserum may be on a limited scale.

Anti whole human serum from any source is invariably of a good quality and is useful for screening for paraproteinemia of either the IgG, IgA, or IgM type. An experienced eye is essential to interpret the pattern.

Anti k serum is moderately good from most suppliers; on immunoelectrophoresis of a paraprotein of the k type, reasonable constant abnormal lines are obtained, although pooling of anti k sera from various sources is recommended. Anti lambda (λ) antiserums from commercial suppliers are generally poor in quality, and it is highly recommended that pooling several different batches is essential if consistent results are to be obtained. Figures 7–10 represent immunoelectrophoretic patterns that may be obtained on immunoelectrophoresis.

Mono specific antiserum to IgG, IgA, and IgD are of reasonably good quality from most manufacturers, but here again it is advantageous to use pooled monospecific antisera from different sources.

The immunoelectrophoretic results should be interpreted in conjunction with interpretation of cellulose acetate electrophoresis of both the serum and urine from patients who are suspected of having multiple myelomatosis. Indeed, it should be mentioned that no single pattern of immunoelectrophoretic precipitin line is typical of patients with multiple myelomatosis, and it can be said that these vary from patient to patient and must be interpreted with care. Frequently it may be essential to repeat

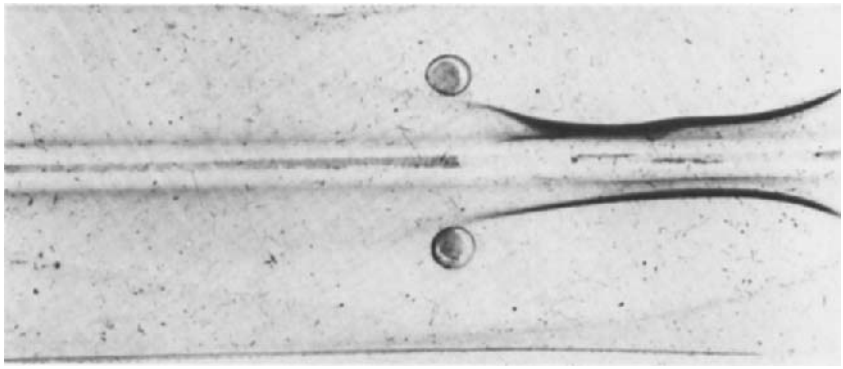


FIG. 7. Immunoelectrophoretic pattern of serum from a patient with IgG myelomatosis. Note the double curvature of the precipitin line in the top part. Frequently the thickest portion of the abnormal line may be completely dissolved, leaving behind only the short normal portion of the precipitin line. Monospecific IgG antiserum was used in the trough.

an immunoelectrophoretic run of a patient's serum against different sources of antiserum, since a particular antiserum may not contain the antigenic idiotype which is characteristic of the particular myeloma protein under investigation.

It should be mentioned that up to 20% of serums from patients with multiple myelomatosis may not contain a clearly visible paraprotein

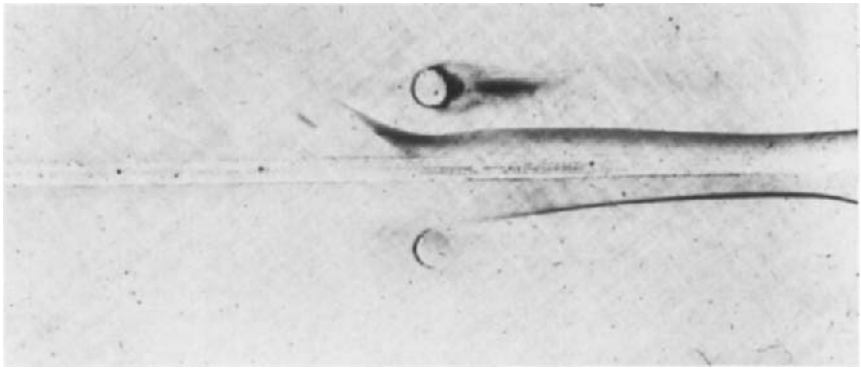


FIG. 8. Immunoelectrophoretic pattern of serum from a patient with IgG myelomatosis. Note the area next to the top well, where there is a spontaneous precipitation of cryoglobulins in the agar gel, which remained in the gel even after extensive washing with saline. The massive increase of the abnormal precipitin line of the patient's serum in the top well is distinctly different from that of the normal precipitin line below antiserum as in Fig. 7.

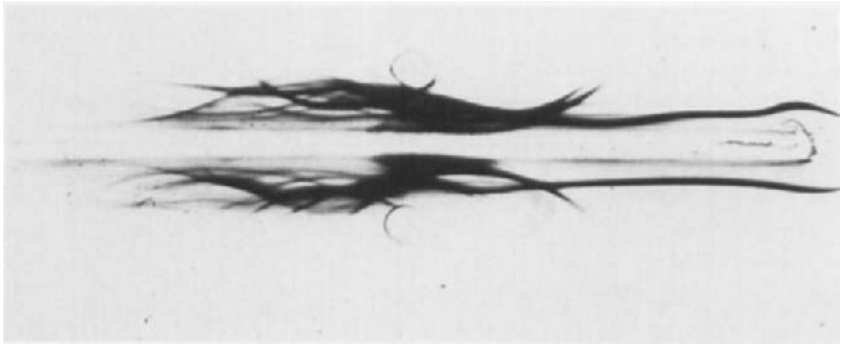


FIG. 9. Immunoelectrophoretic patient with IgA multiple myelomatosis. The antiserum used here was anti whole human serum. Note the abnormally doubly curved precipitin line of the patient's serum in the top portion.

either in the serum or urine by cellulose acetate electrophoresis. In such serum the application of immunoelectrophoresis can provide an invaluable aid to allow a conclusive diagnosis to be made.

8.1.9. *"Normal" Serum Immunoglobulins in Patients with Multiple Myelomatosis*

The majority of patients with multiple myelomatosis, although having a markedly elevated level of the paraprotein, may suffer from a severe hypogammaglobulinemia of their other immunoglobulins. Thus, a patient with considerable levels of abnormal serum IgG may have only 10% of

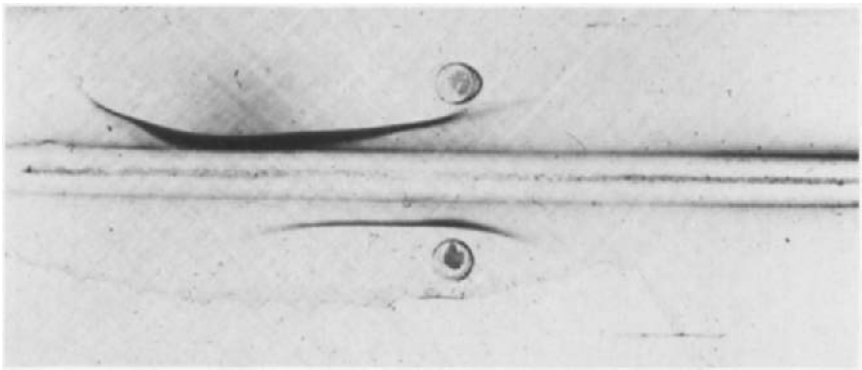


FIG. 10. Immunoelectrophoretic pattern of serum from a patient with IgA multiple myelomatosis. Monospecific anti IgA was used in the trough. Note the marked thickened abnormal precipitin line at the top portion.

normal serum IgG and a severe reduction of all or one of IgA, IgM, IgD, and IgE. This marked reduction of the normal Ig's in patients with multiple myelomatosis contribute to the rate of infection which frequently characterizes the terminal stages of the disease. In some patients in Jamaica with an IgG myeloma the author has noticed very low levels or no detectable levels of one or other immunoglobulins. For instance, the IgA or IgM may be completely absent from the serum of such a patient, giving rise to a secondary antibody deficiency syndrome. In these patients only serial determination of their immunoglobulin levels can tell whether on treatment there is a progressive return to normal levels of the decreased serum immunoglobulin or whether, as occurs in the terminal stages, there are further decreases (Fig. 11).

8.1.10. *Cellular Immune Responses in Patients with Multiple Myelomatosis*

Patients with multiple myelomatosis have a proliferation of abnormal plasma cells, both in their bone marrow and in their peripheral blood. In

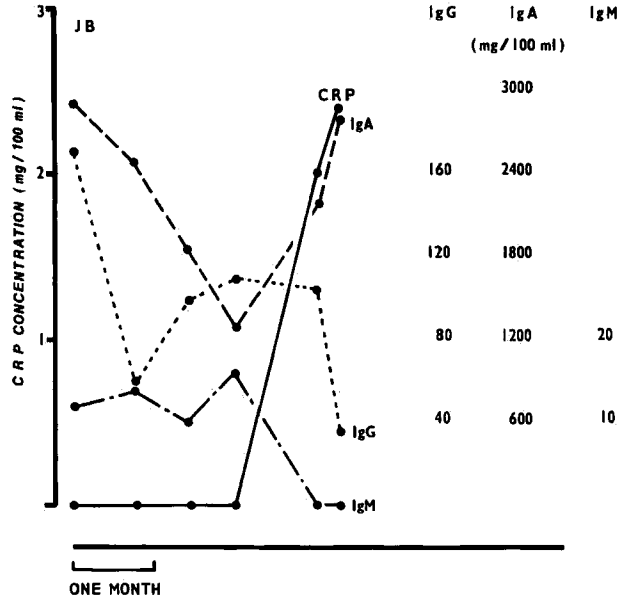


FIG. 11. Typical sequential results that may be obtained for the serum immunoglobulin concentrations in a patient with IgA-type myelomatosis. Note (a) the subsequent marked increase of the IgA, representing an escape phenomenon of the paraprotein, (b) the marked reduction of the serum IgG and IgM, and (c) the concentrations of the C-reactive protein (CRP), also heralding that treatment was no longer effective. This patient died 2 months after these results were obtained.

cell culture studies, peripheral blood lymphocytes from patients with multiple myelomatosis respond far more than normals to pokeweed mitogen (PWM) with a greatly subnormal response to phytohemagglutinin (PHA). Thus the PWM:PHA ratio of peripheral blood lymphocytes is greatly elevated, suggesting the B cell nature of the disease and the depressed T cell function in patients with multiple myelomatosis.

In a recent study of lymphocyte transformation, we have observed that 75% of the initial peripheral blood lymphocyte cultures from patients with multiple myelomatosis showed a high PWM-induced but subnormal PHA-induced lymphocyte transformation. The other 25% of patients had normal PHA-induced lymphocyte transformations. In contrast, the majority of the peripheral blood lymphocyte cultures from normal subjects showed a higher PHA-induced than PWM-induced lymphocyte transformation. Serial lymphocyte transformation tests in 8 out of 18 patients revealed that a subnormal PHA-induced lymphocyte transformation could be restored in some patients, but the significance of this requires further investigation (Fig. 12). Eighty-two percent of the bone marrow cell cultures showed a higher PWM-induced than PHA-induced lymphocyte stimulation, confirming the B cell nature of the disease. In dose response curves, lymphocytes from different patients responded differently to equivalent doses of both PWM and PHA. In some patients the maximum lymphocyte transformation to both PWM and PHA occurred simultaneously either on days 3, 5, or 7, although in other patients the

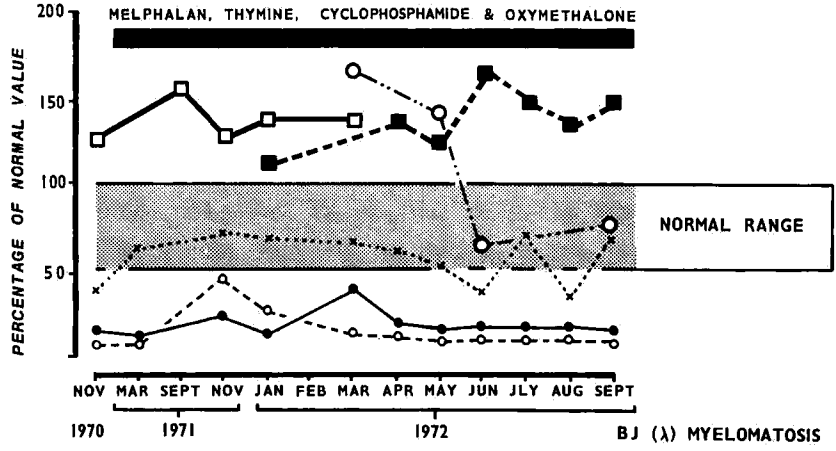


FIG. 12. Summary of sequential investigations carried out on a patient with Bence Jones myelomatosis. Note the high blood urea, high proteinuria, and the early elevated pokeweed mitogen (PWM) response instead of elevated phytohemagglutinin (PHA) response. Urine protein; □, blood urea; ○, PWM:PHA ratio; ○, IgM, ●, IgA; ×, IgG.

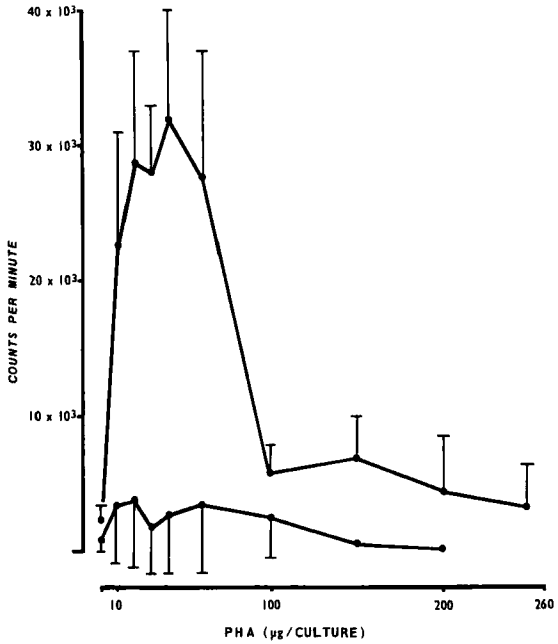


FIG. 13. Phytohemagglutinin (PHA) dose response curves of 10 patients with multiple myelomatosis and 10 controls. Peripheral blood lymphocytes were cultured and stimulated with PHA in increasing concentrations. Transformation was assessed by counting the uptake of radiolabeled thymidine.

maximum PWM-induced response did not coincide on the same day with that of the maximum PHA-induced response (Figs. 13 and 14).

8.2. WALDENSTRÖM MACROGLOBULINEMIA

Waldenström macroglobulinemia occurs in all races, and its incidence in the tropics appears to be the same as in other parts of the world. The author has seen at least two cases in Negroes in Jamaica and two cases in Nigeria (M4). Rowe (R8) has written a Ph.D. thesis describing the occurrence of primary Waldenström macroglobulinemia and secondary macroglobulinemia in Jamaicans. In this disease the most characteristic feature is the high concentration of heavy molecular weight protein in the serum of affected patients. This high molecular weight protein belongs to the 19 S class of IgM, although 7 S IgM may also be a feature of the disease, and is responsible for the clinical symptoms of affected patients, such as the generalized bleeding diathesis, epistaxis, occasionally jaundice and anemia. The statement that Waldenström macroglobulinemia does

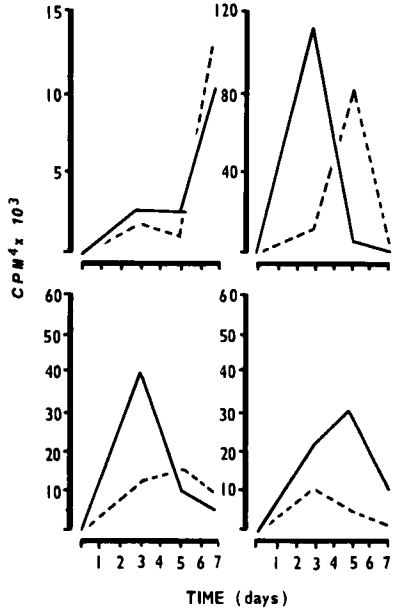


FIG. 14. Phytohemagglutinin (———) and pokeweed mitogen (- - -). Time lapse myelomatosis. Note the different types of patterns (conditions similar to those in Fig. 13).

not occur in Negroes is no longer tenable, and that the disease was not found in any of the Bantu populations (M35) may be owing to the fact that patients with this disease in Africa may not seek medical attention very frequently.

8.2.1. *Diagnosis*

In Waldenström macroglobulinemia the serum IgM value is invariably above 1000 mg/100 ml, and the serum should be diluted before any attempt is made to quantitate the IgM protein by immunochemical testing since antigen excess may obscure the precipitin ring and give falsely low results.

8.2.1.1. *Immunoelectrophoresis.* In Waldenström macroglobulinemia the IgM precipitin line invariably has a pronounced double curvature, and when compared with a normal control serum the IgM pattern of the former is much more distinct. After washing the agar gel with saline for 2 to 3 days, there usually still remains a massive amount of protein (IgM) spontaneously precipitated around the point of application. This feature is also characteristic of cryoglobulinemia—frequently seen in populations who live in areas where parasite diseases are common.

8.2.1.2. *Sephadex Gel Filtration.* The most common type used is the Sephadex G-200. The emergence soon after the void volume of a massive peak which is more prominent than the other peaks is compatible with a diagnosis of Waldenström macroglobulinemia. The test is a very useful aid in the diagnosis of Waldenström macroglobulinemia.

8.2.1.3. *Ultracentrifugation.* In those laboratories where an ultracentrifuge is available, the pattern obtained of the serum of a patient with Waldenström macroglobulinemia is very characteristic. Frequently more than 30% of the total serum proteins could be accounted for by the 19 S peak. Bence Jones protein is not an uncommon finding in patients with Waldenström macroglobulinemia and was detected in patients both in Nigeria and Jamacia with the disease.

