

IMMUNOCHEMICAL TECHNIQUES IN CLINICAL ANALYSIS



By

HON KUEN TANG, B.Sc.

A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Master of Science

McMaster University

IMMUNOCHEMICAL TECHNIQUES IN CLINICAL ANALYSIS

MASTER OF SCIENCE (1980)

McMASTER UNIVERSITY

(Chemistry)

Hamilton, Ontario

TITLE: Immunochemical Techniques in Clinical Analysis

AUTHOR: Hon Kuen Tang, B.Sc. (McMaster University)

SUPERVISORS: Dr. A. Corsini (Department of Chemistry)

Dr. J. Gauldie (department of Pathology)

NUMBER OF PAGES: x, 119

## SCOPE AND CONTENTS

In an attempt to develop a new sensitive immunoassay procedure to measure biologically important small molecular weight compounds in human plasma or serum, the principle of inhibition of antigen-antibody precipitation by free antigen as detected by automated nephelometry was investigated. The assay procedure was optimized for maximum sensitivity. The characteristics of the optimized system are described and with the available instrumentation, the lower level of detection of free digoxin (a cardiac glycoside) was shown to be 2 nanograms. The results demonstrate the successful application of the principle, but fall short of providing a reasonable alternate to the radioimmunoassay of digoxin in human plasma or serum for routine clinical use.

A fast and cheap method for the separation of free and antibody-bound ligand, as applied to established radioimmunoassay techniques, was developed using chemically treated and heat-killed *Staphylococcus aureus* bacteria. In application to the measurement of a small molecular weight compound (digoxin) and a serum protein (ferritin), it was demonstrated to have good correlation with currently utilized techniques. This alternate method is shown to be suitable for routine clinical diagnostic purposes.

### ACKNOWLEDGEMENTS

I do want to express my deep appreciation to both of my supervisors, Dr. A.Corsini and Dr. J.Gauldie, for their, invaluable advice, guidance and patience throughout the course of this work. Their warmth and friendliness will always be remembered.

I take this opportunity to thank the Department of Chemistry, McMaster University for the financial assistance granted me.

Last but not least, I wish to thank Miss Y.H.Kung who did an excellent job. in drawing the figures. Without her patience and constant encouragement, the completion of this thesis would have been unlikely.

## TABLE OF CONTENTS

	Page
I. PURPOSE	1
II. INTRODUCTION	2
A. Digoxin	2
1. Sources and Structure-Activity Relations	2
2. Clinical Administration	4
3. Intoxication	5
4. Serum Level Measurements	5
B. Ferritin	6
1. Structure and Function	6
2. Clinical Implications	8
3. Quantitative Methods for Iron Stores	8
C. Antibody and Antigen	10
1. The Humoral Immune Response	10
2. Antigen-Antibody Interactions	13
3. Hapten as Antigen	16
D. Nephelometry	18
1. Light-Scattering Measurement of Antigen-Antibody Complexes	18
2. Historical Development	21
3. The Technicon AutoAnalyzer II	23
4. Automated Immunoprecipitin Technique	31

TABLE OF CONTENTS (Continue)

	Page
5. Automated Nephelometric Inhibition Immunoassay	31
E. Radioimmunoassay	34
1. Principle of Radioimmunoassay	34
2. Disadvantages of Radioimmunoassay	39
F. Staphylococcal Protein A	41
III. EXPERIMENTAL SECTION	43
A. Apparatus	43
B. Reagents	44
C. Protein Determination by Folin's Method	45
D. Purification of Keyhole Limpet Hemocyanin	46
E. Synthesis of Protein-Hapten Conjugates	47
1. Bovine Serum Albumin-Digoxin	47
2. Bovine Serum Albumin-Digoxin- <sup>3</sup> H	49
3. Keyhole Limpet Hemocyanin-Digoxin	49
F. Immunization of Rabbits	50
G. Radioimmunoassay of Digoxin	51
1. Titre of Antisera	51
2. Radioimmunoassay Procedure	52
a. Digoxin- <sup>3</sup> H Tracer and Ammonium Sulphate Separation	52
b. Digoxin- <sup>125</sup> I Tracer and Second Antibody Separation	53
H. Radioimmunoassay of Ferritin	55
I. Growth of Staphylococcus aureus	56

TABLE OF CONTENT (Continued)

	Page
IV. RESULTS AND DISCUSSION	59
A. Automated Nephelometric Inhibition Immunoassay of Digoxin	59
1. Optimization of the Analytical System	59
a. Hapten-Protein Ratio of Developer-Antigens	59
b. Temperature of Incubation	65
c. Immune Response and Antiserum Dilution	69
d. Preincubation versus Simultaneous Addition of Hapten	74
2. Optimum Conditions for Automated Inhibition Immunoassay of Digoxin	76
3. Extraction of Digoxin from Plasma	79
B. Solid Phase Radioimmunoassay System	84
1. Characterization of S.aureus-IgG Binding	84
a. Acid-Precipitable Protein Associated I-125	84
b. Binding Capacity of S.aureus for Rabbit IgG	84
c. Reactivity of S.aureus for IgG of Various Species	85
d. Reactivity of S.aureus Harvested at Different Phase of Growth	85
e. Binding Competition between Rabbit and Human IgG	89
2. Radioimmunoassay Comparisons	91
a. Ferritin	91
b. Digoxin	92
V. CONCLUSIONS	102
VI. SUGGESTIONS FOR FURTHER WORK	104
VII. APPENDIX	105
VIII. BIBLIOGRAPHY	111



LIST OF TABLES

Number	Title	Page
I	Estimation of digoxin/BSA ratios of various conjugates	60
II	Precipitability of various BSA-digoxin conjugates	62
III	Sensitivity to inhibition of various BSA-digoxin conjugates	62
IV	Variation of temperature on sensitivity to inhibition (Simultaneous addition)	66
V	Variation of temperature on sensitivity to inhibition (Preincubation)	66
VI	Correlation of RIA and AIP function of antisera	70
VII	AIP response of bleeds with various antigen concentrations	71
VIII	Preincubation versus simultaneous addition on sensitivity to inhibition	77
IX	Recovery of digoxin by extraction	82
X	Acid-precipitable protein-associated I-125	86
XI	Binding capacity of S.aureus for rabbit IgG	86
XII	Binding of IgG of various species by S.aureus	88
XIII	Binding of IgG by S.aureus from different phase of growth	90
XIV	Determination of ferritin (ug/litre) at two volumes	94
XV	Determination of digoxin (ng/ml) by three methods	97

LIST OF FIGURES

Number	Title	Page
1.	Structure of digoxin	3
2.	The humoral immune response	12
3.	Antigen-antibody precipitin curve	15
4.	Diagrammatic illustration of use of carrier proteins	18
5.	Schematic diagram of AutoAnalyzer II continuous flow system	24
6.	Diagram of regulatory section of pump	26
7.	Light path diagram of fluoronephelometer	28
8.	Sideview of flowcell used in fluoronephelometer showing debubbling mechanism	30
9.	Recorder tracing of nephelometric peaks	32
10.	Diagrammatic representation of the principle of radioimmunoassay	34
11.	Plotting of the standard curve in Radioimmunoassay	38
12.	Synthesis of protein-digoxin conjugate	48
13.	Precipitability versus moles digoxin per mole BSA	63
14.	Sensitivity to inhibition of various BSA-digoxin conjugates	64
15.	Variation of temperature on sensitivity to inhibition (Simultaneous addition)	67
16.	Variation of temperature on sensitivity to inhibition (preincubation)	68

LIST OF FIGURES (Continued)

Number	Title	Page
17.	Precipitin curves by AIP technique	72
18.	Reverse precipitin curves	73
19.	Precipitin curves for various dilutions of antiserum	75
20.	Preincubation versus simultaneous addition of hapten	78
21.	Standard curve for automated inhibition immunoassay of digoxin	80
22.	Binding capacity of S.aureus for rabbit IgG	87
23.	Correlation between second antibody and S.aureus separation methods for assay of ferritin	93
24.	Determination of ferritin (ug/litre) at two volumes	95
25.	Determination of digoxin (ng/ml) at two volumes	98
26.	Correlation between S.aureus and ammonium sulphate methods for assay of digoxin	99
27.	Correlation between ammonium sulphate and second antibody methods for assay of digoxin	100
28.	Correlation between S.aureus and second antibody methods for assay of digoxin	101
29.	Diagrammatic representation of IgG molecule	110

## I. PURPOSE

The purpose of the present investigation is two-fold:

- A. to provide, with digoxin as the model compound, a nephelometric inhibition immunoassay technique that has comparable sensitivity and precision to that of radioimmunoassay but that attains advantages such as the by-passing of the use of radioactive materials, ease of automation (to meet the high-volume demand of clinical analysis), and substantial cost savings;
- B. to improve present radioimmunoassay methods by development of an alternative separation technique using Staphylococcal protein A which is economical and independent of plasma matrix effects; two models chosen are the assay of a protein (ferritin) and that of an hapten (digoxin).

The ~~INTRODUCTION~~ which follows provides the necessary information as background to the present study. A glossary of terms is provided in the APPENDIX.

## II. INTRODUCTION

### II.A. Digoxin

#### 1. Sources and Structure-Activity Relations[1,2]

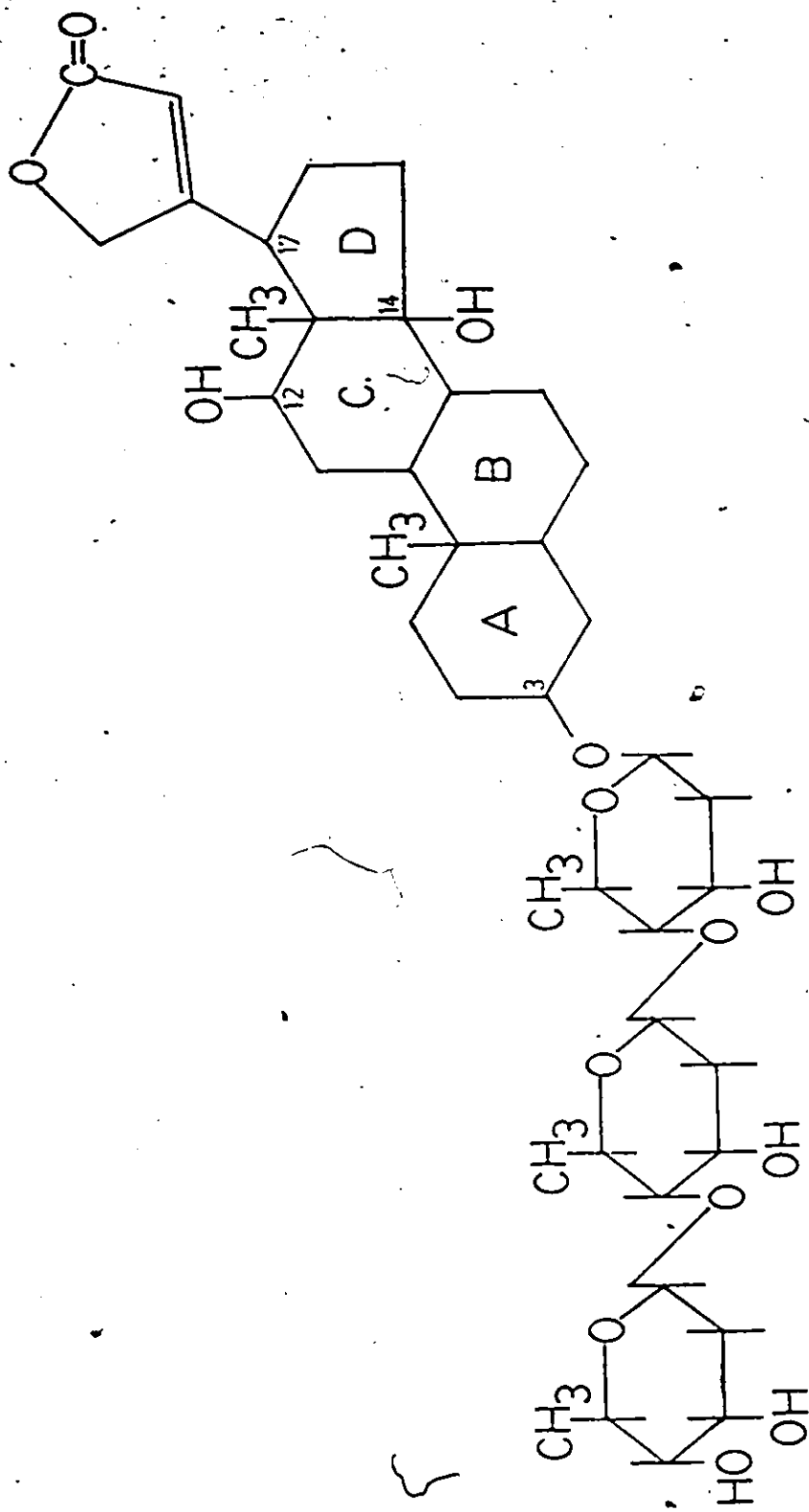
Digoxin (Figure 1) is one of the digitalis glycosides, the term being generally used to refer to any of the cardioactive steroid or steroid glycoside compounds that have characteristic positive inotropic and electrophysiologic effects on the heart. Most of the drugs of this class currently in common use are steroid glycosides derived from the leaves of *Digitalis purpurea* (digitoxin, digitalin, digitalis leaf) or from *Digitalis lanata* (digoxin, lanatoside-C, deslanoside).