The serum IgM concentration in one of the patients in Nigeria, who was only 19 years of age, was above 4000 mg/100 ml. On immunoelectrophoresis, there was spontaneous precipitation of massive quantities of cryomacroglobulins which could not be removed from the agar gel even after extensive washing of the gels for several days. Clinically this patient was jaundiced and anemic, probably due to hemolysis of his red cells by the massive increase of the serum IgM. The monoclonal protein in both the serum and urine were of the IgM k type. The unusual features in this particular case were his age and the presence of hemolytic jaundice.

8.2.1.4. *Viscosity of the Serum.* Provided the apparatus is available, this is a rather simple test to perform and the information obtained helps to establish the condition. Using an Ostwald viscometer at 20.0°C and 37.5°C in thermostatically controlled water baths, it was found that patients with macroglobulinemia had serum with relative viscosity of 2.40–15.80. The normal is 1.54–1.80.

The author has found from his own observation that the mortality of patients with multiple myeloma in the tropics is greater than 75% after two years from first diagnosis. However, patients with Waldenström macroglobulinemia have survived for much longer periods than this and have even shown signs of complete recovery. In Jamacia, the average survival from the onset of symptoms was 8.4 months in males and 6.4 months in females, and the average survival from the time of diagnosis was 2.4 months in males and 2.1 months in females (T1).

8.3. Burkitt's Lymphoma

Burkitt's lymphoma is chiefly a disease of children and is more common in Africa than in other parts of the world. Within the last ten years or so, this disease has attracted much attention—most likely because (a) most patients with Burkitt's lymphoma have a rapid tumor regression following chemotherapy, (b) some patients with Burkitt's lymphoma

have responded after infusions of immune plasma from cured patients (B12, N4): these cases had clinical improvement lasting up to 2 weeks, (c) reports of spontaneous regression of the tumor, and (d) the serum immunoglobulin IgM appears to be related to the course of the disease (K3).

8.3.1. *The Serum Ig's in Burkitt's Lyphoma*

The first report on the concentration of the serum Ig's in children with Burkitt's lymphoma noted that although the serum IgM was depressed in the majority of the patients, both the serum IgA and IgG were largely within normal limits (N3). Furthermore, those sera with high levels of IgM had an inhibitory effect on the growth of Burkitt tumor cells in culture.

The marked decrease of the serum IgM in patients with Burkitt's lymphoma have been confirmed by Ziegler *et al.* (Z1) in East Africa, who noticed an impairment of the primary antibody response and low serum IgM levels in untreated patients with Burkitt's lymphoma, but not in patients in remission. They postulated a defect in humoral immunity possibly to impaired IgM synthesis.

By means of radiolabeled amino acid incorporation, double-diffusion radioimmunoelectrophoresis, and ultracentrifugation techniques, it was possible to show that freshly isolated cells from Burkitt's lymphoma were capable of immunoglobulin synthesis *in vitro*. Burkitt's lymphoma cells from 4 fresh biopsy materials synthesized IgG *in vitro*. One cell line produced IgG type-k light chains, and one cell line produced only IgA. None of the cell lines produced more than one immunoglobulin type—possibly related to the monoclonal nature of the malignancy (O4). It is of interest that none of the cell lines produced IgM *in vitro*, a finding which was compatible with the low serum IgM of most patients with Burkitt's lymphoma. It has been suggested that children with Burkitt's lymphoma and with low serum IgG had a poor prognosis, and Lowry (L6) recommended the therapeutic use of γ-globulin for children with Burkitt's lymphoma, noting that although pooled γ-globulin obtained from the native population in areas of Africa where the disease is endemic, would presumably have high levels of circulating antibodies, it might be more advisable to use γ-globulin obtained in Europe which may be free from any virus responsible for Burkitt's lymphoma.

8.3.2. *Classification of the Different Types of Immunoglobulin Patterns in Patients with Burkitt's Lymphoma* (M16)

Five different types of serum immunoglobulin patterns may be observed in patients with Burkitt's lymphoma (Tables 15–19): Type 1

TABLE 15
TYPE I IMMUNOGLOBULIN DEFICIENCY IN BURKITT'S LYMPHOMA: LOW IgM WITH NORMAL IgG (MG/ML)[a]

Patient	Sex	Clinical classification	IgM	IgG	IgA	Comment
A.O.	F	Grade III	0.44	17.40	4.2	Died
A.S.	M	Grade III	0.69	27.00	2.29	Long remission
S.J.	M		0.31	19.60	2.40	Long remission
O.S.	F	Grade III	0.61	15.60	3.60	Died
R.M.	M		0.52	19.60	3.60	Died
S.O.	M		0.69	20.50	3.40	—
G.J.	M	Grade III	0.27	16.00	0.91	Long remission
A.S.	F	Grade I	0.27	18.00	1.04	Long remission
A.K.	M	Grade I	0.52	29.00	2.23	Long remission
S.O.	M	Grade III	0.56	20.50	2.31	Long remission
A.O.	M	Grade III	0.31	21.50	2.95	Died
O.M.	F		0.47	23.40	2.30	Died
L.L.	M		0.45	18.30	1.50	Alive
O.A.	M		0.55	22.00	2.80	Died
O.T.	M	Grade III	0.46	17.50	2.50	Died
T.D.	F		0.39	25.00	2.10	Died
L.	M	Grade III	0.50	22.00	4.50	Died
A.F.	F		0.40	30.00	0.80	Died
I.O.A.	M		0.67	19.00	2.30	—
I.I.	M		0.58	24.00	1.80	Acute
A.K.	M	Grade III	0.54	19.00	1.10	Long remission

[a] From McFarlane *et al.* (M16). By courtesy of the publishers of the *African Journal of Medical Sciences.*

patients have an isolated decrease of the serum IgM. Type 2 patients have both serum IgM and IgG decreased. Type 3 patients have isolated IgG decreased. Type 4 patients have elevated levels of serum IgG but low serum IgM. A fifth group of patients have normal levels of all three immunoglobulins.

8.3.3. *Type I Immunoglobulin Deficiency*

In twelve of sixty-three patients, both the serum IgM and the serum IgG concentrations were depressed. Two of these Burkitt's lymphoma children also had low IgA, but in the other 10 children the serum IgA was normal. There were 5 known deaths in children classified with this type II deficiency. Seven of these children had tumor classification of the grade III type III immunoglobulin deficiency. There were 14 children with Burkitt's lymphoma classified with this type III immunoglobulin deficiency with moderately reduced levels of serum IgG and normal levels of IgM. One child in the group had a moderately elevated IgA,

TABLE 16
TYPE II IMMUNOGLOBULIN DEFICIENCY IN BURKITT'S LYMPHOMA: LOW IgM AND LOW IgG (MG/ML)[a]

Patient	Sex	Clinical classification	IgM	IgG	IgA	Comment
O.R.	M	Grade III	0.62	14.00	1.72	Long remission
O.S.	M	Grade III	0.48	8.90	2.34	Long remission
A.T.	F		0.56	10.60	1.11	Long remission
A.O.	F		0.48	10.75	2.95	Died
S.H.	M	Grade III	0.32	12.06	0.97	Died
M.I.			0.34	8.00	1.96	?
L.M.	F	Grade III	0.44	10.00	2.80	Died
A.O.	F	Grade III	0.62	12.40	1.55	Died
A.J.	M	Grade III	0.28	10.00	1.40	Died
B.S.	F		0.30	10.00	2.40	?
S.A.	M	Grade III	0.23	10.00	0.90	?
O.K.	M		0.39	10.50	1.80	?

[a] From McFarlane *et al.* (M16). By courtesy of the publishers of the *African Journal of Medical Sciences.*

TABLE 17
TYPE III IMMUNOGLOBULIN DEFICIENCY IN BURKITT'S LYMPHOMA: NORMAL IgM AND LOW IgG (MG/ML)[a]

Patient	Sex	Clinical classification	IgM	IgG	IgA	Comment
B.S.	F		1.96	14.20	2.55	Died
A.D.	M	Grade III	1.96	13.50	2.75	Died
A.E.	M		1.12	10.75	2.10	Died
A.O.	F	Grade III	0.92	13.50	5.40	Died
A.R.	M	Grade IV	1.24	14.20	1.09	
O.S.	M	Grade III	0.72	11.20	1.25	?
A.O.	F	Grade III	1.46	10.60	—	Died
S.S.	M		1.05	12.40	—	Well
A.O.	F		1.46	10.60	—	?
O.J.	M		1.30	13.50	2.90	?
A.O.	F		1.00	10.00	2.10	
R.O.	M	Grade III	0.90	10.00	2.10	Died
M.A.	M	Grade I	1.55	10.00	1.80	Cured
K.A.	M		0.76	12.50	2.30	Cured

[a] From McFarlane *et al.* (M16). By courtesy of the publishers of the *African Journal of Medical Sciences.*

TABLE 18
TYPE IV IMMUNOGLOBULIN DEFICIENCY IN BURKITT'S LYMPHOMA: HIGH IgG AND LOW IgM[a]

Patient	Sex	IgM	IgG	IgA	Comment
A.A.	M	0.51	39.00	2.50	Alive
G.S.	M	0.48	35.00	2.30	Alive

[a] From McFarlane *et al.* (M16). By courtesy of the publishers of the *African Journal of Medical Sciences.*

but in the others IgA was normal. Six of 14 children belonging to this group are known to have died. There were 2 patients in the group who had tumor distribution of grades I and IV, respectively, but five had the grade III type tumor distribution.

8.3.4. *Type IV Immunoglobulin Deficiency*

There were two children in this group with elevated levels of serum IgG, normal levels of serum IgA, but low serum IgM.

There was a fifth group of patients with Burkitt's lymphoma who had mostly normal levels of serum immunoglobulin. The majority of these patients were in remission from their tumor.

TABLE 19
NORMAL IMMUNOGLOBULIN LEVELS IN BURKITT'S LYMPHOMA: NORMAL IgM AND NORMAL IgG (MG/ML)[a]

Patient	Sex	Clinical classification	IgM	IgG	IgA	Comment
O.A.	M		0.87	17.40	1.50	In remission
O.N.	M	Grade III	0.77	19.60	4.70	Acute
B.E.	M		1.74	17.00	4.10	?
A.T.	F	Grade III	2.67	19.60	1.34	In remission
A.I.	M		1.46	17.00	2.75	?
A.S.	F		0.88	19.20	1.04	In remission
E.O.	M		1.74	17.40	—	Died
A.T.	M		2.10	29.00	2.97	In remission
O.V.	F	Grade III	1.54	42.00	2.55	In remission
L.T.	M	Grade III	1.12	15.20	3.18	Died
D.O.	M		0.80	22.00	2.10	?
O.F.	F		1.00	22.00	2.70	?
A.O.			0.88	19.00	1.40	?
F.	F		1.55	24.00	1.90	?

[a] From McFarlane *et al.* (M16). By courtesy of the publishers of the *African Journal of Medical Sciences.*

TABLE 20

IMMUNOGLOBULINS IN THE CEREBROSPINAL FLUID (CSF) OF PATIENTS WITH BURKITT'S LYMPHOMA[a]

Serial No.	Initials	Age (years)	Sex	Total protein (mg/100 ml)	Albumin (mg/100 ml)	Albumin % Total protein	IgG (mg/100 ml)	IgG % Total protein	IgM[b]	Cells/mm³ Burkitt	Cells/mm³ WBC	Cells/mm³ RBC	Remarks
Burkitt patients													
1a	Om. E.			54	35	66	19	35	+	40	?[c]	?[c]	
1b	Om. E.	5	M	54	40	74	18	33	+	Nil	12	240	
1c	Om. E.			21	15	71	4.0	19	+	10	15	150	
2a	Si	17	F	18	4	22	3.0	17	−	120	20	15	
2b	Si			212	18	8.5	40	19	+	Nil	2	24,000	
3a	Ra	12	M	68	38	56	18	22	+	25	35	16,000	
3b	Ra			10	9	90	3.0	30	−	10	25	400	
4	A. Ay	7	M	45	22	49	9.0	20	−	5	15	?[c]	
5	A. As	3	M	186	120	65	48	26	+[d]	Nil	50	Nil	Burkitt cells in CSF 3 weeks previously; Burkitt cells = 40, WBC = 180, RBC = 360/mm³
6a	Af.	10	M	140	60	43	14	10	−	80	50	5	
6b	Af.			160	65	41	16	10	−	960	100	60	

7	La	12	M	10	4	40	3	30	–	Nil		
8	Ba	5	M	11	3	27	2.5	23	–	Nil		
9	Ag.	6	F	19	12	63	3.5	18	–	Nil		
10	OI.	7	M	20	18	90	7.0	35	–	Nil		
11	Al	5	M	26	8	31	2.0	7.6	–	Nil	1	130
12	On.	8	M	16	12	75	3.5	22	–	Nil	1	2
Controls												
13	As. S.	7	F	950	40	4.2	—		–	Nil	<1	Nil
14	O. F.	6.5	F	38	30	79	4.5	12	–	Nil	70	1
15	O. I.	2.5	F	14	8	57	1.0	7.1	–	Nil	<1	10
16	A. O.	8	M	19	3.5	18	7.5	40	–	Nil	<1	Nil
17	At. O.	5	F	10	8	80	2.0	20	–	Nil	1	44
18	A. A.	5	M	20	—		2.0	10	–	Nil	1	3
19	A. T.	8.5	F	30	—		4.0	13	–	Nil	<1	410
20	Ogun	3	F	50	40	80	5.0	10	–	Nil	<1	296
21	Ala	3	M	225	168	75	12	5.3	–	Nil	2	1
22	Ayin.	4	M	18	12	67	3	17	–	Nil	9	141

Landre-Guillain-Barre syndrome	
Hemophilia influenzae meningitis	
Abdominal pain and vomiting (meningitis?)	
Idiopathic epilepsy	
Malaria with bronchopneumonia	
Convulsions, cow's urine poisoning	
Malaria with severe anemia	
Cerebral malaria	

[a] From Udeozo *et al.* (U1). Reproduced by courtesy of the publishers of the *Journal of Laboratory and Clinical Medicine.*

[b] + = IgM present; – = IgM absent.

[c] Cells present, but not counted.

[d] More than 21 mg of IgM per 100 ml.

8.3.4. *CSF Immunoglobulins in Burkitt's Lymphoma*

The cerebrospinal fluid from patients with Burkitt's lymphoma were examined for immunoglobulins (U1) Table 20. The presence of IgM was demonstrated only in those in whom Burkitt cells had invaded the CSF, suggesting that the IgM could either have been synthesized by the Burkitt cells which invaded the CSF, or released from dead cells in the CSF, or as subunits could have crossed the blood-brain barrier into the CSF. In one patient IgM was detected in the CSF both on admission when Burkitt cells were also present in the CSF and again 3 weeks later when cells had disappeared after treatment with cyclophosphamide. Six weeks later, when Burkitt cells had again invaded the CSF, IgM was still present but in much lower concentration. The precipitin lines on this occasion developed after 72 hours. In a second patient IgM was detected in the CSF 1 week after treatment was commenced, by which time Burkitt cells, present on admission, had disappeared from the CSF. IgM was detected in the CSF in a third patient only on admission and had disappeared 3 weeks later with the disappearance of Burkitt cells following cyclophosphamide therapy. All the patients in whom IgM was detected in the CSF also had elevated levels of CSF IgG. The significance of IgM in the CSF of patients with Burkitt's lymphoma is perhaps related to the grave prognostic sign when the central nervous system becomes involved, and Ziegler *et al.* (1970) have observed that in these patients with CNS involvement aggressive treatment was necessary to prevent relapse (Z2).

It is likely that the IgM in the CSF of patients with Burkitt's lymphoma may be partly derived from subunits of serum IgM crossing the blood-brain barrier, since 4 out of 17 patients with Burkitt's lymphoma had detectable IgM in their urines which emerged from Sephadex G-200 columns in two different peaks, strongly suggesting the occurrence of subunits of the intact molecule (M17).

Klein (K2) reported IgM absorbed to the surface of fresh Burkitt's lymphoma cells, and it is possible that the low serum IgM values may result from absorption to malignant cells. Zeigler *et al.* (Z1) postulated that IgM metabolism may be inhibited by active production of IgG *in vitro* by the Burkitt lymphoma cells (O4), and furthermore, that the IgM defect may be either acquired or inherited or an expression of delayed maturity of immunoglobulin metabolism which contributes to the susceptibility of the individual to the oncogenic agent (N3).

One of the chief causes of early death in patients with Burkitt's lymphoma was renal failure, and it is tempting to suggest that the increased excretion of proteins into the urine may well be related to the damage caused to the kidney (Z2).

A viral etiology has been implicated in Burkitt's lymphoma, and there is some evidence that Epstein-Barr virus causes infectious mononucleosis in Europe and Burkitt's lymphoma in Africa. It has been suggested that if the cause of the Burkitt's lymphoma is viral, then entry of the virus particles into the lymphoid cells of the body may derange a part of the cell immune process, subsequently affecting the production of the IgM antibody, a theory which is compatible to the dysproteinemia sometimes seen in multiple myeloma and other lymphomas (N2). Also in support of this is the observation that C-reactive protein was markedly elevated in the serum of patients with Burkitt's lymphoma, and disappeared entirely from the blood when they were cured (M10). The relationship between malarial infection and Burkitt's lymphoma has been dealt with in a previous section on malaria.

8.3.5. *Cellular Responses*

The majority of patients with Burkitt's lymphoma showed an intact delayed hypersensitivity response to DCNB and lymphocyte transformation to phytohemagglutinin was also normal in the majority of patients (Z3). Fass *et al.* (F2) reported that 12 patients with Burkitt's lymphoma when tested with autologous tumor extracts showed measurable cellular responses of the host against his own tumor. In contrast to patients with Burkitt's lymphoma who have impaired antibody production but normal cellular immunity, patients with other forms of neoplasia of the reticuloendothelial system frequently have impairment of both the humoral and cellular aspects of immunity.