All cardiac glycosides contain a steroid nucleus to which an  $\alpha,\beta$ -unsaturated lactone ring is attached at the C-17 position. This portion of the molecule without attached sugars is called the agenin, and is generally more transient and less potent in its myocardial effects than the parent glycoside.

Digitoxin differs from digoxin only in the absence of the C-12 hydroxyl group. Other cardiac glycosides differ in substituent groups on the steroid nucleus as well as in the structure and number of sugars attached at the C-3 position.

Molecular requirements for typical cardiac effects include the unsaturated lactone ring, the C-14 hydroxyl group and an unusual cis-fusion of the C and D rings of the steroid nucleus that is absent

Figure 1. Structure of digoxin



in endogenous steroids from mammalian species. The addition of one or more sugar residues at C-3 modifies potency and duration of action but has no effect on the fundamental pharmacologic properties of this class of compounds.

## 2. Clinical Administration[1,2]

Though all the digitalis glycosides are effective towards congestive heart failures and cardiac rhythm disturbances, digoxin has become predominantly used in hospitalized patients because of flexibility of route of administration and intermediate duration of action. It may be given orally, intravenously and intramuscularly. After administration its action is prompt, and when it is given intravenously, its effects is usually rapid enough to meet all but the most unusual situations. Furthermore, it is eliminated more readily, and thus if toxicity does occur, it is much less prolonged than with digitoxin.

Administered either orally or intravenously, about 20% of the digoxin is bound by serum protein and excreted unmetabolized. A normal person, including children, will eliminate 37% of that in the body daily, primarily by glomerular filtration and excretion. A steady state is reached in maintenance therapy when the same amount is replaced each day.

The serum levels of digoxin rises linearly with dosage, and the ratio of serum to myocardial concentration is relatively constant after completion of uptake and distribution of the drug. Thus, the serum concentration would be expected to reflect the myocardial



concentration.

### 3. Intoxication[1,2]

The sensitivity of individuals to cardiac glycosides is influenced by a number of factors, including serum potassium, calcium and magnesium concentrations, the adequacy of tissue oxygenation, acid-base balance, thyroid status, renal function, autonomic-nervous-system tone, other drugs concurrently received and type and severity of the underlying heart disease. Intoxication is manifested by nausea, vomiting, nervous system effects, visual symptoms, gastrointestinal symptoms, allergic skin lesions and cardiac arrhythmias. Since there are no unequivocal electrocardiographic features that distinguish digitalis toxic rhythms from those due to intrinsic cardiac disease, determination of serum levels has been resorted to for differentiation between normal and toxic groups of patients.

In general, there is significant correlation between the clinical state of the patient and his serum digoxin concentration. The mean serum level tends to be two or three times higher in patients with digoxin intoxication. Despite the significantly different mean levels, overlap has been observed between the ranges of the two groups, and no arbitrary level can be chosen that clearly differentiates toxic from nontoxic serum digoxin concentrations. Thus, it requires the interpretation of serum level data in the overall clinical context.

### 4. Serum Level Measurements

Knowledge of plasma or serum digoxin concentrations is useful

in assessing possible digoxin intoxication, confirming that adequate but not excessive concentrations are being maintained in the blood of patients predisposed to abnormally high values (e.g., patients with renal failure), evaluating patient compliance with dosage instructions, managing some supraventricular arrhythmias, diagnostically evaluating patients who are unable to give adequate histories and who present cardiac arrhythmias compatible with digoxin intoxication, and providing additional information regarding the clinical pharmacology of digoxin.

Methods applicable to evaluation of digoxin levels in patient serum or plasma samples include: (i) physicochemical separation methods such as gas-liquid chromatography[4,5]; (ii) inhibition of sodium/potassium dependent adenosine triphosphatase[6-12] and (iii) competitive protein binding methods such as radioimmunoassay[13-21] or sodium/potassium dependent adenosine triphosphatase enzymatic isotopic displacement[22].

Regarding ease of application in the clinical laboratory, methods dependent on sodium/potassium dependent adenosine triphosphatase inhibition are somewhat more complex and difficult than those based upon competitive protein binding, and radioimmunoassay methods are easily adapted to laboratories accustomed to such methods. Only small volumes of serum or plasma are used directly without prior extraction, necessitating a minimal number of manipulations.

## II.B. Ferritin

### 1. Structure and Function[23,24]

Of all the essential trace elements required by higher animals, iron is by far the most important because of its presence as a structural component of hemoglobin. Although about 70% of the total iron of the human body is present as hemoglobin and about 3% in the muscle as myoglobin, most of the remainder is found in the protein ferritin, which, together with the protein hemosiderin, is the major storage form of iron. Ferritin is present mainly in the reticuloendothelial cells of the liver, spleen and to a lesser extent, bone marrow[25]. A small amount is normally found in circulating plasma[26,27].

Ferritin consists of a shell of protein subunits surrounding a micelle of ferric hydroxyphosphate which is present in varying amounts. The nature of this colloidal iron complex has not been exactly elucidated, though it is known to be a polymer with a probable composition of  $(FeOOH)_8 \cdot (FeO \cdot PO_3H_2)$ [28,29]. Fractions of ferritin with different iron contents have the same iron-phosphorus ratio. The diameter of the iron micelle is about 70 to 75 Å whereas that of the protein is of the order of 120 Å[30-33].