The serum immunoglobulins, in particular the IgM concentrations, are depressed in patients with Burkitt's lymphoma. All patients with central nervous system (CNS) involvement had IgM in their CSF, and subunits of IgM were detected in the urines of some Burkitt's lymphoma patients. The cellular immune responses were reported to be intact, but the humoral immunity was depressed in most patients with Burkitt's lymphoma.

In summary, it can be said that the majority of patients with Burkitt's lymphoma have a significant decrease of their serum IgM levels. Others have deficiencies of either IgG or both IgG and IgM. The CSF and urinary IgM may be related to CNS and renal involvement in patients with Burkitt's lymphoma.

8.4. Ewing's Sarcoma

In India, Gupta has described a marked increase in the serum IgM levels in 6 patients (ages between 4 and 7 years, and one 20 years old) with Ewing's sarcoma (G9). Host reactions to the tumor or the tumor-producing particulate antigens were thought to be the contributing fac-

tors to the elevated serum IgM. β-Lipoproteins and α_2-macroglobulin as well as an increase in the numbers of intranuclear periodic acid–Schiff-positive granules were also other unusual features in patients with Ewing's sarcoma. On the other hand, in Nigerian children of the same age group and having Burkitt's lymphoma there is a significant reduction of the serum IgM as well as the serum α_2-macroglobulin levels. If these two malignant conditions are caused by viruses, it would appear that the differences of the serum immunoglobulin patterns may be the results of two different abnormal effects of the viruses on protein synthesis in the hosts.

8.5. Serum Ig's in Other Lymphoid Neoplasia—Hodgkin's Disease, Reticulum Cell Sarcoma, Lymphosarcoma, and Chronic Lymphatic Leukemia

The serum immunoglobulin patterns in patients with the above malignancies in subtropical and tropical populations are similar to those in the temperate regions, and since several publications about these are already available they will be treated briefly here. Summarizing the patterns in these diseases in the Bantu, Michaux noted no constant immunoglobulin level for any single disease: some patients had one or other of the immunoglobulins in the serum markedly reduced, or markedly elevated, or all three of them normal. In many, the serum IgM showed a pattern similar to that of Burkitt's lymphoma, and secondary antibody deficiencies were just as common. Table 21 shows typical immunoglobulin results from Jamaican patients with various forms of malignant diseases of the reticuloendothelial system.

Antigenic typing of the immunoglobulins against monospecific light and heavy chain antisera was not as clear-cut as was the case with patients with multiple myeloma or macroglobulinemia. On several occasions the immunoprecipitin lines were normal, although a few showed patterns that were characteristic of a monoclonal origin of the malignancies.

As mentioned previously, serial estimations of the immunoglobulin levels in patients with malignancies are useful indices in the assessment of response to treatment and of the course of the disease. They are therefore recommended. It is more than likely that the subnormal transformation of peripheral blood lymphocytes to plant mitogens which indicate impaired cellular immune responses coupled with the antibody deficiencies are responsible for the infection which frequently occurs in these patients.

8.6. Reynaud's Phenomenon

Patient's with Reynaud's phenomenon have been described in both the West Indies and Africa, and it is my impression that it occurs there

TABLE 21
SERUM IMMUNOGLOBULIN CONCENTRATIONS IN VARIOUS MALIGNANT DISEASES OF THE RETICULOENDOTHELIAL SYSTEM IN JAMAICANS

No.	Age	Sex	Diagnosis	Total protein	Albumin (g/100 ml)	Globulin (g/100 ml)	Immunoglobin (mg/100 ml) IgM	IgA	IgG
1	26	M	Hodgkin's	9.8	1.2	8.6	260	800	2760
2	29	F	Hodgkin's	8.0	4.6	3.4	77	110	670
3	36	M	Hodgkin's	7.1	4.0	3.1	61	108	3300
4	30	M	Hodgkin's	7.7	2.3	5.4	—	390	2300
5	30	M	Hodgkin's	7.0	4.6	2.4	17	77	1400
6	33	M	Hodgkin's	7.8	3.5	4.3	92	108	1500
7	37	M	Hodgkin's	7.1	28	4.3	60	160	390
8	49	M	Hodgkin's	7.8	2.8	5.0	51	275	2300
9	66	M	Hodgkin's	7.3	4.5	2.8	150	380	850
10	51	M	Reticulum cell	7.1	2.3	4.8	60	80	900
11	67	M	Reticulum cell sarcoma	8.8	5.1	3.7	27	252	2700
12	44	M	Lymphosarcoma	10.2	3.0	7.2	275	Nil	6000
13	47	M	Lymphosarcoma	6.9	3.1	3.8	32	140	2200
14	50	M	Lymphosarcoma	5.8	2.5	3.3	—	275	960
15	55	M	Lymphosarcoma	6.4	3.1	3.3	—	250	1220
16	64	M	Lymphosarcoma	7.4	2.5	4.9	110	380	1820
17	78	M	Lymphosarcoma	8.6	1.6	7.0	125	460	800

with the same or greater frequency as in other parts of the world. The frequency of cryoglobulinemia among these populations is probably related to the frequency with which Reynaud's phenomenon occurs. In Jamaica Rowe (R8) has described a patient with classical Reynaud's phenomenon both from the clinical and laboratory angles—with gangrenous distal phalanges which became black for periods lasting for 3 weeks at a time. The laboratory tests confirmed the clinical diagnosis. Bone marrow biopsy revealed a marked increase of plasma cells, which were more than likely responsible for the production of the cold agglutinins which form precipitates when the temperature of the extremities has fallen slightly below that of the standard body temperature.

9. Rabies Antibody

Hospital employees, caring for a patient with rabies, were given 14 days of daily injections followed by booster shots on days 10 and 20 after the fourteenth injection of duck-embryo derived rabies vaccine (DEV). Rabies antibodies first appeared in the serum on day 6 of the injection course, and antibody titers fell significantly after the serum

samples were treated with mercaptoethanol, which is known to inactivate IgM antibody. Sephadex G-200 molecular filtration showed also that the rabies antibodies were mainly IgM, but a smaller proportion might also have been IgG and IgA. Throughout the immunization schedule, IgM antibody was constantly elevated. For instance, at 41 days after the rabies vaccine was given, 100% of the antibody was IgM; at 10 days, 79% was IgM, and between days 13 and 27, 45% was IgM (R9). Since 80–90% of the total body pool of IgM exists in the intravascular space and cannot get to the tissue sites where it is needed, the authors suggested that the immunization schedule against rabies infection should be modified to permit more rapid appearance of high levels of IgG-type antirabies antibodies which could readily diffuse across the tissue spaces. Another possibility that must be borne in mind is the occurrence of antigenic variation in rabies infection which may hinder the existence of the whole spectrum of rabies antibodies at any one time in the infected host—a phenomenon resembling that which occurs in malaria or trypanosome infection.

10. The Immunoglobulins in Cerebrospinal Fluid and Urine in the Tropics

The normal cerebrospinal fluid (CSF) especially contains total protein ranging from 10 to 60 mg/100 ml, 70% of which is made up of albumin. The remaining 30% consists of small quantities of IgG and transferrin. Ceruloplasmin is detectable in trace amounts in some normal CSF; IgA and IgM are not usually detectable in the normal CSF. The total proteins in Nigerian CSF was higher than the corresponding figures quoted for persons living in temperate regions.

In various infectious diseases involving the central nervous system, the CSF protein may reflect gross abnormalities.

In diseases like syphilis and the large variety of meningitis and Burkitt's lymphoma, the total protein concentration in the CSF is markedly elevated. In tuberculosis meningitis in Africans, all immunoglobulin types were detectable in elevated concentrations in the CSF (U1): IgG ranged from 10 mg/100 ml to 350 mg/10 ml. IgM and IgA were detectable especially in those patients with markedly elevated total proteins in their CSF. In East Africans we were also able to detect elevated levels of all three immunoglobulins in the CSF of patients with *Staphylococcus* meningitis. In Burkitt's lymphoma, we detected high IgM as well as IgG and IgA in the CSF of those patients with central nervous system (CNS) involvement.

In patients with African sleeping sickness, it is common to find high IgM in their CSF, but no IgM could be detected in the CSF of children

with either convulsions due to cerebral malaria or cow's urine poisoning.

Finally, it can be stated that in the tropics the commonest proteins that may be detected in the CSF of normal persons are albumin, IgG, transferrin, and ceruloplasmin, the most consistent finding being the albumin. There seems to be some relationship between infections and transferrin in CSF in affected patients, and this could be fruitful ground for further research.

In conclusion, the following observations must be considered.

IgM is frequently detected in the CSF of patients with trypanosomiasis, tuberculous meningitis, *Staphlococcus* meningitis and in patients with Burkitt's lymphoma with CNS involvement and could very well be of diagnostic significance.

The total protein excreted in the urine of normal persons in the tropics ranged from 50 to 100 mg/day. This was made up of albumin, IgG, and IgA. There is some evidence that both the IgA and IgG in the urine are subunits as well as intact molecules. When normal urine is passed through columns of Sephadex G-200, both IgA and IgG emerge from the column in at least two to three peaks. Some of the urinary IgA appeared to be of very large molecular weight and may be related to secretory IgA. Small quantities of light chains have been detected in normal urines in the tropics as well as in large quantities of the urines from some patients with multiple myeloma and Waldenström macroglobulinemia.

In all forms of glomerular nephritis in tropical populations in Africa, there was increased protein in the urine. In the selective proteinuria of the nephrotic syndrome most of the protein was mainly albumin and transferrin, with lower concentrations of IgG and other globulins. On the other hand, the urine of the nonselective proteinuria of the nephrotic syndrome is characterized by much higher concentrations of IgG, IgA, α_2-macroglobulins, and some IgM. Differential protein clearance to establish selectivity of proteinuria has been discussed above.

The author has observed a marked hypogammaglobulinemia in patients with the nephrotic syndrome in both the West Indies and Nigeria. In these patients the serum IgG level may be less than 500 mg/100 ml, and the urine electrophoretic pattern may resemble that of a normal serum. The striking hypogammaglobulinemia which so frequently accompanies chronic glomerular nephritis is responsible for the superimposed infection that may occur in this condition.

The total proteins in the urine of patients with various lymphomas was higher than in normals. The reason for this was most likely an increased excretion of light chain proteins due to abnormal immunoglobulin synthesis. Most of these patients have secondaries in the kidneys, and this may well be a cause and effect phenomenon.

11. Applicability of Immunoglobulin Estimations in Populations of Subtropical and Tropical Countries

It has been emphasized herein that only limited information can be obtained from single measurements of the immunoglobulins. Nevertheless their serial estimates can provide invaluable diagnostic and prognostic information in several of the following diseases, which occur in populations of subtropical and tropical countries:

Group A includes diseases in which patients have one or other of their immunoglobulin concentrations lower than normal:

1. Malignancies of various types—multiple myelomatosis, Waldenström macroglobulinemia, chronic lymphocytic leukemia, reticulum cell sarcoma, Hodgkin's disease, Burkitt's lymphoma and other malignancies of the reticuloendothelial system
2. Renal diseases, especially the nephrotic syndrome
3. Liver disease, especially infective hepatitis patients who may have a transient and substantial decrease in IgG, IgA, or IgM
4. Primary hypogammaglobulinemia, e.g., Bruton's sex-linked hypogammaglobulinemia and secondary hypogammaglobulinemia
5. Malnutrition in children who have continuous infection, especially those in whom the malnutrition developed before the age of 7 months
6. Diseases during pregnancy—infections, infective hepatitis, amebiasis
7. Prematurity, especially babies with frequent respiratory or gastrointestinal infections
8. Cholera

Group B includes diseases in which patients have one or other of their immunoglobulin concentrations higher than the normal:

1. Suspected malignancies listed in A 1 above; and also Ewing's sarcoma
2. Malaria
3. Trypanosomiasis
4. Amebiasis
5. Tuberculosis
6. Leprosy
7. Syphilis
8. Splenomegaly
9. Rabies
10. Cholera
11. Schistosomiasis
12. Rheumatoid arthritis

13. Polyarthritis nodosa
14. Amyloidosis
15. Indian childhood cirrhosis
16. Rubella

Group C includes diseases in which patients have CNS involvement, hence immunoglobulin concentrations in their CSF:

1. Meningitis
2. Burkitt's lymphoma
3. Trypanosomiasis
4. Syphilis

Group D includes malignant diseases involving the kidneys, hence Bence Jones protein estimation is needed. Urines from all patients with malignancies should be tested. The simplest method is to test the urine first for protein by the Albustix or simple heating to boiling. If protein is present, then concentrate urine by dialysis against sucrose followed by electrophoresis on cellulose acetate. The presence of a band in the γ-globulin region is diagnostic of Bence Jones protein excretion.

Where facilities are available for cell cultures, then the quantitation of immunoglobulin concentration may be supplemented with investigations on peripheral blood lymphocyte transformation with phytohemagglutinin and pokeweed mitogen as a quick guide to cellular immune response. Whole blood cultures, without the need for separating the lymphocytes, can be performed in any laboratory where an incubator, a centrifuge, a hot-air oven, and a microscope are available. Lymphocyte transformation can be readily assessed by counting the percentage of blast lymphocytes among the total lymphocyte populations. In those laboratories so fortunate as to possess isotope-counting equipment, lymphocyte transformation can be assessed by tritiated thymidine uptake. Most patients with malignancies, parasitic diseases, and malnutrition, apart from having impaired antibody synthesis, may also have a concomitant impairment of their cellular immune responses. Cell culture studies need not be an expensive undertaking, and I would recommend that one lymphocyte transformation index in triplicate be carried out before and after (6 weeks or so) treatment is commenced.

Finally in those areas of the subtropics and tropics where animal-house facilities and staff are not readily available, it is advisable to purchase all antiserum reagents from commercial suppliers rather than attempt to prepare them, bearing in mind that such reagents differ greatly in potency from supplier to supplier. Nowadays there are several suppliers of commercial antiserum.

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CRITIQUE OF THE ASSAY AND SIGNIFICANCE OF BILIRUBIN CONJUGATION

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1. Introduction

The present review covers a description of methodology and properties of UDP-glucuronyltransferase and of related UDP-glycosyltransferase activities (assayed with bilirubin as the acceptor substrate) and attempts to delineate applications to human disease. Studies with other hydrophobic acceptor substances will be discussed as far as relevant to the subject matter.

Bilirubin derives largely from senescent erythrocyte hemoglobin [for reviews, see references (F9, G7, L6, S3, W11)]. When transported in plasma the pigment is bound to albumin (B9, O4). Dissociation from albumin precedes rapid uptake by liver tissues (B4, B11, B29). This uptake and the transfer of bilirubin to its sites of metabolism may be mediated by cytoplasmic binding proteins (G8, G11, L8, M9).

In most normal adult animals, conjugation of bilirubin with various sugars probably represents the major mechanism for its transformation (Fig. 1). The reaction products are eliminated from the organism by excretion into the bile. Glucuronyl transfer has been investigated in

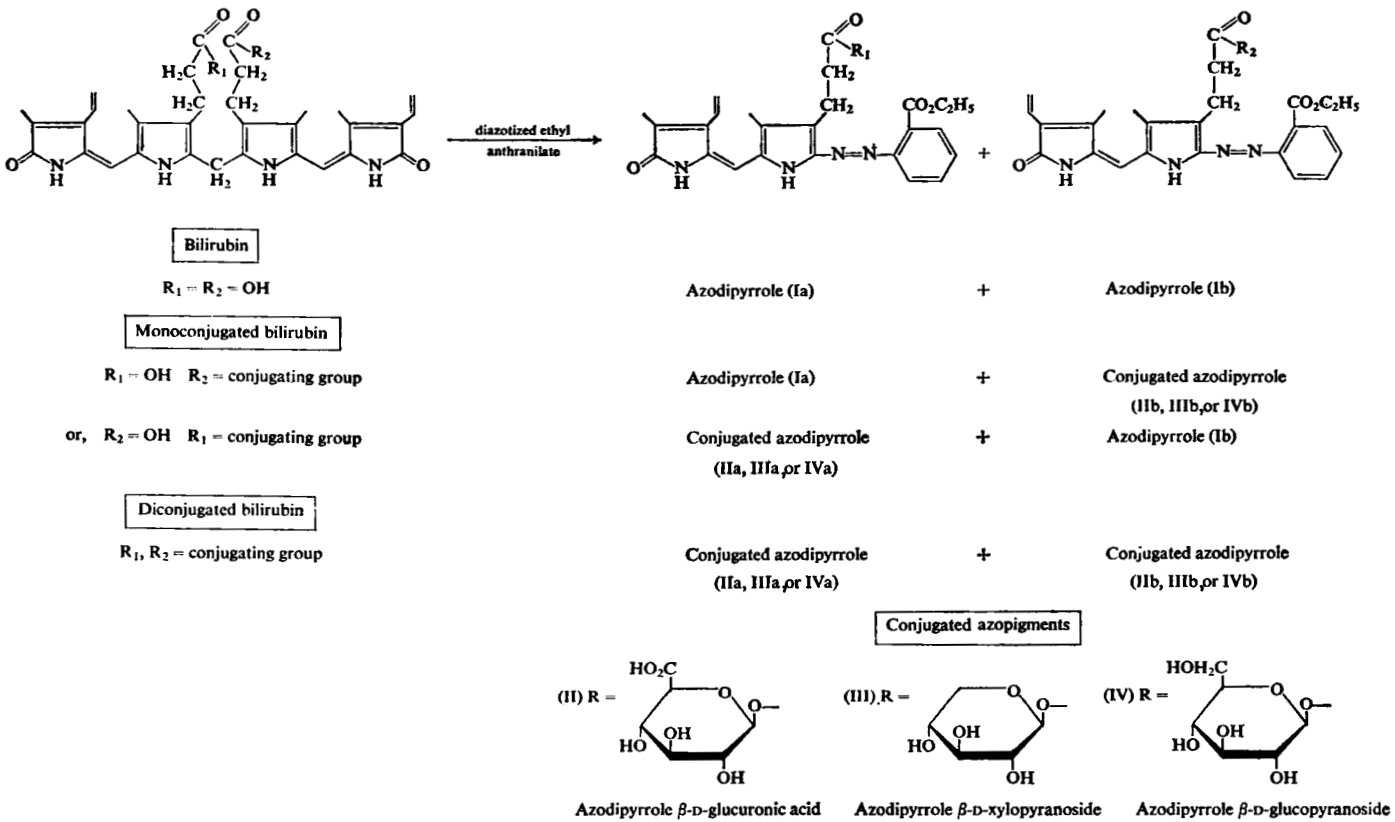

FIG. 1. Structures of some bilirubin conjugates and nature of the azo derivatives obtained by treatment with diazotized ethyl anthranilate. From Fevery *et al.* (F5), by kind permission of the Editor of *The Biochemical Journal.*

greatest detail (A8, G9, L4, S3) and is of predominant importance in the biotransformation of bilirubin in man and rat (B13, F6, H11, O3, S1, S6, T1). The bilirubin glucuronosides may be accompanied by small amounts of bilirubin sulfate (I2, N2) and other conjugates (F7, T3). Relatively large amounts of hexuronic acid-containing conjugates, distinguishable from the bilirubin glucuronosides, have been detected in bile, serum, and urine of patients with conjugated hyperbilirubinemia (F6, H11, K3) and in bile of rats immediately after relief of biliary obstruction (F6, V1).