Ferritin molecules that are completely filled with iron have a molecule weight of about 900,000[34] and contain approximately 5,000 atoms of ferric iron per molecule. Apoferritin has been found to have a molecular weight in the range of 430,000 to 480,000 and consists of 24 subunits of molecular weight of the order of 18,500[35,36]. Ferritin is capable of aggregation, predominantly through hydrophobic interactions. Unfractionated ferritin normally contains between 80 and 90% of monomers, and most of the remainder as dimers, though oligomers

are possible[37,38].

Three electrophoretically distinct forms of human ferritin specific to spleen, liver and reticulocytes have been identified [39]. In bone marrow, two types of ferritin have been found, one with the electrophoretic mobility of spleen ferritin and one with the mobility of reticulocyte ferritin. Although the subunit molecular weights of these apoferritins are around 18,500, the amino acid analysis of the proteins show marked differences. Fingerprinting of the tryptic peptides from human liver and spleen and from horse spleen apoferritin show that more than half the peptides are identical in all three proteins, whereas the others vary[40]. It is clear that ferritin in liver and spleen of human and horse is organ specific. Microheterogeneity is also shown by many ferritins on isoelectric focusing and represent a structural heterogeneity in the ferritin population within the tissue[41,42]. Moreover, the physical characteristics of serum ferritin in anion-exchange chromatography are clearly distinct from the ferritin of human spleen, liver, heart or kidney[43], although circumstantial evidence derived from clinical studies suggests that ferritin circulating in the serum is normally closely related to reticuloendothelial ferritin[45].

## 2. Clinical Implications

Serum ferritin concentrations correlate with total body iron stores in normal males and females[44]. Total body iron store is determined by quantitative phlebotomy, the standard reference method for assessing iron stores and measuring the amount of mobilizable iron

available for hemoglobin synthesis. The same correlation holds in patients with iron deficiency or transfusional iron over-load[45-47], except in some patients suffering from idiopathic hemochromatosis[48,49]. The normal range in humans is between 12 and 250 ug per litre. In patients with iron-deficiency anemia, concentrations are below 12 ug per litre and in patients with iron overload, the concentration may be as high as 10,000 ug per litre[45,46,50,51].

In most situations the serum ferritin concentration appears to reflect reticuloendothelial storage iron fairly accurately. Changes in reticuloendothelial iron are followed rapidly by changes in the serum concentration of ferritin. In both hemochromatosis and secondary iron overload, serum ferritin can be used to monitor therapeutic removal of excess storage iron. It has been suggested that 1 ug per litre of serum ferritin represents about 8 mg of storage iron[44].

In spite of the fact that high serum ferritin concentrations inappropriate to the amount of storage iron have been found in patients with hepatic disease, increased cellular turnover, inflammatory conditions or certain malignant conditions, a decrease in the serum concentration can be interpreted as strong evidence for iron depletion[46,47,51].

### 3. Quantitative Methods for Iron Stores

The most accurate method for measuring iron stores is by quantitative phlebotomy, which is the standard method for measuring the amount of mobilizable iron available for hemoglobin synthesis[44]. But

in practice it is a lengthy and inconvenient procedure for which indirect, but more convenient, methods are usually substituted. Some, like the visual assessment of iron in marrow or liver biopsies, cannot be quantitated. Methods like the estimation of desferrioxamine-induced urinary excretions and non-heme iron concentration in liver involve the patient in some inconvenience. The former requires 6 to 24 hours of urine collection and the latter requires a liver biopsy. These procedures have to be compared to the single sample of venous blood required for measurement of ferritin concentration which has been shown to correlate well with total iron stores[44].

An immunoradiometric assay technique in which the antibody is labelled has been used extensively in a one-site form by those following the work of Addison et al.[26] and a two-site immunoradiometric assay was introduced by Miles et al.[52].

The alternate radioimmunoassay approach, in which the ligand rather than the antibody is labelled, has been described by Marcus and Zinberg[53] and Niitsu et al.[54]. These workers used the second antibody method of separation. In a further refinement, Luxton et al.[55] used a second antibody with polyethylene glycol to accelerate the separation of bound and free ferritin, allowing the entire assay to be completed in 24 hours. Optimization of the assay and its performance are also described.

## II.C. Antibody and Antigen

### 1. The Humoral Immune Response

The injection of a single dose of a foreign substance into an immunocompetent animal will cause specific antibody to appear in the serum after a period of time. First exposure to an immunogen evokes the primary response (Figure 2). Immediately after introduction of the immunogen, little or no specific antibody is detected in the serum. This period is referred to as the inductive phase. It is during this period of time that the immunogen is recognized as foreign and processed, and an unknown signal is transferred to the appropriate cells destined to make antibody. The duration of this period is variable and depends upon (i) the immunogenicity, quantity, form and solubility of the stimulant; (ii) the animal species into which it is injected; (iii) the route of immunization; and (iv) the sensitivity of the assay used to detect the newly formed antibody.

There is then a time of active biosynthesis of antibody that can be further subdivided into three phases: (i) the logarithmic phase when the antibody concentration increases logarithmically until it reaches a peak; (ii) the steady state (which is highly transitory) in which the level of circulating antibody remains constant as the rates of synthesis and catabolism of the antibody are the same; and (iii) the decline phase in which the rate of antibody catabolism is greater than that of its synthesis.

The early primary response to most immunogens is characterized by the predominance of IgM antibody; the IgG class of antibody appears somewhat later but reaches a higher level of concentration in the serum. Administration of the immunogen in adjuvant usually results in the continued synthesis of both IgM and IgG antibodies for prolonged