UDP-glucuronyltransferase is a microsomal enzyme (D8, D9) with probably an exclusively parenchymal localization in liver tissues (B10, H14). Glucuronyl transference to bilirubin (H4, W4) and to many other acceptor substrates (G5, L14, M14, P4, W4), with the exception of phenolphthalein (G5), is higher in the rough endoplasmic reticulum than it is in the smooth endoplasmic reticulum. Bilirubin UDP-glucuronyltransferase has also been detected in preparation of gastrointestinal tract (S9, T4) and kidney (G9, G10, T4); however, in normal adult animals conjugation of bilirubin in these organs is probably of little importance. Studies following surgical removal of the liver, kidney, or intestine in dogs and rats (F15, H9, S6, S7) suggest that extrahepatic conjugation can play a role when the uptake and/or conjugation capacity of the liver is either deficient or exceeded.

Much less is known about processes in mammalian tissue leading to conjugation of hydrophobic acceptor substances with sugars other than glucuronic acid. Considerable amounts of glucose and xylose conjugates of bilirubin are present in bile of normal dogs (F7). Microsomal preparations from the livers of various animals catalyze the conjugation of bilirubin with glucose in the presence of UDP-glucose (F3, W13, W14); with UDP-xylose as the sugar donor, xylosyl transfer to bilirubin has been demonstrated with microsomal material from rat liver (F3). Glucosyl transfer from UDP-glucose to steroids and 17α-estradiol 3-glucuronosides (C5, W5), ceramide (B5), isoflavones (L1), and *p*-nitrophenol (G2), galactosyl transfer from UDP-galactose to steroids (W5), and *N*-acetylglucosaminyl transfer from UDP-*N*-acetylglucosamine to several steroid 3-glucuronosides have also been described (L5).

Conjugation with glucuronyl residues is of great importance for the metabolic fate of bilirubin (S3), steroids (L5, M2, R8), catecholamines (W17) and other hydrophobic compounds (D8, D9). Neonatal accumulation of bilirubin in man and rats may trigger maturation of UDP-glucuronyltransferase (B1, B2, T6). Delayed maturation of the enzyme, or its partial or total deficiency, are critical factors in the development of kernicterus (P6). Compared to other species partial deficiency of the

TABLE 1
ACTIVITY AT 37°C OF BILIRUBIN UDP-GLUCURONYLTRANSFERASE FROM LIVER

Enzyme preparation		Bilirubin carrier[c]	Enzyme activity[a] in liver from: Rat		Other species		Species, strain, sex	References
Source	Treatment[b]		A_P	A_L	A_P	A_L		
Slices	None	HS(2.8)	—	16	—	—	Wistar rat	(L4)
	None	HS(2.8)	—	9	—	—	Rat	(H8)
	None	HS(2.8)	—	20	—	—	Wistar rat, male	(B12)
	None	HS(2.8)	—	28	—	—	Hooded rat, male	(A1)
	None	HS(2.8)	—	18–26	—	—	Wistar rat, male	(A3)
	None	BA(0.2)	—	—	—	2.8	Guinea pig, female	(F11)
Homogenates	None	HS or RS(2.8)	—	69–82	—	—	Sprague-Dawley rat, male	(M6)
	None	HA(1.0)	—	43–83	—	—	Wistar rat, male	(M5)
	None	HA(1.0)	—	23–49	—	—	Wistar rat, female	(M5)
	None	HA(1.1)	—	55	—	—	Wistar rat, male	(H10)
	None	HA(1.0)	—	—	—	63–91	Guinea pig	(M5)
	None	HS or RS(2.8)	—	—	—	63	Man	(M7)
	Dialysis	BA(4.4)	—	380	—	—	Sprague-Dawley rat	(H2)
	Dialysis	BA(4.4)	—	774	—	—	Fisher rat, male	(H3)
	Dialysis	BA(4.4)	—	675	—	—	Fisher rat, female	(H3)

	Dialysis	BA(2.8)	—	350	—	—	Rat	(O1)
	Digitonin	HA(2.1)	—	425	—	—	Sprague-Dawley rat	(B17)
	Digitonin	HA(2.1)	—	655	—	—	Wistar rat	(T6)
	Digitonin	HA(1.1)	—	731	—	—	Wistar R-rat, male	(H10)
	Dialysis	BA(4.4)	—	—	—	206	Rabbit	(H2)
	Dialysis	BA(4.4)	—	—	—	83	Guinea pig	(H2)
	Dialysis	BA(2.8)	—	—	—	324	Man	(O1)
	Digitonin	HA(2.1)	—	—	—	314	Man	(B17)
Microsomal material	None	HS(2.8)	0.86	—	—	—	Hooded rat, male	(A2)
	None	HA(1.1)	1.49	—	—	—	Wistar R-rat, male	(H10)
	None	A	—	—	0.82	—	Rabbit	(T8)
	UDPNAGA	BA(1.5)	—	203	—	—	Wistar rat, male	(S10)
	UDPNAGA	BA(1.5)	—	185	—	—	Wistar rat, female	(S10)
	UDPNAGA	BA(1.5)	—	222	—	—	Sprague-Dawley rat, male	(S10)
	Digitonin	HA(1.1)	17.0	—	—	—	Wistar R-rat, male	(H10)
Purified preparations		HS(2.8)	8.1	—	—	—	Hooded rat, male	(A2)
		HA(2.6)	—	—	6.0	—	Hartley guinea pig	(M13)

[a] Enzyme activities are expressed as nanomoles of bilirubin conjugated in 10 minutes per milligram of protein (A_P) or as nanomoles of bilirubin conjugated in 10 minutes per gram wet weight equivalent of liver (A_L).

[b] Enzyme preparations pretreated by dialysis at pH 9.2 (dialysis) or with digitonin (digitonin), or UDP-*N*-acetylglucosamine (UDPNAGA) added to enzymatic incubation mixtures.

[c] Only results obtained with albumin-solubilized bilirubin are given: human serum (HS), rat serum (RS), albumin (A), human serum albumin (HA), bovine serum albumin (BA). Figures between parentheses indicate the molecular ratio, bilirubin:albumin.

enzyme in cat liver may be counteracted to some extent by normal transglucosylation activity (W14). Changes in the amounts and composition of diazo-positive bile pigments in serum, urine, and bile are of considerable diagnostic and prognostic importance in liver pathology.

Attempts to evaluate the role of conjugating processes have instigated many valuable studies on glucuronyl transference with xenobiotic acceptor substrates (D8, D9). However, extrapolation of observations from a foreign to a natural substrate is hazardous. For some species and tissues the activity of UDP-glucuronyltransferase toward xenobiotics may not at all reflect its specificity toward the natural substrates (D9). Significantly, the long-standing question whether or not one or several UDP-glucuronyltransferases exist, each with its own substrate specificities, has not yet been settled (D9). Further, diconjugate formation, a routine process demonstrated *in vitro* with steroids (L5) and with bilirubin (F5, H4, J5, R9, S6, S10), can rarely be studied with xenobiotics, if at all. For these reasons more intensive studies of bilirubin UDP-glucuronyltransferase and of related glycosyltransferases may be rewarding in relation to their roles in normal and pathological processes.

Studies of UDP-glucuronyltransferase with bilirubin as the acceptor substrate are technically difficult. This is indicated by frequent modification of the initial assay systems (A8, G9, L4, S4) and by the wide range of reported enzyme activities (Table 1). Possible causes of these discrepancies, which are manyfold, will be discussed in some detail below. The conclusions drawn should be helpful in the design of assays of conjugate formation of bilirubin and of the synthesis of mono- and diconjugates.

An effort has been made to cover the literature from January 1963 to October 1972. Extensive referencing to studies on bilirubin metabolism (L6, S3, W11) and on glucuronoconjugation (D8, D9), including earlier work, can be found in the review articles cited.

2. Nomenclature

The general term UDP-glycosyltransferase or the more specific ones UDP-glucuronyl-, UDP-glucosyl-, and UDP-xylosyltransferase will be employed as convenient. They are used primarily to denote enzyme activities observable in broken cell preparations. This practice does not imply any expression of opinion with regard to the existence of either a single polyfunctional enzyme system, or of several enzymes with different specificities toward the sugar donor (Section 7).

As bilirubin can be converted *in vitro* into mono- and diconjugates of glucuronic acid (H4, R9, S6, S10) and of xylose (F5), it is useful to distinguish the "bilirubin conjugation rate" (BCR) from the "group trans-

fer rate" (GTR). On a molar basis GTR equals twice BCR when diconjugates are being synthesized; the quantities are identical only in case of monoconjugate formation (Fig. 1). In general, inspection of the procedure used to assay the conjugate synthesized will indicate whether values proportional to BCR or GTR are obtained (Table 2). Knowledge of the nature of the reaction products is required to derive BCR from GTR, and vice versa.

To quantitate and analyze conjugated bile pigments, azo derivative formation is applied most frequently (Fig. 1). Such terms as "azobilirubin," or "azobilirubin conjugate," erroneously suggest tetrapyrrolic structures. The following trivial names appear to be more appropriate: "dipyrromethene azo derivative," "azodipyrrole," "dipyrrolic azo pigment," and the like. The term "azodipyrrole" will be employed in the present review.

3. Enzymatic Incubation and Control Incubation

Compared to work with soluble enzymes, it is more difficult to meet standard kinetic requirements in studies with membrane-linked systems, e.g., derivation of reaction rates from the straight portions of the velocity-time curves, and incubation at optimal pH and at near-saturation with respect to substrates and activators. Endogenous sources of reactants, involvement of multienzyme systems, changes in enzyme conformation induced by variations in the composition and structure of the phospholipid environment, etc., may complicate the establishment of generally valid working conditions and interpretation of the final results. In the present section an attempt will be made to delineate some factors that should be taken into account in applying and developing assays of bilirubin UDP-glycosyltransferase activities. Considerations will be kept as practical as possible, references being mentioned only as far as required to substantiate recommendations or to help making a choice among alternative approaches.

3.1. Enzymatic Incubation

In work with bilirubin UDP-glycosyltransferases, only so-called discontinuous or sampling methods are employed.

3.1.1. *Determination of Initial Reaction Velocities*

It may be difficult to conserve initial velocities over a sufficient period of time to warrant appropriate sensitivity of the assays, especially with unactivated enzyme preparations. With carrier-free bilirubin substrate, decrease of the initial velocity may occur earlier than with albumin-solubilized bilirubin (M16). Spontaneous activation (W7) of enzyme

TABLE 2

RECOMMENDED DIAZO PROCEDURES FOR ASSAYING SYNTHETIC BILIRUBIN GLUCURONOSIDE

Assay principle	Derived result	Remarks	References
Nonradioactive methods determining total amount of synthetic conjugate			
(A) Diazo-coupling in a two-phase system using diazotized sulfanilic acid; the azo color, obtained in the aqueous phase, is determined photometrically	BCR[a,b]	Ratio BMC:BDC obtainable by chromatographic analysis of the azo derivatives; low blank	(H2, H4, S10)
		A micro version assays artificially activated enzyme	(O1)
(B) The azo color is read photometrically after diazo-coupling of BC in a homogeneous acid medium with		High blank if color read in reaction medium	
Diazotized sulfanilic acid (5 minutes, room temperature)	BCR	After terminating the coupling reaction with ascorbic acid, reaction mixture clarified by precipitating protein with ethanol	(A2, L4)
Diazotized sulfanilic acid (10 minutes, room temperature)	BCR	Turbidity decreased by final addition of caffein, sodium benzoate, and Teepol; micromethod	(F17)
Diazotized ethyl anthranilate (pH 2.6, 30 minutes, room temperature)[c]	BCR[b]	After the coupling reaction with ascorbic acid is terminated, azo color is extracted and read; low blank; the extract can be applied directly to a thin-layer plate and be separated rapidly to determine the ratio BMC:BDC	(H10, H11, V2)
		Micro versions assay artificially activated enzyme	(B17, H1)

Nonradioactive methods determining isolated conjugated azodipyrrole			
Conjugated azo pigment, derived from synthetic BC, is determined photometrically after its chromatographic isolation[c]	GTR	Versatile; TLC can be applied	(B28, W12)
Methods assaying labeled conjugated azodipyrrole			
(A) Enzymatic incubation mixtures contain [U-^{14}C]UDP-glucuronic acid; the derived azo pigments are separated by TLC, and the conjugated pigment is counted	GTR	[U-^{14}C]UDP-glucuronic acid of known specific activity is used; versatile method	(W12)
(B) Synthetic BC and excess B are coupled with [^{35}S]sulfanilic acid diazo reagent in presence of ethanol; azo derivatives are extracted in presence of cold carrier pigments, separated chromatographically and counted by radio scanning	GTR	Enzymatic incubation mixtures contain a known amount of B; the percentage of radioactivity in conjugated azodipyrrole, and the total amount of bilirubin are used for calculations; sensitive micromethod	(M7)
(C) Solutions of standard BDC and of unknown are treated with [^{35}S]sulfanilic acid diazo reagent; the conjugated azo derivatives are purified, then hydrolyzed; the derived unconjugated azo pigments are further purified, then counted	GTR	GTR is calculated from the known concentration of the standard solution, and from the specific activities of the labeled azodipyrrole derived, respectively, from the standard and unknown solution	(B22, B24, F11)

[a] Abbreviations: bilirubin (B), conjugated bilirubin (BC), bilirubin monoconjugate (BMC), bilirubin diconjugate (BDC), bilirubin conjugation rate (BCR), group transfer rate (GTR).

[b] The conclusion that BCR is obtained is supported by chromatographic analysis of azo derivatives.

[c] System is also applied to assay bilirubin UDP-xylosyl- and/or bilirubin UDP-glucosyltransferase activity.

during incubation may lead to upward curvature of the product formation–time curves. The latter complication is less likely with enzyme preparations, previously activated with, e.g., Triton X-100 or digitonin. If spontaneous activation or denaturation of enzyme had measurable effects, it is possible that its speed could depend on the value of the parameter investigated, e.g., a substrate that protects the enzyme against denaturation. In establishing activity–pH curves of bilirubin UDP-glucosyl- and bilirubin UDP-xylosyltransferase one broad optimum was found at pH 6.6–7.2 (F5) at two relatively short incubation times. At prolonged incubation, maxima at pH 6.6 and 7.1, or one maximum at pH 7.1 and a shoulder at pH 6.6 appeared. Lowering of the usual incubation temperature, 37°C to 30°C or 25°C, may reduce potential complications (H10), but this has not yet been investigated.

In assessment of optimal incubation conditions it is advisable, as is frequently done in work with less costly substrates, to measure the activities as a function of the parameter considered at two time points along the velocity–time curves. A survey of available techniques for assaying conjugated bilirubin (Section 4) shows that the possibilities of miniaturization, e.g., by reducing the total volumes of the incubations mixtures without changing the relative concentrations of their components, have seldom been exploited.

3.1.2. *Stability of Substrates and Reaction Products*

Enzymes hydrolyzing UDP-glucuronic acid (UDP-glucuronic acid pyrophosphatase) and β-D-glucuronosides (β-glucuronidase) are present in microsomal material. Their activities in tissues depend on the species examined (G4, P2, W15).

The activity of β-glucuronidase at the usual pH of the transferase assays (pH 7.4–8.2) is very low. The enzyme, most likely, has no role in the conjugation process itself (G4). If potentially important, e.g., in work with homogenates or cell extracts containing partially lysed lysosomes, the specific inhibitor saccharo-(1 → 4)-lactone (L9) can be added to the incubation mixtures.

Approximate conservation, during the finite incubation periods, of the degree of saturation with UDP-glucuronic acid, prevalent at zero incubation time, may be hampered by hydrolysis of the pyrophosphate bond (A2, S10). Addition of UDP-*N*-acetylglucosamine, a known inhibitor of UDP-glucuronic acid pyrophosphatase (A2, P2, S10), to enzymatic incubation mixtures stimulated untreated bilirubin UDP-glucuronyltransferase (A2, H10, S10, W8) but had virtually no effect on enzyme fully activated by aging (S10, W8) or by treatment with Triton X-100 (W7) or digitonin (B17, H10). Detergents do not inhibit the pyro-

phosphatase (G4). Unless a direct structural interaction between the transferase and the pyrophosphatase exists, a system competing for donor substrate should not affect conjugation rates, as long as the total concentration of sugar donor remains large compared to the enzyme-bound fractions. It is possible that, to some extent, activation of untreated transferase by UDP-*N*-actylglucosamine, was due to inhibition of extentive breakdown of UDP-glucuronic acid. However, other phenomena must intervene. In cell extracts from liver of mouse and rat, the activating effect of UDP-*N*-acetylglucosamine on UDP-glucuronyltransferase activity (tested with various xenobiotics) was abolished by previous treatment with *N*-ethylmaleimide (W8). With bilirubin as the acceptor substrate, a similar but less pronounced effect was observed with preparations from rat liver (W8). Also, depending on the aglycon tested, addition of UDP-*N*-acetylglucosamine to cell extracts from the livers of mouse and rat, either had no effect or stimulated glucuronyl transfer up to 10-fold (W8). These observations seem to be incompatible with inhibition of breakdown of UDP-glucuronic acid as the main cause of activation of transferase. The antiparallelism between the activities of the pyrophosphatase and the transferase, observed with liver preparations from various animals (G4, W15), does not prove the existence of a structural link between both enzymes.

No data are available regarding effects of competing enzymes on the activities of transferases other than glucuronyltransferase. For assays of native transglucuronylation rates, it may be useful to run parallel incubations with UDP-*N*-acetylglucosamine-fortified (S10) and unfortified preparations, particularly when species are compared.

Enzymatic destruction of the acceptor substrate, bilirubin, has not been reported, but see (B23). It is likely to occur somewhere in Gunn rats (S5), probably in liver, and is a possibility in preparations from animals with partial deficiency of UDP-glucuronyltransferase. However, in the presence of microsomal material of normal adult rat, albumin-solubilized bilirubin in the absence of added UDP-sugar underwent no measurable destruction during prolonged incubation in air at 37°C in the dark (F3). Under similar conditions, carrier-free bilirubin could be more susceptible to oxidative destruction. If so, incubation mixtures could be provided with an oxygen-free atmosphere. In general, incubation should be done in the absence of light, manipulations being done under subdued light. Photooxidation at about neutral pH is more pronounced with albumin-bound than with carrier-free bilirubin (O2).

For preparation of the substrate solution, it is common practice to dissolve bilirubin at alkaline pH (pH 11–12.5) and subsequently to lower the pH to the required value, usually pH 7.4–8.2. The bilirubinate solu-

tion should be prepared immediately before use. Its stability can be improved by addition of about 1 mM disodium EDTA (F13). When albumin is incorporated in the incubation mixtures, a small volume of a concentrated bilirubinate solution can be added without delay to a larger volume of albumin solution, with immediate mixing. In the absence of light this procedure confers stability to the substrate. In work without bilirubin carrier, freshly prepared bilirubinate solution, eventually after appropriate dilution, may preferentially be added as the last component to the incubation mixtures. Determination of the bilirubin concentration of the initial alkaline solution should be avoided (V3). Total bilirubin can be determined (H12) by assaying, immediately after the addition of the pigment, either bilirubin-albumin substrate solution or incubation mixture.

3.1.3. *The Physical State of the Acceptor Substrate, Bilirubin*

At 25°C in phosphate buffer (ionic strength 0.1), the solubility of bilirubin is 0.1 μM at pH 7.4 and 34 μM at pH 8.2 (B25); see also earlier work (O5). Various methods have been employed to increase the solubility of bilirubin in the incubation mixtures. These include the use of a slightly alkaline pH (J5, P3, P5, S4, W12) or the addition of either organic solvents (A2, V2) or binding protein (F3, G9, H2, H10, L4, M5, M7, S10, V2).

Most frequently, binding protein is added to the incubation mixtures as either serum or purified serum albumin. With human serum albumin, at equilibrium, the acceptor substrate will largely be protein-bound, when the bilirubin:albumin molecular ratio is smaller than one (the dissociation constant of the first binding site of purified human serum albumin is approximately $7 \times 10^{-9} M$ with $2 \times 10^{-6} M$ for two additional binding sites) (J2). The first binding site of albumin, measured with rat serum, has a dissociation constant of about $5 \times 10^{-8} M$ (M8). The unbound fraction will normally not exceed the very low solubility of the pigment. Addition of albumin to an alkaline solution of bilirubin, or its addition immediately after neutralization, prevents colloid formation, if the bilirubin:albumin molecular ratio is smaller than one (B25). However, colloidal bilirubin, once formed, cannot be redissolved by the addition of albumin (B26).

At a bilirubin:albumin molecular ratio below one the added binding protein will thus act as a kind of "buffer," keeping the concentration of unbound substrate sufficiently low to inhibit colloid formation (B25) or precipitation onto bound bilirubin (B26), and will prevent aspecific binding to cell particulates. The binding protein can also be thought of as a "reservoir" providing a continuous stream of molecularly dispersed sub-

strate to the incubation medium. Bilirubin bound to albumin does not isomerize (M4) and, in the dark, is protected against oxidation (O2).

The use of binding protein involves a number of side effects that should be duly evaluated. Bilirubin UDP-glucuronyltransferase from rat liver is stabilized by human serum albumin against denaturation at 37°C, whereas the xylosyltransferase is not (F3). This may explain stimulation by albumin of glucuronyl transfer, observed with the probably more labile, purified enzyme preparations (H2) and during prolonged enzymatic incubation (P2). Inhibition by albumin of glucuronyl transfer to phenolphthalein at low substrate concentration and a biphasic stimulation-inhibition effect at high concentration (M15) are probably due to decreases of enzymatically available substrate, with relief at high concentration of substrate-inhibition. Albumin-binding of monoconjugates of bilirubin may permit isolation of the first conjugation step (J5) and may decrease product inhibition. Conversely, once pure samples of monoconjugates of bilirubin become available, diconjugate formation should be studied with due regard to protein-binding of the acceptor substrates. As yet, affinity constants for protein-binding of conjugated bilirubins are not known.

Mixtures of water and dimethyl sulfoxide (A2), or of water and ethylene glycol (V2), have been used to increase the solubility of bilirubin. Promotion of solubilization by such solvents as methanol, propylene glycol, or detergents is common practice in work with enzymes acting on steroids, ceramide, cholesterol, and other hydrophobic substrates. Such systems may be useful when, besides bilirubin, other hydrophobic substances are incorporated in the incubation mixtures, e.g., in inhibition (A2) or mixed substrate experiments. Albumin-containing systems may be less suitable in such cases because of possible binding to carrier protein of all components, eventually to common binding sites. Unsuspected, or difficult to control, activation of enzyme by water-miscible solvents, with eventual inhibition at high concentrations (V2), is a potential drawback.

Some authors have used carrier-free enzymatic incubation mixtures at pH 8.0–8.3 (J5, P3, W12). In general, the final concentrations used (incubation at 37°C) were 5–10-fold higher than the solubility of bilirubin at 25°C (B25). Although solubility data at 37°C are not available, it is likely that in most instances the solubility was exceeded. It is not known whether, and to what extent, bilirubin is solubilized in an aspecific way, e.g., by dissolution in lipid membrane regions. Formation of colloidal bilirubin is possible (B25). Aging of the initial, supersaturated (B25) bilirubinate solution is expected to depend (B26) on the procedure of initial solubilization, the time elapsed between lowering the alkaline

solution to the required pH and the onset of incubation, the final pH value adopted, etc. It is likely that careful programming of manipulatory steps may lead to adequate reproducibility. It should also be noted that components promoting solubilization of bilirubin may unknowingly be incorporated in the incubation mixtures, e.g., bilirubin-binding cytosol protein (V2) added with tissue homogenate or cell extract, or detergent used to activate the transferases (J5, P5).

With albumin-solubilized bilirubin, pH optima of microsomal bilirubin UDP-glucuronyltransferase were 7.4–8.0 for rat (H2, H10, S10) and 7.4 for guinea pig (M13) and rabbit (T8). Above pH 8 the enzyme activity decreased abruptly (H10). In absence of carrier protein, optima were at pH 8 and 8.2 with preparations from liver of guinea pig (P3) and rat (W12), respectively. The activity–pH curve with optimum at pH 8.2 (W12) showed pronounced skewing, with a steady and rather rapid increase of enzyme activity from pH 7.4 to 8.2. One may wonder whether such measurements were influenced by the rapid increase of solubility of the acceptor substrate occurring over the same pH range (B25).

For the present, systems containing albumin-bound bilirubin appear to be of greatest practical value. The fraction of fully hydrated substrate can be known more accurately than with the other systems. Carrier-free systems or the addition of water-miscible solvents may be quite useful for specific purposes. Comparative studies with the three modes of substrate addition would be helpful to allow assessment of their merits.

3.1.4. *The Sugar Donors*

UDP-sugars are suitable external sugar donors (Section 7). Apart from endogenous UDP-sugars (G1, K1, W16, Z3), tissue homogenates may contain other forms of active sugar that can act as sugar donors in transfer reactions with bilirubin (H10, L4, M5), steroids (C9, W5), and *o*-aminophenol (D10) as the aglycons. The latter endogenous sugar donors have been detected in microsomal material (A4, B6, B7, B8, D2, M11, R7, T5, W5); the former are probably confined to cytosol.

With fresh rat liver homogenates that were not fortified with UDP-sugar, appreciable conjugate formation of bilirubin occurred (H10, M5). The process occurred in the absence of added Mg^{2+} and was inhibited by digitonin (H10). The conjugation rates at 37°C were constant for the first 3- to 5-minute period, then decreased gradually to zero (M5).

Correction for endogenous substrate present in enzyme preparations is difficult. Measuring the "endogenous rate" (obtained by omitting the substrate involved) and subtracting this from the overall rate is generally incorrect (R5). Due to the nonlinearity of the substrate saturation curve, the "endogenous rate" will be more important in the control mixture than

is its contribution to the overall rate; in the test mixture its relative importance will decrease as the concentration of exogenous substrate increases. In the present case, even qualitative prediction is impossible because of the presence of true substrate(s) [UDP-sugar(s)] and of intermediate or alternate substrate(s) (active sugars different from UDP-sugar). In tissues the relative amounts of both types of substrate probably vary with species and nutritional state.

The following approach is suggested. By comparison of bile pigment present in duplicate incubation mixtures (UDP-sugar omitted), at zero incubation time and after incubation, respectively, a value of the "endogenous rate" is obtained. If the value is negligible, i.e., smaller than 1% of the overall rate, it can be ignored. If not so, one of two procedures can be adopted: (1) The control incubation mixture is treated under exactly the same conditions as the test mixture with the exception of omission of UDP-sugar, or (2) additional steps are taken to suppress "endogenous synthesis" in the control mixture (see Section 3.2). Procedure (1) leads to underestimation, procedure (2) to overestimation, of the transglycosylation rate. The authors prefer the second procedure for determining enzyme activities, as, at high concentration of the exogenous substrate, "endogenous synthesis" is expected to be suppressed largely in the test incubation mixture. Preparation and incubation of control incubation mixtures is discussed in Section 3.2.

3.1.5. *Endogenous Bilirubin Conjugates*

Bile pigment deposits are frequently present in liver biopsy specimens from human patients with conjugated hyperbilirubinemia (D3), but not in liver of obstructed rats (D4). Nonperfused liver homogenates or cell extracts from animals with experimentally induced liver disease or obstruction are also likely to contain conjugated bilirubin as a consequence of contamination with blood. Conjugated bilirubin is absent from tissues and blood of normal animals. To our knowledge eventual inhibition, by bilirubin conjugates, of UDP-glycosyltransferases has not yet been investigated. The pigments can easily be detected in enzyme samples by assaying zero-time controls (UDP-sugar also omitted). The absence of inhibitory effects will be assumed for the present discussion.

3.1.6. *Activation by Bivalent Metal Ion*

The effects of Mg^{2+} on UDP-glucuronyltransferase depend on preparations and substrates (D9, L14). Bilirubin UDP-glucuronyltransferase in untreated (F17, W12) and detergent-activated microsomal preparations from rat liver (H10, V2) and in purified fractions (A2, H2), is stimulated by Mg^{2+}. Employing purified enzyme (probably still linked to a piece of

membrane) from the hooded rat, Adlard and Lathe (A2) noted an absolute requirement for Mg^{2+} when dimethyl sulfoxide-solubilized bilirubin was used as the acceptor substrate. Mg^{2+} showed little stimulation when washed microsomal material from guinea pig liver was tested (P5).

With bilirubin UDP-glucuronyltransferase from rat liver, Mn^{2+} was more (H10), and Ca^{2+} less, stimulatory than Mg^{2+} (A2, F17, H10). The behavior was similar when either UDP-glucose or UDP-xylose was used as the glycosyl donor (F3). Enzyme activities were also stimulated by Fe^{2+} and Co^{2+} (F3, H10); Pb^{2+} activated glucuronyl transfer but was inhibitory with the other UDP-sugars. The effects of Mg^{2+}, Mn^{2+}, and Co^{2+} are in accordance with work of Lucier *et al.* (L14) on the catalysis of glucuronyl transfer to *p*-nitrophenol and 1-naphthol by Triton X-100-activated and untreated microsomal material from rat liver.

With washed microsomal preparations from the liver of guinea pig, rat, rabbit, mouse, and cat, conjugation of bilirubin also occurred at appreciable rates in the absence of added bivalent cation (P3). With digitonin-activated preparations from rat liver, glycosyl transfer rates were, respectively, 16–33%, 0–38%, and 58–78% of the values found at near-saturation of Mg^{2+} when UDP-glucuronic acid, UDP-xylose, or UDP-glucose were assayed (F3, H10). The great variability of the rates could point to an artifact.

It is not obvious whether cation-stimulated or cation-independent enzyme activities, or both, should be assayed. The apparent Mg^{2+}-independent activities cannot be explained (H10) by endogenous Mg^{2+} and Ca^{2+} ions present in microsomal material (C1, M17), particularly since identical activities were obtained in the presence of a large excess of disodium EDTA (F3, H10). Analysis of enzyme saturation with respect to Mg^{2+} supports this conclusion (F3, H10). It thus seems likely that part of the enzymes either shows no requirement for bivalent cation or contains its share in a strongly bound form. The latter hypothesis is attractive in view of the tendency of microsomal lipoprotein to bind Ca^{2+} and Mg^{2+} (C1). It is noteworthy that microsomal drug-metabolizing enzymes similarly consist of bivalent ion-dependent and -independent fractions (P1). The observation that Mg^{2+} and Mn^{2+} frequently stimulate enzymes acting on phosphorylated substrates (D5) increases the probability that assays of bivalent cation-stimulated bilirubin UDP-glycosyltransferases are physiologically significant.

In studies of species variation different conclusions may be reached when the transferases are incubated with or without activating ion. Until the significance of the cation-dependent and -independent activities is better understood, parallel assays of both may be preferable. No firm recommendations can be made.

3.1.7. *Saturation with Substrates and Bivalent Cation—Determination of Enzyme Activities*

With the exception of a recent bisubstrate kinetic analysis of bilirubin UDP-glucuronyltransferase (P5), saturation with either one of the substrates was investigated at some rather arbitrarily fixed concentration of the other substrate. The results, therefore, have to be interpreted with caution.

In kinetic studies of UDP-glucuronyltransferase with albumin-bound bilirubin as the aglycon, bilirubin:albumin ratios varied from 0.4 (F11) to 4.4 (H2) with values below 3 in 6 of 7 procedures (A2, F3, H1, H10, M5, S10, V2). At high substrate concentration, inhibition of glucuronyl transfer was evident (A2, H10, T8). When the concentration of total bilirubin was the variable parameter, Michaelis-Menten kinetics were obeyed (F11, H2, M13, V2), but this has not been found by others (A2, H10). The relatively low conjugation rates observed at low concentrations of total bilirubin by the latter authors are compatible with the hypothesis that only the unbound fraction of substrate is available to the enzyme. If so, the unbound fraction, rather than the total bilirubin concentration, should be used in analyzing enzyme saturation curves. The complicating effect of the binding equilibrium on the interpretation of the kinetic results can easily be dealt with mathematically as long as the bilirubin:albumin ratio is smaller than one.

To avoid future difficulties in comparing and interpreting kinetic results, the source of enzyme and the source and concentration of carrier protein used, and also the range of concentrations of the acceptor substrate explored, should be stated unequivocally, preferentially in direct relation to the presentation of the experimental results. In some of the studies the range of bilirubin concentrations used has neither graphically nor numerically been indicated, making it impossible to determine whether eventual adherence to Michaelis-Menten kinetics could have been due to the exploration of a limited range of concentrations that could be fitted to a rectangular hyperbola.