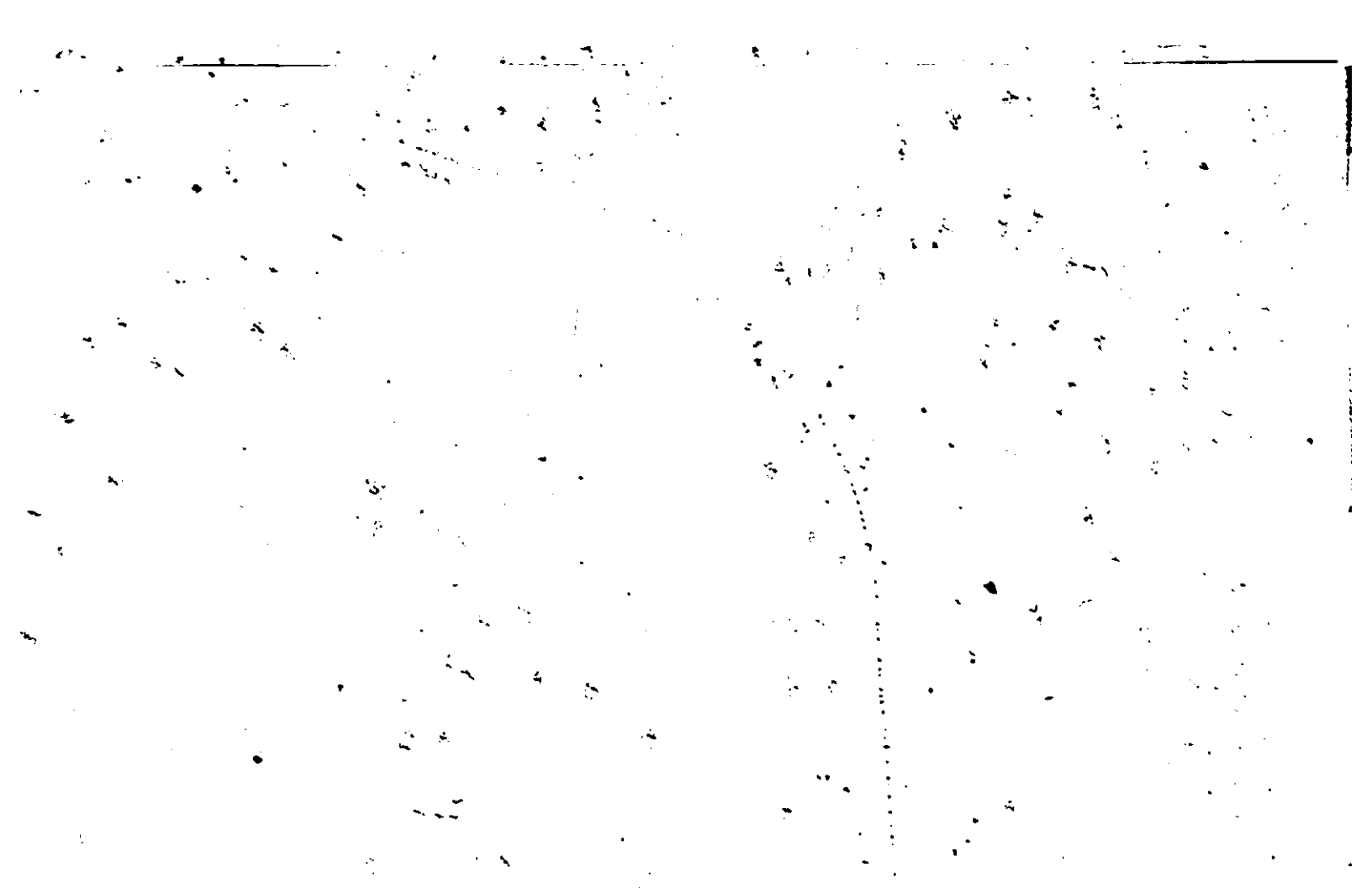
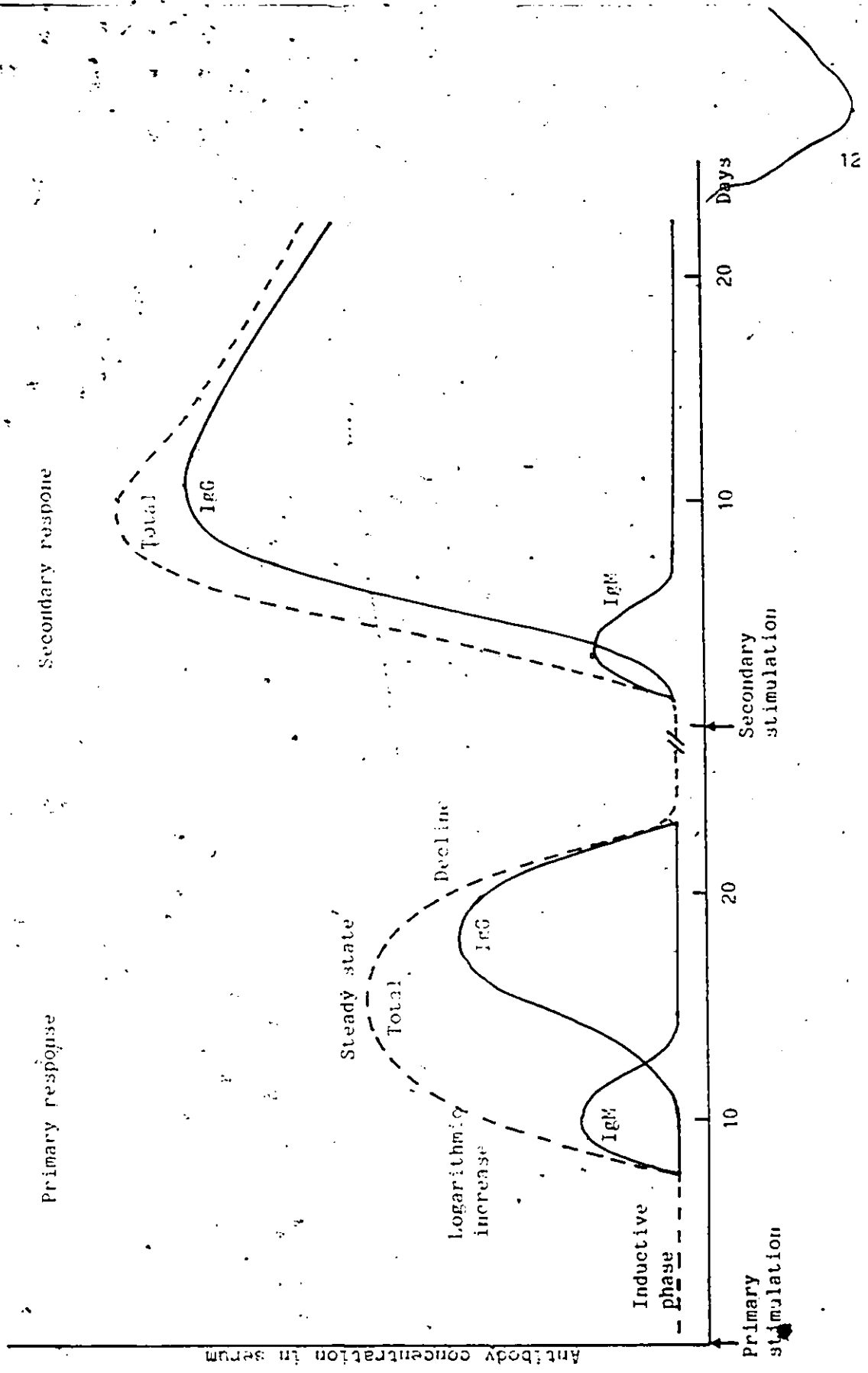


Figure 2. The humoral immune response





Antibody concentration in serum

Primary stimulation

Secondary stimulation

Days

Primary response

Secondary response

periods of time.