With UDP-glucuronic acid as the variable substrate and bilirubin at constant concentration, Michaelis-Menten kinetics were obeyed (A2, H2, H10, P5, V2, W12). Rat liver microsomal preparations treated in different ways yielded apparent K_m for UDP-glucuronic acid of 0.37–0.70 mM (A2, H2, H10, V2), with albumin-bound bilirubin as the aglycon; a higher value (1.66 mM) was found in a carrier-free system (W12). These values probably do not represent K_m for UDP-glucuronic acid at saturation with bilirubin (P5, V6). Under certain conditions of activation, cell extracts from livers of newborn and adult rats, when tested with *o*-amino-

phenol as the acceptor substance, yielded double reciprocal plots of enzyme activity against concentration of sugar donor, showing two intersecting straight lines (W9). This observation should warn against hastily concluding that adherence to Michaelis-Menten kinetics occurred; this may be the case at a limited range of concentrations, but not when a broader range is explored. Sets of intersecting straight lines were also found in double reciprocal plots of bilirubin UDP-xylosyltransferase activity against the concentration of UDP-xylose (F3). In this case the sections correlated with the formation of bilirubin monoxyloside at relatively low concentrations of the sugar donor, with the synthesis of mixtures of the mono- and dixylosides at high concentrations. As with the acceptor substrate, bilirubin, it is imperative, in work studying saturation with UDP-sugar, to indicate clearly the range of nucleotide concentrations investigated.

When appreciable cation-independent transferase activities were found (F3, H10), double reciprocal plots of enzyme activity against concentration of added Mg^{2+} were nonlinear, but became linear when only the stimulated enzyme fractions were plotted. The apparent K_m values so obtained were independent of the concentration of UDP-glucuronic acid, and vice versa (H10), suggesting that K_m for Mg^{2+} represents the dissociation constant of an Mg^{2+}–enzyme complex (D5).

In determining enzyme activities, it is usually assumed that at a fixed set of so-called saturating substrate concentrations a sufficiently accurate value of V_{max} is obtained. Bisubstrate kinetic analyses of UDP-glucuronyltransferase [assayed with bilirubin (P5) and *p*-nitrophenol (V6), respectively] indicate that a true measure of the amount of enzyme can be obtained only by suitable extrapolation procedures. This restriction applies in particular to bilirubin (A2, H10, T8) and other aglycons (M15, V6) because of substrate inhibition. UDP-glucuronic acid was inhibitory at concentrations only about 10-fold higher than the apparent K_m value (H10); this was most pronounced at relatively short incubation times. Mg^{2+} was noninhibitory at concentrations equal to 20 times the apparent K_m values (F3, H10).

Obviously, extrapolation procedures are impractical for routine determination of enzyme activities. When substrate saturation-curves conform to rectangular hyperbolas, reasonable concentrations of substrates should equal 10 to 20 times the respective K_m values. As outlined above, application of this rule to assays of bilirubin UDP-glycosyltransferase activities is hampered by substrate inhibition and by occasional deviation from Michaelis-Menten kinetics. The best alternative in such cases may be to choose the concentrations at optimal enzyme activity. However, great care should be exercised in interpreting the results. When a bio-

logical parameter is changed, e.g., pre- or postnatal development time, progressive organization of the endoplasmic reticulum may occur, accompanied by changing interaction between enzyme molecules and their lipid environment (see Section 7). Developmental variation of kinetic parameters other than amount of enzyme would remain undetected, as they would have an indistinguishable effect on the activity measurements (V6). Such changes do occur with other hepatic microsomal enzymes (G6) and are likely for UDP-glucuronyltransferase when assayed with *o*-aminophenol as the acceptor substrate (W9). The same limitation should be kept in mind when species or differences in physiological state are investigated.

For determination of total activity of tissues, it may be preferable to use tissue homogenate instead of a microsomal preparation. When only very small samples can be obtained, e.g., needle biopsy specimens, this may even be the only possible way. A brief centrifugation of homogenate to remove cell debris and nuclei facilitates manipulation and may eliminate subsequent analytical difficulties. Comparable transferase activities in homogenates and cell extracts have been found (F3, H10). Whenever feasible, and in particular in comparative work (species, tissues, etc.), assays should be performed on homogenates (or cell extracts) and on derived microsomal material. Endogenous inhibitors may be present in tissue homogenates, as was observed in work on kidney retinoic acid UDP-glucuronyltransferase (L10). Conversely, factors present in the soluble phase of the cell, e.g., NADPH (T2), may stimulate glucuronyl transfer to some acceptor substrates.

3.1.8. *Should Artificially Activated or Untreated Enzyme be Assayed?*

With bilirubin as the acceptor substance, UDP-glucuronyltransferase is activated by aging (S10, W7), by alkaline dialysis (H2), or by treatment with Triton X-100 (M16, P5, W8, W9, W10), deoxycholate (V2) or digitonin (H10, W7). Comparable maximum activities were found. Studies with xenobiotic acceptor substrates yielded the same conclusion (H13, L14, L15, V4, W7). Very rapid and maximum activation of *p*-nitrophenol UDP-glucuronyltransferase was obtained at pH 9.8–10.5 (V4). Digitonin-activation of UDP-glucosyl- and UDP-xylosyltransferase activities (both assayed with bilirubin as the acceptor substrate) has also been reported (F3).

The question may be raised whether it is preferable to assay artificially stimulated enzyme. Uncontrollable activation of enzyme samples, e.g., due to variation in homogenization and preparation procedures, or to addition of unsuspected activating agents, may occur, contributing to variation of enzyme activities of native preparations (Table 1). In con-

trast, full activation of enzyme before or during incubation increases the sensitivity of the assays and may lead to more consistent results within (H10) and among laboratories (Table 1).

According to Zakim and Vessey, the microsomal enzymes *p*-nitrophenol UDP-glucuronyltransferase (V4, V5, V6, Z2) and glucose-6-phosphatase (Z1) are constrained, in the native state, in conformations of low activity by interaction with their phospholipid surroundings. Activating treatments, such as mentioned above, could produce changes in the lipid phase causing relaxation of the constraints; maximum stimulation would reveal the full conjugation potential of the enzymes. Enhancement and relaxation of the constraints by endogenous compounds have been implied in regulation of enzyme activities *in vivo* (H5, J6, N3, V4, W6, Z1). If this theory applies to bilirubin UDP-glucuronyltransferase, assays on fully activated enzyme would measure an important biological parameter. Conversely, activities of untreated enzyme would more closely reflect the native situation (as influenced by the nutritional and hormonal state, etc.?).

As yet it cannot be excluded that the observed latency is a preparation artifact resulting, e.g., from vesiclization (E2, D1). If so, artificial activation would yield values closer to the native situation. Support for this hypothesis is found in the observation that maximum bilirubin excretion rates correlated well with the UDP-glucuronyltransferase activity of fully activated liver homogenate (H2, H10).

Until the significance of this latency is better understood, it seems justified to consider the activities of both activated and untreated enzyme as potentially useful parameters. In doing so, quite different conclusions were reached regarding the effects of age and sex (W9) and of *in vivo* treatment with phenobarbital (W10).

3.2. Design of Incubation Controls

Most authors use a mixture of the same composition as the test mixture for the incubation control, except for omission of UDP-sugar. As outlined above, complete suppression of endogenous synthesis is preferred by the present authors for determination of enzyme activities.

Some procedures allow the relative importance of endogenous conjugate formation to be decreased. Omission of UDP-sugar is an obvious requirement. Replacement of activating ion by disodium EDTA (B17, H10) is indicated in case: (a) some fraction of the transferase depends on bivalent cation for its activity, and (b) appreciable amounts of UDP-sugars are present in the enzyme samples. In determining the enzyme activities it may be advantageous to allow maximum prolongation of the incubation time, taking care to remain within the straight portion

of the velocity–time curve. Aging of homegenates at 0°C, or fasting of the experimental animals, can be investigated as potentially useful procedures to decrease the amounts of endogenous sugar donors. Digitonin may inhibit endogenous conjugation of bilirubin in tissues other than rat liver (H10). In stubborn cases incubation of the control mixture at 0°C will probably be effective. The effectiveness of the procedure adopted, and its compatibility with the experimental aims and with the analytical determination of bilirubin conjugates, should be assessed in each particular case.

Admitting that endogenous synthesis in the control mixtures is negligible or can be adequately suppressed, the difference in response of test and control samples toward the final detection system (Section 4) should be such that it is exclusively due to the bile pigment conjugate synthesized in the test incubation mixture. The latter requirement depends, to some extent, on the final assay procedure adopted (Section 4).

4. Procedures for Determination of Conjugated Bilirubin Applicable to the Assay of Bilirubin UDP-Glycosyltransferase

In general, during incubation of bilirubin and UDP-glucuronic acid with an appropriate source of enzyme, only relatively small fractions of the aglycon are conjugated with sugar. Preferentially, assays should be based on the quantitation of the reaction product(s). This has proved rather difficult, as evidenced by the variety of assay procedures that have evolved (Table 2). Recently, some of the methods have been applied to assaying UDP-glucosyltransferase (F3, W13) and UDP-xylosyltransferase (F3).

Owing to lack of studies with pure reference compounds, published procedures can, at best, be incompletely evaluated, and frequently only in an indirect way. Pure preparations of mono- and diglucuronosides of bilirubin have only recently become available (J1, O3). Normal rat bile predominantly contains the latter pigments and is more suitable as a source of reference material than the more complex biological fluids from patients with conjugated hyperbilirubinemia (F6, H11). For the present, procedures for determining conjugates other than glucuronides can in some cases be evaluated indirectly with complex bile pigment preparations free of phospholipids and other contaminants (L12, O3), or, when this is economically possible, with synthetic bile pigment.

Most assays are based on the transformation of bilirubin conjugates into azo derivatives (Section 4.3). In evaluating such methods, chromatographic analysis of the azo derivatives is a useful supplement to the study of color yields. A review on the diazo-coupling of bile pigments in various biological media, and on the separation and structure elucidation of the

derived azo pigments will be published elsewhere (H12). Attempts to determine conjugated bile pigment without the intervention of diazo-coupling should be encouraged. However, methods available at present (F15, M5) are unsatisfactory (Sections 4.1 and 4.2). In all procedures, a single control incubation mixture, treated in an appropriate way (Section 3.2), allows the blank value to be obtained; with the exception of the radioactive assay of Wong (W12), correction for endogenous bile pigment is necessary.

In discussing the basic approaches used to assay bilirubin UDP-glucuronyltransferase activity, potential extension to the UDP-glucosyl- and UDP-xylosyltransferases will be outlined. Determination of the rates of synthesis of diconjugates and of nonglycosidic conjugates will be dealt with in Section 6.

4.1. Direct Spectrophotometry

Spectral differences between conjugated and unconjugated bilirubin have been exploited to determine bile pigments in albumin-containing fluid (F12). In its present state this method cannot be recommended. The preparation of conjugated bilirubin used to establish the reference spectrum was assumed to consist solely of bilirubin diglucuronoside. However, depending on the species examined and on its physiological state (F6, H11, K3), bile pigments show considerable heterogeneity with respect to conjugating groups. Further, mono- and diconjugated bilirubins occur in biological fluids (F5, H11, O3, S10, V2).

Even if differences in the conjugating groups are assumed to have negligible influence on the spectra, it remains very likely that the spectra of mono- and diconjugated bilirubin are significantly different. In reassessing the assay system (F12), at least terms for unconjugated and for mono- and diconjugated bilirubin should be included. An experimental reevaluation of the method has indicated considerable overestimation of bilirubin and interference of hemoglobin (B20). The former observation could indicate that the unconjugated dipyrrole moieties of monoconjugated bilirubin show, to a first approximation, the same spectrum as the dipyrrole moieties of bilirubin itself. If so, the method of Fog (F12) would estimate the number of conjugated dipyrrole moieties, and thus, by calculation, GTR.

4.2. Direct Separation of Bile Pigments

Quantitation of synthetic, conjugated bilirubin, after its direct separation from the incubation mixtures, offers the most valid approach to the determination of conjugation rates. However, for the present, such methods are unlikely to find wide application because of instability of

the conjugated pigments and difficulty in obtaining suitable reference preparations.

In an attempt to develop a sensitive micromethod, Menken *et al.* (M5) separated synthetic bilirubin glucuronoside directly. Using [^{14}C]bilirubin as the acceptor substrate and UDP-glucuronic acid as the sugar donor, they separated conjugated bilirubin from the incubation mixtures by partition in a solvent system composed of water, ethyl acetate, lactic acid, and chloroform. Pigment recovered in the aqueous phase was counted. Pure bilirubin added to this solvent system is recovered largely in the chloroform-rich phase; however, the distribution coefficients of the mono- and diglucuronosides are not known. In an acid medium with the carboxylic acid groups probably largely undissociated, the solubilities of the conjugates in the organic phase may be appreciable.

The suitability of the extraction conditions cannot be validly assessed by investigating the distribution of derived azo pigments, as attempted by the authors mentioned (M5). Diazotized sulfanilic acid was employed. The strongly acidic, sulfonic acid group will presumably be largely in the ionized form in a lactic acid-containing solvent system. This should promote transfer of the azo pigments into the aqueous phase, creating the impression that the system is more suitable for extracting bilirubin conjugates than it really is. The only acceptable evaluation consists in investigating the distribution coefficients of pure reference material. Diazo-coupling, if applied at some stage, should be preceded by direct separation of the fluid phases.

4.3. Diazo-Coupling of Enzymatic Reaction Products

A variety of methods employ diazo-coupling of the synthetic conjugates (Table 2). To derive the maximum information from the assays, it is essential to have some knowledge about the mechanism of reaction. Diazo-coupling of conjugated and unconjugated bilirubin involves scission at the central methylene bridge (B21, C6, C7, F8, J3, K3, L12, O6, T9). If the reaction goes to completion, two dipyrrolic azo derivatives are obtained from each bile pigment molecule provided no selective destruction of azo pigments occurs (Fig. 1.). Obviously, equal amounts of azodipyrrole and of monoconjugated azodipyrrole are then derived from synthetic monoconjugated bilirubin, diconjugated bilirubin yielding only conjugated azo pigment(s).

4.3.1. *Nonradioactive Methods*

The available procedures all rely on photometric determination of azo pigment color.

4.3.1.1. *Methods Not Involving Separation of Azo Pigments.* The basic

procedure is simple. Conjugated bilirubin is converted into colored azo derivatives and is determined photometrically. Most frequently the diazonium salt of sulfanilic acid or ethyl anthranilate is employed. Reaction is allowed to proceed in aqueous acid medium to suppress reaction of excess bilirubin substrate as far as possible.

4.3.1.1.1. *Blank values—reaction conditions.* Blank values may be due to (a) endogenous conjugate formation, (b) endogenous bile pigments, (c) partial reaction of excess bilirubin substrate, (d) diazo-coupling of nonbilirubin compounds, (e) colored, nondiazopositive substances, absorbing measurably at the wavelength of assay, or (f) turbidity.

To obtain blank values it is not sufficient to treat a duplicate incubation mixture with so-called diazo-blank reagent ($NaNO_2$ omitted from the diazo reagent), as is common practice in assaying serum or bile (H12). Contributions (b)–(d) would be obtained with the test, not with the control mixture. A control incubation mixture, treated with diazo reagent in parallel with the text mixture, will adequately measure contributions (b)–(f). Whether endogenous conjugate formation in the incubation control (contribution a) is suppressed completely or not, must depend on the preference of the reader (Sections 3.1.4 and 3.2).

Completeness or near-completeness of coupling of conjugated bilirubin is a basic requirement for obtaining maximum useful information. Partial reaction can eventually be tolerated (e.g., for semiquantitative localization of enzyme activity in an effluent of a separation system) but cannot be recommended. The degree of advancement of the reaction depends on a number of factors which may exert either accelerating or decelerating effects (L3, W11). Some factors are probably still unknown and might vary from one enzyme sample to another, e.g., detergents or water-miscible organic solvents, introduced into the incubation mixtures to activate enzyme or to solubilize bilirubin, are likely to accelerate diazo-coupling.

Determination of the so-called 1-minute direct-reacting bilirubin (D6) probably leads to pronounced underestimation of conjugated bile pigment (F4). The extent of coupling is expected to depend less critically on the composition of the reaction mixtures and on the exactness of timing when longer reaction periods are allowed. Potentially useful information about the synthesis of mono- and/or diconjugates, obtainable by further chromatographic analysis of azo derivatives (Section 5), is likely to be lost with incomplete coupling of conjugates.

The only valid alternative is to choose the diazo reagent and the reaction conditions in such a way that diazo-coupling is reasonably complete. However, promotion of coupling, e.g., by increasing the concentration of

diazonium salt or the duration or temperature of the reaction, will also stimulate diazo-coupling of the relatively large excess of bilirubin remaining after enzymatic incubation. This will result in an undue increase of azo color obtained from the control incubation mixture. In some procedures turbidity is important.

In contrast to the effects of reaction parameters enumerated above, changes in pH allow diazo-coupling to be influenced in a more specific way (L3, M10, N4, V2, W11). The establishment of color formation–pH curves with samples of bilirubin and of conjugated bilirubin, is therefore a useful preliminary to the adoption of any method for the determination of bilirubin conjugates. The assay mixtures should simulate the actual enzymatic incubation mixtures as closely as possible. Incubation mixtures (UDP-sugar omitted) provided with pure bilirubin conjugate are to be preferred. At present purified preparations of bilirubin conjugates containing, glucose, xylose, or other conjugating groups are not available. In such cases conjugates can be synthesized *in vitro*. Next, adequate portions of the incubation mixture are subjected to diazo-coupling, varying the reaction conditions as required. The pH-dependence of the assay can be established, e.g., by addition of buffers. A less satisfactory alternative, although of value in many cases, consists of using a mixture of conjugated bilirubins that is free of contaminants (L12, O3).

Diazotized ethyl anthranilate may be used to illustrate the prime importance of choosing the optimal pH for diazo-coupling. With albumin-solubilized bilirubin, coupling was negligible from pH 2 to 2.8, whereas it increased abruptly above about pH 4 (V2). In contrast, with purified conjugated bilirubin (V2) and with bile samples from man and rats (H11), diazo-coupling was complete from pH 2.4 to 3.1. Maximum color yields from conjugated bilirubin, with minimal diazo-coupling of excess substrate, should, therefore, be found at pH about 2.6.