Upon a second exposure to the same immunogen, weeks, months or even years later, there is a markedly enhanced secondary response that is characterized by the accelerated appearance of antibody (Figure 2). This enhanced response serves as the principle for giving booster doses of immunogens. In contrast to the primary response, the secondary response is characterized by a shorter latent period, a more rapid rate of antibody synthesis, and a higher level of antibody concentration that persists for a longer period of time. Antibody concentration may reach as high as 10 to 12 mg/ml and is predominantly of the IgG class.

In both responses and in particular the secondary response, the antibody produced late in the response has higher affinity and avidity for antigen than that synthesized earlier. These changes are related to the diverse antigenic determinants on the immunogen that give rise to a variety of antibody specificities, which appear after different latent periods. Also there may appear antibodies against certain antigenic determinants that only weakly stimulate the formation of antibodies against them earlier in the response (the minor antigenic determinants). All these factors together exemplifies the fact that the humoral immune response is heterogeneous, producing a population of antibodies with differences in immunoglobulin class, affinity, avidity and specificity.

## 2. Antigen-Antibody Interactions

The combination of an antibody with an antigen is a stereochemical linkage which is specific and involves a combining site

14

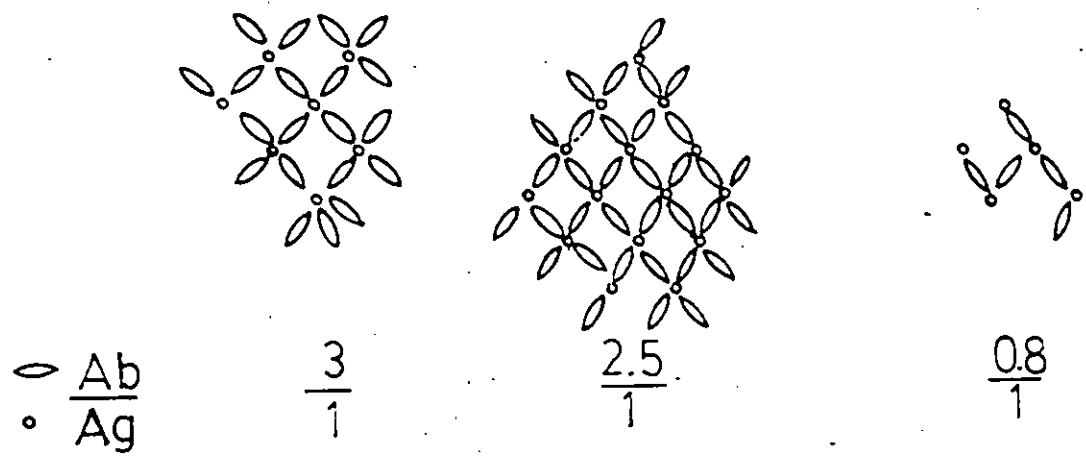
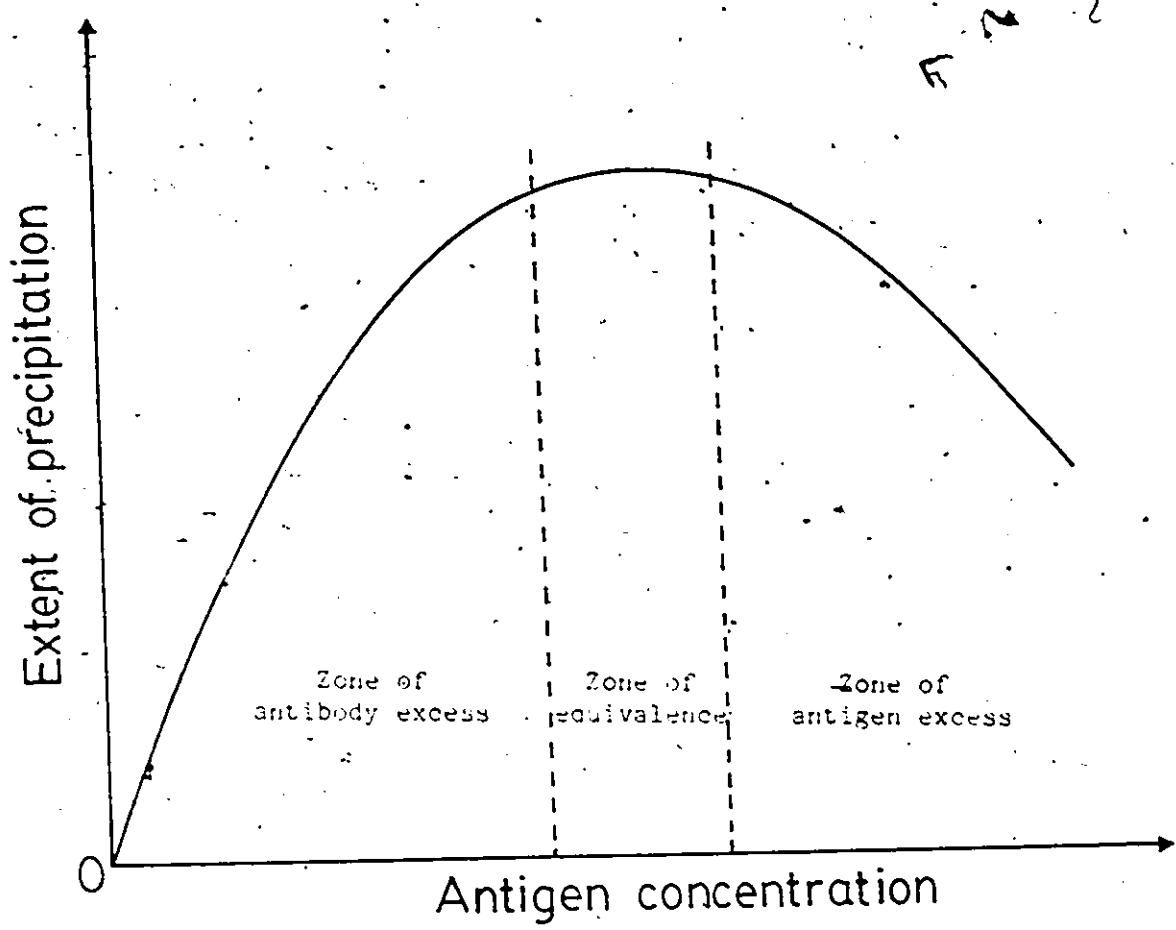
on the antibody linking with a determinant group of a small area on the antigen. This is the primary interaction. The linking between the antibody and the antigen is by weak chemical forces (hydrogen bonding, ionic interaction and van der Waal forces) which are very dependent on geometric complementation between the determinant groups and the corresponding combining sites. The interaction has a high equilibrium constant and is reversible.

Antibody molecules have two identical combining sites because in an antibody molecule, the two pairs of heavy and light chains that form the antigen binding sites are identical. Antigen molecules bear several determinant sites (from a few to over 200 per molecule). Thus antigen and antibody molecules can combine in varying proportions to form complexes. These complexes may remain in solution or they may precipitate if they attain a large enough lattice size. Such precipitation is one of the many manifestations of the secondary interaction.

The reaction of antibody with antigen occurs over all concentrations of both reagents. The resulting complex or precipitate varies in composition according to the proportions of antigen and antibody in the reaction mixture. For maximum precipitation, the antigen and the antibody must be mixed in optimal proportions, this being dependent on the relative immunological valencies of the two molecules.

The amount of precipitation is related to the antibody/ antigen ratio and is described by the precipitin curve (Figure 3). In

Figure 3. Antigen-antibody precipitin curve, with diagrammatic representation of antigen-antibody complexes in the corresponding zones



In the zone of antibody excess, many antibody molecules will remain uncombined and a precipitate will form only slowly. As the concentration of antigen is increased, the zone of equivalence is reached, resulting in the formation of a large lattice and rapid precipitation. The precipitate contains all the available antibody and antigen so that neither is present in the supernatant. With antigen in excess, a less perfect lattice is formed and precipitation is slower and less complete. Free antigen is present in the supernatant. Extreme excesses of antigen will result in complexes that fail to precipitate.

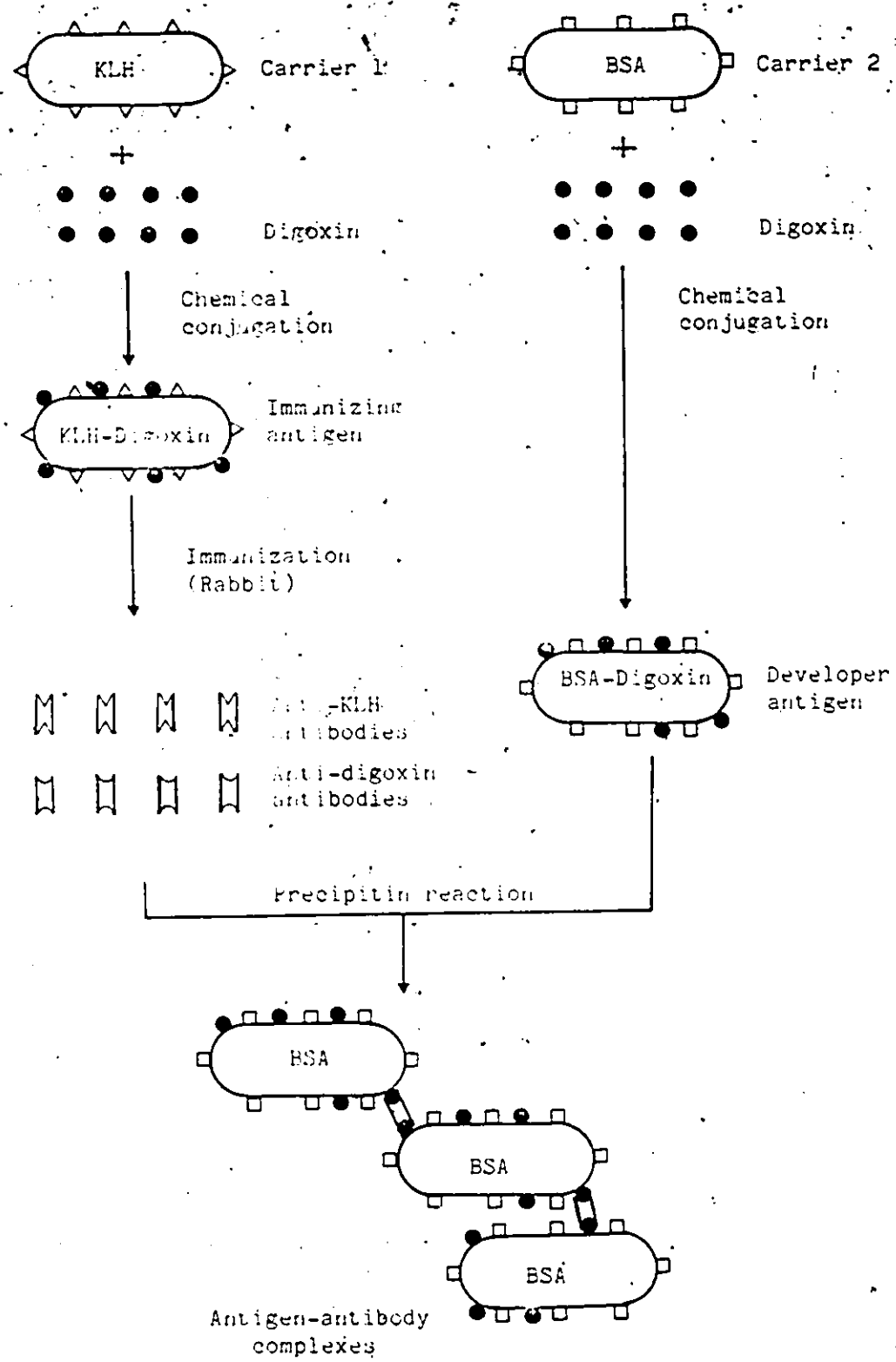
### 3. Hapten as Antigen

Hapten is a term used to describe small molecular weight compounds, which by themselves are incapable of eliciting the formation of a detectable amount of antibody. However they can interact with the combining site on an antibody molecule possessing suitable specificity. In order to elicit the formation of antibody against a hapten, it is necessary to covalently bond the hapten to a large molecule (the carrier) such as a protein on which the hapten acts as one of the many antigenic determinants. The antibody response will be against the whole protein-hapten conjugate (the immunizing antigen) and will include a response against the hapten itself (Figure 4). The antibody response stimulated will be better if the carrier protein used is isolated from a species from that which is being immunized.