As a simple test for the completeness of coupling, identical incubation mixtures (containing synthetic conjugated bilirubin or provided with purified material) can be subjected to diazo-coupling for various periods at two to three temperatures. The curves should have identical and stable maximum color levels. Making allowance for a reasonable safety margin, the finally adopted reaction time should not be extended unnecessarily to avoid undue coupling of excess substrate. With diazotized ethyl anthranilate identical color levels were reached at 25° and 37°C (B17). Occurrence of an optimum in the color formation–time curves may indicate destruction of azo pigment by excess diazonium salt (L12). With diazotized *p*-iodoaniline such behavior was observed at 37°C; however, at 0°C and at 25°C identical and stable maximum color levels were obtained (V3).

If (a) conjugate formation in the control incubation mixture is unimportant or can be suppressed (Section 3.2), (b) blank contributions are adequately determined, and (c) diazo-coupling of synthetic conjugates is reasonably complete, the difference in extinction of test and control (usually measured at 530–550 nm) allows the amount of pigment synthesized, and thus the value of BCR, to be obtained.

4.3.1.1.2. *Assay systems.* In two-phase systems (H2, O1, P3, P5, S10), based on the diazo method of Weber and Schalm (W1), parasitic reaction of excess bilirubin is avoided by transferring the unconjugated pigment into a chloroform-rich phase before the addition of diazotized sulfanilic acid. After allowing sufficient time for the coupling of the bilirubin conjugates to proceed, one can determine the azo color of the aqueous phase by optical reading. Occasional turbidity can be removed by centrifugation after mixing the aqueous phase with chloroform (H2). A full description of the method has been given by Strebel and Odell (S10); a micro version has been developed (O1).

The other procedures utilize reaction of conjugated bilirubin in a homogeneous aqueous medium with the diazonium salt of either sulfanilic acid (F17, G9, L4) or ethyl anthranilate (B17, H10, V2). The method of Grodsky and Carbone (G9), which measures the 1-minute direct-reacting bilirubin (D6), shows turbidity in the final samples, but see (S8). Diminishing turbidity by diazo-coupling of the supernatant, obtained after centrifuging the enzymatic incubation mixtures at 22,000*g* for 20 minutes (W2), entails the risk of loosing pigment entrapped in sedimented cell organelles. Reaction of conjugated bilirubin is probably incomplete (F4).

A 5-minute reaction period and protein precipitation by addition of ethanol before optical reading have been applied by Lathe and Walker (L4). According to Lathe and Ruthven (L3) the allotted reaction time is adequate. The same method has been used to assay conjugated bilirubin excreted by liver slices (A1, A3, B12, H8, L4). Conjugated pigment remaining in the slices can be determined by the method of Hargreaves (H6). In a micro version of the 10-minute diazo method of Michaëlsson (M10), turbidity was decreased by adding detergent to the final assay mixtures (F17).

Recently, diazotized ethyl anthranilate has been introduced as a reagent for conjugated bilirubin (V2). Selection of the reagent was based on a comparative study which suggested that the discriminatory power of diazo reagents may be improved by the presence of a bulky activating group *in ortho* to the diazo group (V2). The effect may be due to different conformational states of bilirubin and of conjugated bilirubin (F14). Some properties of the ethyl anthranilate reagent have already

been discussed. The organic-soluble azo derivatives are easily extracted into pentan-2-one (H10), yielding water-clear organic phases. The relatively low and reproducible blanks allow the sensitivity of assays of conjugated bilirubin to be increased by decreasing the extraction volume: sample volume ratio and by optical reading in microcuvettes of increased optical path-length. Micromodifications of the method (B17, H1) and assays of UDP-glucosyl- and UDP-xylosyltransferase activities (F3) have been developed. Without further processing, the organic extracts can be applied to thin-layer plates for further analysis (Sections 5 and 6).

4.3.1.2. *Methods Involving Isolation of Unlabeled Conjugated Azo Pigment.* In principle, the methods consist of diazo-coupling of conjugated bilirubin, followed by extraction of the azo derivatives and quantitation of chromatographically isolated conjugated azo pigment (B28, W12) (Table 2). More references about chromatographic systems are given below (Section 5). Obviously, only values of GTR can be obtained.

Completeness of reaction of conjugated bilirubin is of key importance. Promotion of coupling is permissible, e.g., by prolonging the treatment with diazo reagent or by addition of a reaction accelerator (ethanol, etc.). The increased amounts of azodipyrrole derived from excess bilirubin are of no concern, as they will be removed from the conjugated azo pigment in the separation stage. Rapid and quantitative thin-layer separation of the azo pigments is possible (F5, F7, H10, T3, W12, W13). The solvent systems are sufficiently flexible to allow removal of most other contaminating pigments. Determination of azodipyrrole conjugates other than azodipyrrole glucuronoside (W12), and thus of corresponding values of GTR, should be easy (W13).

Correction for endogenous bile pigment, yielding conjugated azodipyrrole with the same R_f value as the azo derivative obtained from the enzymatic incubation mixture, should be applied. Test and control incubation mixtures are treated with diazo reagent. Extracts obtained from equal volumes of both mixtures are separated in parallel on the same paper strip or thin-layer plate. From both tracks equal chromatographic areas, corresponding to the R_f value of the azopigment of interest, are eluted and read photometrically.

In essence, simple quantitation of conjugated azodipyrrole renders any conclusion with regard to the extent of formation of mono- and diconjugates impossible. A conclusion (W12) pertinent to the question is untenable and may have been caused by confusing use of terminology.

4.3.2. *Methods Assaying Labeled Conjugated Azo Derivatives*

Radioactive conjugated azo pigment, derived from conjugated bilirubin, is counted, after separation from other labeled compounds (Table

2). In discussing the procedures (F11, M7, W12) it will be assumed that removal of contaminants was complete, as the present authors have no personal experience with these methods. In essence, they can only yield values of GTR.

In the procedure of Wong (W12) bilirubin is incubated enzymatically with [U-^{14}C]UDP-glucuronic acid of known specific activity. The derived azo pigments are transferred quantitatively to a thin-layer plate and are separated. The spot of conjugated azo pigment is eluted and counted. With other radioactive UDP-sugars, extension of the procedure to the corresponding transfer processes is obvious.

In the method of Metge *et al.* (M7) a known amount of bilirubin is incubated enzymatically with UDP-glucuronic acid. The bile pigments (conjugated and unconjugated) are subjected to diazo-coupling with diazotized [^{35}S]sulfanilic acid in the presence of ethanol. The azo derivatives are extracted after the addition of cold conjugated and unconjugated azodipyrrole and are separated by paper chromatography. After determination of the ratio (A) of radioactivity found in the conjugated azo pigment spot to that present in both conjugated and unconjugated azo pigments, results (D) are given by

$$D = m_B{}^T \times A \tag{1}$$

where $m_B{}^T$ is the number of moles of bilirubin present in the incubation mixture at zero time. By definition the ratio (A) is given by

$$A = m_{ca}/(m_{ca} + m_{ua}) \tag{2}$$

with m_{ca} and m_{ua} the numbers of moles of conjugated (ca) and unconjugated (ua) azo pigments, respectively, obtained by diazo-coupling of the enzymatic incubation mixture. The significance of the D values depends on whether mono- or diconjugated bilirubin (both tetrapyrroles) were synthesized.

In case of monoconjugate (BX) formation, the following relations are assumed to hold at any time

$$m_B{}^T = m_B + m_{BX} \tag{3}$$

$$m_{ua} = 2m_B + m_{BX} \tag{4}$$

$$m_{ca} = m_{BX} \tag{5}$$

where m_B and m_{BX} are the numbers of moles of bilirubin and monoconjugated bilirubin, respectively, present in the enzymatic incubation mixture. Substitution of Eqs. (2)–(5) into Eq. (1) indicates that D equals 0.5 m_{BX} The experimental results correspond to half the number of moles of monoconjugated bilirubin synthesized.

In case of diconjugate (BX_2) formation, one has at any time

$$m_B{}^T = m_B + m_{BX_2} \quad (6)$$

$$m_{ua} = 2m_B \quad (7)$$

$$m_{ca} = 2m_{BX_2} \quad (8)$$

with m_{BX_2} the number of moles of diconjugated bilirubin synthesized. Substitution of Eqs. (2) and (6)–(8) into Eq. (1) indicates that D now equals m_{BX_2}. As, in essence, the method cannot give any information about the extents of mono- and diconjugate formation, the experimental results (D values) are uncertain by a factor 0.5–1. However, the significance of the product $2 \cdot D$ is unequivocal. In both cases GTR (in moles of sugar transferred/unit of time) is given by

$$\text{GTR} = 2D = 2m_B{}^T A/\text{incubation time} \quad (9)$$

The method requires careful determination of the amount of bilirubin ($m_B{}^T$) added to the incubation mixtures. The assumptions implicit in Eqs. (3) and (6) are probably closely verified, as albumin-solubilized bilirubin is stable in the dark at 37°C in air (F3), under conditions of enzymatic incubation as used by Metge *et al.* (M7). Partial hydrolysis of conjugated bilirubin (a somewhat unlikely possibility) would not invalidate the above conclusions. Equations (4), (5), (7), and (8) presuppose (a) completeness of diazo-coupling of conjugated and unconjugated bilirubin, and (b) absence of selective destruction of azo derivatives (Section 4.3.1.1.1). Eventual extension of the method to glucosyl and xylosyl transferring enzymes will require the preparation of the corresponding azodipyrrole conjugates (a) to serve as carrier pigments, and (b) to allow evaluation of completeness of extraction and separation from interfering labeled compounds. Dog bile is an adequate source of these conjugates (F7).

In comparative experiments on the same enzyme samples (sugar donor UDP-glucuronic acid) satisfactory agreement was found between D values (obtained as indicated above) and conjugation rates estimated by a 1-minute direct-reacting diazo method (M7). This can signify either that exclusive diconjugate formation was obtained or that, in the case of monoconjugate formation, diazo-coupling of the conjugates is only about half complete during the 1-minute period. The latter explanation is likely. With icteric sera partial diazo-coupling was demonstrated (F4). Further, with incubation mixtures of similar compositions to that used by Metge *et al.* (M7) exclusive synthesis of the monoglucuronoside (H10, V2), or only partial conversion into the diglucuronoside, was found (H4, S10).

The radioactive method used in enzymatic studies by Flodgaard and Brodersen (F11) is based on work of Brodersen (B22) and Brodersen and Jacobsen (B24). Samples of a solution of bilirubin diglucuronoside

(at known concentration C_0) and of the incubation mixture (at unknown concentration C) are treated in parallel with diazotized [^{35}S]sulfanilic acid. Excess diazonium salt is destroyed with sodium azide. Duplicate samples of both solutions are identically treated with nonradioactive reagent). The radioactive solution C_0 is combined with nonradioactive solution C, and vice versa. By a number of extraction steps, pure preparations of conjugated azodipyrrole are obtained containing radioactivity derived from the standard and from the unknown, respectively. The conjugated azo pigments are hydrolyzed and led through another series of purification steps. The two final azodipyrrole solutions are counted, and their extinctions are determined. The specific activities A_0 and A are obtained for reference and unknown solutions, respectively.

Obviously, in case of formation of diconjugate, its molar concentration C_{BX_2} is given by

$$C_{BX_2} = C_0 (A/A_0) \tag{10}$$

where C_0 is the molar concentration of bilirubin diglucuronoside in the standard solution. In case of monoconjugate formation (BX), the molar concentration C_{BX} is equal to

$$C_{BX} = 2C_0 (A/A_0) \tag{11}$$

An approximate form of Eq. (10) has been used by the authors mentioned (B22, B24, F11). By the very nature of the procedure it cannot be known whether Eq. (10) or (11), or some combination of both, should be applied. However, in both cases values of GTR (in moles of glycosyl residues transferred per unit of time) are given by

$$\text{GTR} = 2C_0 (A/A_0)\ V/\text{incubation time} \tag{12}$$

where V is the volume (in liters) of the incubation mixture.

4.4. Concluding Remarks

Most diazo methods (Table 2), with the exception of a 1-minute diazo procedure, are acceptable. For a number of reasons (Section 3) firm recommendations regarding the conditions of enzymatic incubation could not be made. Therefore, it is gratifying that, to a large extent, the choice of the analytical assay system is independent of previous enzymatic incubation.

Methods involving quantitation of conjugated azopigment directly measure the number of groups transferred and thus, by calculation, GTR. Expression of the results in function of bilirubin equivalents conjugated is equivocal.

5. Analysis of Enzymatic Reaction Products

To our knowledge only two attempts have been made to characterize conjugates, synthesized *in vitro,* by direct separation (R9, S6). The derived dipyrrolic azo pigments are far more stable than the parent bile pigments. For the time being, azo pigment analysis seems to offer the only practical tool for analyzing the structures and relative amounts of bile pigments synthesized.

Analysis of sulfanilic acid azo derivatives by chromatography on paper (H4, S4, S6) and on thin-layer plates (S10, W12, W13) and of ethyl anthranilate azo derivatives on thin-layer plates (F5, F7, H11) has been applied most frequently. Analysis of ethyl anthranilate azo pigments instead of the more hydrophilic derivatives obtained with diazotized sulfanilic acid, allows easier differentiation according to differences in the hydrophilic character of conjugating groups (F7, H11). In enzymatic assays using diazotized ethyl anthranilate (F3, H10, V2) the solutions used for optical reading can be applied without further processing to the thin-layer plates. Considerably more labor is involved in preparing sulfanilic acid azo pigment for chromatography (H4, S10). Structurally characterized ethyl anthranilate azodipyrrole conjugates of glucuronic acid, glucose, and xylose are available (C6, C7). 2,4-Dichloroaniline (R2) has probably the same advantages (R9) as ethyl anthranilate. Sulfanilic acid azo derivatives may be more suitable for testing hydrolysis of conjugated azo pigments with enzymes. A review of methods applicable to structure elucidation of diazo-positive bile pigments will be given elsewhere (H12).

Some useful applications follow:

a. Conjugated azo pigments are easily separated from unconjugated azodipyrrole. Their formation from control incubation mixtures (UDP-sugar omitted), in amounts greater than those present at zero time, demonstrates unequivocally that endogenous synthesis occurs (Section 3.2).

b. Occasional checks on the nature of the conjugating group attached to the aglycon are easy. The use of reference products of known structure will indicate whether the conjugated azodipyrrole obtained moves with the expected R_f value. Correspondence of R_f values is a necessary, though insufficient, proof of identity; isomerization of sugar when still attached to uridine diphosphate is possible and might, if glycosyl transfer occurs, lead to azodipyrrole derivatives with very nearly the same R_f values as the expected product. In any case, the test will reveal gross structural changes.

c. The relative amounts of mono- and diconjugated bilirubin synthe-

sized *in vitro* can be calculated from the ratio R, azodipyrrole:azodipyrrole conjugate. As far as published procedures go, only those involving diazo-coupling and optical reading of total conjugated bile pigment (Section 4.3.1.1) yield suitable starting material. The diazonium salts of sulfanilic acid (H4, S10) and of ethyl anthranilate (F5, J5, V2) have been used. After chromatographic separation of the azo derivatives, the ratio R is determined by either densitometry (F7) or optical reading of eluted azo pigment spots (F5, H4, J5, S10). From R one calculates the percentages of monoconjugates $[200 \cdot R/(1 + R)]$ and of diconjugates $[100 \cdot (1 - R)/(1 + R)]$.

Corrections should be applied: (a) for azodipyrrole derived from coupling of excess substrate, and (b) for foreign compounds eventually present in the control incubation mixture. Azo pigment solutions derived from equal volumes of test and control incubation mixtures are separated, in parallel, on the same thin-layer plate or paper strip. For each azo pigment spot on the test track a corresponding area on the control track is evaluated. In general, the most important correction term corresponds to the azodipyrrole spot. It can be kept to a minimum by careful control of the conditions of diazo-coupling (Section 4.3.1.1). Its importance will decrease on prolonging the enzymatic incubation.

6. Assays of Related Transferring Enzymes

In vitro enzymatic synthesis of sulfate (I2, N2, T3), phosphate (T3), and oligosaccharidic conjugates (K3) detected in bile has not yet been reported. If appropriate incubation systems are worked out with bilirubin as the acceptor substrate, there should be no major difficulty in obtaining values of BCR by nonradioactive diazo methods not involving separation of azo pigments. The analytical validity of the procedure adopted can be tested as outlined in a previous section (Section 4.3.1.1.1). For determination of GTR the assay principles of Wong (W12) and of Metge *et al.* (M7) may be applied, provided adequate techniques for chromatographic separation are found. Very probably the TLC systems referred to in Section 5 would be suitable with minimal adaptation.

An analytical route to the study of glycosyl transference to mono- and diconjugated bilirubin consists of quantitative analysis with TLC of test and control incubation mixtures. In this way conversion of bilirubin monoglucuronoside into the diglucuronoside has been investigated by Jansen (J5), using ethyl anthranilate azo pigments.

7. Investigation of Metabolic Pathways

Considering the large variety of glycosidic conjugates of bilirubin demonstrated in normal bile of man (F6, H10), dog (F7), and rat (F7,

H10, V1), it seems likely that a considerable number of pathways are involved in the metabolism of the aglycon. The predominant sugar moieties were, in the order of quantitative importance, D-glucuronic acid, D-glucose, and D-xylose. Unpublished work from this laboratory demonstrates that the following classes of conjugates can be recognized in bile of normal dogs: (1) various bilirubin β-D-monoglycosides, (2) bilirubin β-D-diglycosides containing identical sugar moieties, and (3) mixed conjugates (e.g., with D-glucuronic acid and D-xylose attached to different carboxylic acid groups of the same bilirubin molecule) (Fig. 1). Synthesis *in vitro* and *in vivo* of homogeneous and mixed diconjugates of steroids containing various combinations of sulfate, D-glucuronic acid, D-glucose, and *N*-acetylglucosamine has recently been reviewed by Layne (L5).

The few enzyme activities, relevant to bilirubin conjugation, that have been studied experimentally can be called bilirubin UDP-glycosyltransferases. In broken cell systems, conjugation of bilirubin does not occur with free sugars (F3) but requires UDP-glucuronic acid (A8, G9, L4, S4), UDP-glucose (F3, W13), or UDP-xylose (F3). ADP-, CDP-, and GDP-glucose are inactive as glucosyl donors (W13). Similar specificity toward UDP-glucose has been noted in transfer reactions involving ceramide (B5), 17α-estradiol or 17α-estradiol 3-glucuronoside (C5), as the acceptor substrates. In the case of glucuronyl and xylosyl transfer, little is known of transferase specificity toward nucleotide; the few relevant data concerning glucuronyl transfer are discussed by Dutton (D8, D9).

Glucuronic acid-, glucose-, and xylose-conjugates of bilirubin isolated from bile (F7, H10), and corresponding conjugates synthesized *in vitro* (F5), are glycosidic structures with the β-D-configuration at C-1 (Fig. 1). The sugar moieties are attached in ester linkage to the carboxylic acid groups of bilirubin (F5). Glucuronyl transfer to either of these groups has been suggested in the case of monoconjugate synthesis (J3). UDP-sugars that occur naturally or that are used in enzymatic incubation mixtures have an α-link; therefore, conjugation of bilirubin must be accompanied by inversion at C-1. This follows the well established pattern of glucuronoconjugation of other aglycons, observed with animal tissues *in vitro* and *in vivo* (A13, D8, M1). Similarly, biosynthetic steroid conjugates containing glucose, galactose, or *N*-acetylglucosamine are in the β-D-configuration (L5). A contradictory report (A5) has been refuted (M3).

As yet only a limited number of bilirubin conjugates can be synthesized *in vitro*. Mono- and diglucuronosides of bilirubin predominate in rat bile (F6, H11, O3, S6). In accordance with these observations, isolated liver preparations and liver slices from rat (S6), and rat hepatoma cells (R9)

are each capable of transforming bilirubin into the diglucuronoside. However, with homogenates and digitonin-activated and untreated microsomal material from rat liver, bilirubin β-D-monoglucuronoside was obtained (B17, F5, V2). The enzymatic incubation mixtures contained bilirubin solubilized with human serum albumin. Conjugation could not be brought beyond the monoconjugate stage by prolonging the incubation time or by the addition of UDP-*N*-acetylglucosamine or large excesses of UDP-glucuronic acid to the incubation mixtures (F5). In contrast, mixtures of the mono- and diconjugates were obtained in similar systems with microsomal preparations activated by alkaline dialysis (H4) or by adding UDP-*N*-acetylglucosamine (S10). Inspection of the relevant publications does not suggest any preponderant effects of differences in homogenization media, enzyme activation procedures, or strains of rat employed. Synthesis *in vitro* of bilirubin β-D-monoglucoside and of mixtures of the mono- and dixylosides has been demonstrated (F5).

From *in vitro* studies the synthesis sequence bilirubin $\xrightarrow{\text{step 1}}$ bilirubin monoconjugate $\xrightarrow{\text{step 2}}$ bilirubin diconjugate is likely. Diconjugate formation with UDP-xylose increased with increasing concentrations of sugar donor, decreasing concentrations of bilirubin and prolonged enzymatic incubation times (F5). With microsomal preparations from cat livers that had been incubated in albumin-free media, the diglucuronoside could be synthesized either from bilirubin or from the monoconjugate (F5). The latter pigment accumulated in the presence of added bovine albumin. Selective inhibition of the conversion of mono- to diglucuronoside by *p*-nitrophenol (J5) and exclusive formation of the monoconjugate by smooth endoplasmic reticulum from rat liver (H4), favor participation of separate enzymatically active sites in conjugation steps 1 and 2. The widely different water-solubilities of bilirubin and its monoglucuronoside also render a single-site hypothesis unlikely. It is noteworthy that transfer of *N*-acetylglucosamine to some 3-hydroxy steroids by UDP-*N*-acetylglucosaminyltransferase from rabbit tissues requires prior conjugation of the 3-hydroxy group with glucuronic acid (C4, C5) or sulfate (C3).

The properties of bilirubin UDP-glucosyl- and bilirubin UDP-xylosyltransferase from rat liver closely parallel each other with regard to activation by digitonin and dependence on pH and bivalent metal ions. Considerable fractions of the enzyme activities (especially of the glucosyltransferase) functioned independently of added bivalent cation (F3). The observations are compatible with identical enzyme locations, at least for the metal ion-stimulated activities.

Bilirubin UDP-glucuronyltransferase, in contrast to the xylosyltransferase, is markedly stabilized by human serum albumin against inactivation, and shows a different activity–pH curve (F3, H10). The effect of albumin on the stability of the glucosyltransferase was not determined. With microsomal material from rabbit liver, different substrate profiles were found with UDP-glucuronyl- and UDP-glucosyltransferase (L1), and the ratio of both activities (determined with bilirubin as the acceptor substrate) showed pronounced species variation (W14). The available evidence thus favors different locations of the UDP-glucosyl- or UDP-xylosyl-, and the UDP-glucuronyltransferases. However, liver homogenates from Gunn rats, characterized by hereditary deficiency of bilirubin UDP-glucuronyltransferase (S5) also lack the ability to conjugate bilirubin with glucose or xylose (F3). This would rather support identical enzymatic locations. Alternatively, some link between different locations, or a common step in polyenzyme systems, could be defective.

Kinetic investigation of glucuronyl transfer has scarcely begun. With bilirubin (P5) or *p*-nitrophenol (V6) as the acceptor substance, with microsomal material as the source of enzyme, in systems not provided with bivalent cation, bisubstrate kinetic analyses support a sequential, rather than a Ping-Pong, enzymatic mechanism. With *p*-nitrophenol, the easier substrate to work with, product inhibition and isotope exchange studies were compatible with a "rapid equilibrium, random order" catalytic process (V6). The analysis provides a test of the identity or nonidentity of UDP-glucuronyltransferase, which is still a crucial problem. If two aglycons are glucuronidated according to the proposed mechanism (V6), K_m for UDP-glucuronic acid should be identical when determined with either aglycon. With bilirubin as the aglycon a "Bi Bi sequential ordered catalytic process" was considered more likely than any other conceivable mechanism (P5). Inhibition studies with bilirubin β-D-monoglucuronoside have still to be done. The authors (P5) determined "bilirubin conjugation rates" (BCR); the extent of mono- vs diconjugate formation was not assessed. However, if conjugation of bilirubin involves the monoglucuronoside as an obligatory intermediate, values of BCR represent, apart from sign, the bilirubin disappearance rate, and thus equal the rate of monoconjugate formation. According to work of Jansen (J5) the total conjugation rate (BCR) is not inhibited by *p*-nitrophenol, but the conversion of mono- into diglucuronoside is. More work is needed to clarify the significance of these kinetic studies.

A close dependence of the activity of UDP-glucuronyltransferase (A12, G3), glucose-6-phosphatase and enzymes related to microsomal electron transport to NADPH (W6), on the structural integrity of the

membranal phospholipid environment, follows from lipid-depletion studies (A12, G3, W6). This conclusion is also supported by the characteristics of artificial activation of microsomal enzymes. With UDP-glucuronyltransferase (see Section 3.1.7 for references) and other enzymes (R3, R4), blending, or sonication, or a variety of chemical treatments (treatment with deoxycholate, digitonin, synthetic detergents, alkaline solution, phospholipases, etc.) produce comparable biphasic effects on the enzyme activities, an optimum being found at some intermediate value of the parameter investigated, e.g., duration of sonication or concentration of detergent. The striking similarity between the activation–pH curves of UDP-glucuronyltransferase and glucose-6-phosphatase (V4) may be cited as an example. The nonspecificity of the response with regard to treatments and enzymes, points to sites of action situated outside the protein moieties of the enzyme molecules, i.e., in the surrounding phospholipid phase. The implication that the expression of enzyme activity (in the cases mentioned) results from interaction with the phospholipid environment has been analyzed in some detail by Zakim and Vessey (V4, V5, V6, Z1, Z2) (see also, Section 3.1.7).

In assays of UDP-glucuronyltransferase with albumin-solubilized bilirubin deviation of substrate-saturation curves from rectangular hyperbolas (A2, H10) can conceivably be explained by postulating that only the unbound bilirubin fraction is active in the transfer process. It is evident that in the *in vitro* assays the binding equilibrium has to be taken into account, even when bilirubin would migrate to the enzyme in protein-bound form and would be transferred to the enzyme only at the moment the aglycon–albumin complex makes contact with it. It is worth mention that, in enzymatic oxidation, unbound bilirubin is the active species (B2). Binding equilibria probably also affect the interpretation of kinetic studies with enzymes acting on other strongly hydrophobic substances, e.g., steroids. Owing to the presence in liver and other tissues of cytosol proteins with high affinities for endogenous hydrophobic compounds (K2, L11, M12), studies of the influence of protein binding of substrates, on their enzymatic transformation are of potential interest for relating observations made *in vitro* and *in vivo*. Comparable rates of glucuronyl transfer to bilirubin have been obtained with either albumin or cytosol from the liver of either Wistar or Gunn rats as the substrate carriers (V2).

Glycosyl transfer to hydrophobic acceptors by polyenzyme systems is a possibility. Transfer of sugar to endogenous lipid intermediates present in microsomal material from liver has been implied in the incorporation of glucose, mannose, and *N*-acetylglucosamine into endogenous glycoprotein (A4, B6, B7, B8, D2, M11, R7, T5). Dolichol monophosphate

appears to have a prominent role in these processes (B7, B8). Similarly, preliminary studies of the conjugation of steroid (W5) and bilirubin (H10) suggest that intermediate acceptor substances play a role (see also Section 3.1.4). The apparent duality of Mg^{2+}-dependent and -independent bilirubin UDP-glycosyltransferase activities (F3, H10) may be related to the functioning of such polyenzyme systems. Indeed, in biosynthesis of endogenous glycoprotein only the first step, the glucosyl transfer to dolichol monophosphate, was stimulated by bivalent cation (B6).

Solution of many of the problems touched upon should be greatly facilitated if soluble transferase were available. However, claims of obtaining truly soluble UDP-glucuronyltransferase (A2, H2, I1, L2, L7, M13, P2) have remained unconvincing (A12, H10); but see (H17). This failure, and their own lipid-depletion and -replacement studies (A12, G3), led Attwood *et al.* (A12), to express the view that attachment of transferase to a piece of membrane is a prerequisite for activity. As yet, it cannot be decided whether the lipid sheet, required for enzyme activity and eventually modulating its expression, is strongly enzyme bound or belongs to the lipid ground structure of the supporting membrane. Interestingly, the natural membranes may not be the only ones suitable, as lipid-depleted UDP-glucuronyltransferase could be reactivated by adding either phosphatidylethanolamine or cationic detergents (A12).

A completely unknown area is presented by the conjugation pathways operating in liver pathology. Compared to normal bile, that from patients with conjugated hyperbilirubinemia contains an increased number of bilirubin conjugates (F6). Various types of disaccharidic conjugating groups have been demonstrated in fistula bile of patients with cholelithiasis (K3). Similar conjugates may be present in small amounts in normal bile (F6, F7, H11). These observations suggest that either conjugating processes that have little importance in normal humans are stimulated in liver disease or transient intermediates accumulate. In rats hindrance to bile flow (produced by hydrostatic or mechanical obstruction) induces similar changes and may offer a useful tool for investigating the effects of obstruction on the conjugation of bilirubin *in vivo* (F6, V1).

8. Applications Related to Medicine and Clinical Research

Partial or total deficiency, or inhibition of bilirubin UDP-glucuronyltransferase may cause unconjugated hyperbilirubinemia. Increased production (hemolysis, ineffective erythropoiesis) should be excluded by investigating hematologic parameters. Determination *in vitro* of bilirubin UDP-glycosyltransferase activities can contribute to a differential diagnosis. To minimize the effect of cytoplasmic carrier proteins, in *in vitro*

assays with homogenates or cell extracts, albumin-bound bilirubin can be used as the acceptor substrate.

Progressive unconjugated hyperbilirubinemia is observed in neonatal children with the Crigler-Najjar syndrome. *In vitro* determination of bilirubin UDP-glucuronyltransferase activity in liver yielded no measurable or very low values (A11). However, in some cases (type II disease) bilirubin conjugates could be demonstrated in bile, and the serum bilirubin concentration decreased under phenobarbital treatment (A11, C11), indicating that some conjugation occurred *in vivo*. In other cases (type I disease), which, as far as is known, always develop kernicterus, deficiency of bilirubin conjugation was complete (A11). Earlier studies in patients with Crigler-Najjar's disease showed impaired *in vivo* metabolism of sodium salicylate and cortisol (C2, F16, H15), trichloroethanol (C2), menthol (A6), *N*-acetyl-*p*-aminophenol (S2), and sulfadimethoxine (J4).

So-called physiological jaundice of the neonate is related to immaturity of UDP-glucuronyltransferase at the time of birth, as demonstrated for experimental animals (D7, L4, S10). In some neonates with congenital pyloric stenosis and severe vomiting, unconjugated hyperbilirubinemia was found. The pathogenesis of the increased bile pigment concentrations is not understood. Delayed maturation of UDP-glucuronyltransferase due to undernutrition is documented in neonatal rabbits (F10) and fasting hyperbilirubinemia may contribute. The latter syndrome, demonstrated in patients with Gilbert's disease (F1), as well as in normals (B19), may be due to decreased "handling" in the liver cell (B19) and/or increased production of bilirubin, caused by enhanced microsomal heme oxygenase activity (B3). *In vitro* assays of the bilirubin UDP-glucuronyltransferase activity in liver biopsy material (eventually taken during surgery) may help to unravel the possible contributing factors.

Bilirubin UDP-glucuronyltransferase activity in liver biopsy specimens was decreased in patients with Gilbert's syndrome (A8, B15, M6) and in Wilson's disease (B15). In most instances, diagnosis can be established on clinical and clinicochemical grounds alone; however, when a liver biopsy is taken, measurement of transferase activity is decisive. Activities were normal or increased in most of the patients with hepatitis, cirrhosis, or obstructive jaundice (B15), as well as in hemolytic jaundice (F2). In 3 patients with acute hepatic failure, the activity still present was proportional to the number of liver cells detectable histologically. This suggests that conjugation of bilirubin is a long-lasting function.

In general, for diagnostic purposes in human liver disease, biochemical tests on serum, urine, and eventually duodenal bile, will mostly be suffi-

cient. However, in some cases, determination of bilirubin UDP-glycosyltransferase activities in liver biopsy samples will aid in the diagnosis.

In vitro assays of bilirubin UDP-glycosyltransferase activities are expected to find more frequent application in animal research. The Gunn rat, a mutant of the Wistar rat, is characterized by deficiency of UDP-glucuronyl-, UDP-glucosyl-, and UDP-xylosyltransferase activities (assayed with bilirubin as the acceptor substance) (F3, S5). This animal has a lifelong unconjugated hyperbilirubinemia and is frequently used in metabolic studies of bilirubin. Enzyme activities should be measured periodically to control continuity of the homozygous state of the inbred animals. Heterozygotes have intermediate transglucuronylating activity (T6).

Delayed uptake of bilirubin has been described in mutant Southdown sheep (C10), suggesting a deficiency in cytoplasmic organic anion-binding protein. Measurements of the conjugating enzymes are desirable. Conceivably, a decreased conjugating capacity could influence the uptake, as is suggested from studies on patients with Gilbert's syndrome (B14, B15, B18).

The main field of application of *in vitro* assays of bilirubin UDP-glycosyltransferase activities would seem to be the evaluation of stimulation or inhibition, by drugs or endogenous substances, of the enzyme activities, or of their maturation. Development of unconjugated hyperbilirubinemia in some neonates has been related to breast feeding (A7, N1). Inhibition of bilirubin UDP-glucuronyltransferase activity by addition of breast milk from the mothers was demonstrated with guinea pig liver homogenates (A10), but not with digitonin-activated rat liver homogenates. The transferase activity in purified microsomal preparations from human liver was not inhibited by pregnan-3α,20β-diol (A1), although this steroid has been assumed to be the causative agent (A7). A similar situation is present for sexogenous steroids in sera of some pregnant mothers with icteric babies (A9, H16, L4, L13). Administration of novobiocin caused unconjugated hyperbilirubinemia in neonates (S11) and in adults (E1). The drug diminished *in vitro* conjugation of bilirubin by guinea pig liver (B27, H7). Microsomal enzymes are stimulated by *in vivo* administration of phenobarbital, carcinogens, and some 300 other drugs and substances (C8, R6). Some of them (phenobarbital, glutethimide, coramine, DDT) have been used to decrease serum bilirubin concentrations in humans (B16, C11, R1, T7, W3). To evaluate the effects of these drugs and in developing new drugs, assays of bilirubin UDP-glycosyltransferase activities in human and animal tissues will be necessary.

ACKNOWLEDGMENTS

The authors thank Dr. J. Vandenbroucke (Leuven) and Dr. J. De Groote (Leuven) for their encouragement, Dr. G. J. Dutton (Dundee) for stimulating criticism and Dr. W. E. Stewart II (Leuven) for linguistic help. Thanks are also due to the "Fonds voor Geneeskundig Wetenschappelijk Onderzoek" of Belgium for supporting the work from the authors' laboratory.

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AUTHOR INDEX

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