As an hapten is usually univalent (i.e. only possesses a single unique binding site), it can only participate in a primary interaction



Figure 4. Diagrammatic illustration of use of carrier proteins



Poor Copy



with its antibody. It is necessary to have more than one hapten molecule covalently bonded onto a carrier (forming the developer-antigen) before a three dimensional lattice can be formed in the antigen-antibody reaction. The way in which the hapten molecules are bonded to the carrier for the precipitin reaction must be similar to that in which they are bonded to the carrier used in immunization. This ensures the exposure of the same portion of of the hapten on the developer antigen for interaction with the antibody. Also, different carrier proteins are needed for the elicitation of antibody formation and for the precipitin reaction so that the latter involves only the determinant groups resulting from the covalently bonded haptens (Figure 4).

Keyhole limpet hemocyanin (KLH), a protein of molecular weight about 7.5 million, is known to be highly immunogenic[56,57] and is capable of accepting digoxin conjugation through available amino groups. It was chosen to be the carrier protein for the immunization of animals. Bovine serum albumin (BSA) is a readily available protein of molecular weight about 60,000. It is obtainable in a highly purified form and was chosen to be conjugated with digoxin to act as the developer-antigen in the present experiments.

## II.D. Nephelometry

### 1. Light-Scattering Measurement of Antigen-Antibody Complexes

Nephelometry is an analytical procedure in which the intensity of light scattered from particles suspended in a medium is measured.

A light beam entering a suspension medium is broken up into several populations with different destinies depending on the concentration and nature of the suspended particles. A portion can traverse straight through the medium without interference and emerge from the opposite side. Part of the energy of the beam is lost in the medium. It is either absorbed to reappear as heat or light at another wavelength, or is utilized in the production of new chemical substances. A large portion of the beam encounters reflecting particles and is reflected. The reflected light can encounter additional surfaces and be further reflected. At high particle concentrations, only a small portion of the incident beam is released from the medium. Lower concentrations may allow a small portion to traverse through in the plane of the incident beam but reflect or absorb the great bulk. Low concentrations interfere little with the incident beam or the secondary beams reflected from the small number of suspended particles. Therefore, nephelometry is most suitable when the concentration is fairly low in contrast to turbidimetry which is more applicable with a high concentration of suspended particles.

In nephelometry, it is difficult to relate the scattered intensity ( $I_s$ ) to the concentration (C) of suspended particles by any simple theoretical equation, because the scattered intensity depends in a complicated way on the properties of the scattering suspension. At low concentrations, an approximately linear relationship can be expressed empirically according to Raleigh's law:

$$I_s/I_o = K.C.\sin^2\theta/\lambda^4 \quad (1)$$

where  $I_o$  is the incident intensity,  $\lambda$  is the wavelength of the

incident beam,  $\theta$  is the angle at which  $I_s$  is measured and  $K$  is an empirical constant. As the intensity of scattered light is inversely proportional to the fourth power of the wavelength, a wavelength of around 360 nm is used on most commercially available instruments. This is about the shortest wavelength applicable without strong absorption by the protein particles. Furthermore, these commercial instruments are in general modified fluorometers for dual applications, thus the name fluoronephelometer.

Antigen-antibody complexes formed in dilute solutions can be detected by nephelometry[58]. For the present consideration, the medium is an aqueous liquid and the suspended particles are protein molecules complexed with antibodies. The antigen-antibody reaction follows the law of mass action and under the right conditions, the amount of complexes formed is a function of both antigen and antibody concentrations. If the antibody concentration is kept constant, the amount of complexes formed varies in direct proportion to the concentration of the antigen. Consequently, with the scattered light measured at 90 degrees from the incident beam, equation (1) can be further simplified to express a linear relationship between the intensity of the scattered light and the concentration of the antigen ( $C_A$ ):

$$I_s/I_o = K_A \cdot C_A \quad (2)$$

where  $K_A$  is the proportional constant. Applying this principle, quantitative determination of unknown antigen concentrations has been achieved by comparison with light scattered by standard solutions under identical analytical conditions[59,60].

## 2. Historical Development[61]

Though the phenomenon that precipitates developed on the addition of soluble antigen to specific antibody was observed as early as the turn of this century[62-64], its application in immunochemical analysis depended greatly on the development of the instrumentation. Initially light scattering techniques were used but were replaced by turbidimetry only to revert again in the late 1960's toward nephelometry with the help of greatly improved electronic instruments. The use of optical analysis vacillated between these two techniques as instruments improved.

Nephelometry enjoyed considerable popularity in the early part of the twentieth century[65-69]. Kober[68] used this approach for the first time to measure the concentration of acid precipitated protein. Because of the relative insensitivity of the equipment at that time, the concentration of light scattering materials had to be great, and in 1921, Denis stated that "the amount of light reflected is not strictly proportional to the weight of the precipitate under observation but seems to be influenced by a variety of factors"[70]. For the next 18 years, very few publications dealing with nephelometry appeared.

In 1938, Libby improved the instrument by introducing a collimating light source, the use of a variable resistor to adjust the intensity of the incident beam, a photoreceptive surface and an electronic output in the form of a galvanometer[71]. Subsequent work on comparative serology suggested that nephelometric analysis had several advantages over the then popular quantitative precipitin data[72,73]. The importance of using low concentrations of reactants in nephelometry

was emphasized[74,75] and the relationship between the actual amount of precipitates and the galvanometric readings obtained in photoelectric instruments was established[76]. Moderate changes in particle size distribution in the analyzed precipitates was found to have little effect on nephelometric determinations[77]. It was also noted that the light scattering ability of a system could be divided into two phases[78-80]. First, a very rapid rise in light scattering that lasted only a few minutes and later, a slower increase that could last for hours.

It was not until 1953 that mercury arc lamp and filtered light was introduced to replace unfiltered incandescent light sources[81]. In 1959, Schultze and Schnick illustrated that many human plasma proteins could be accurately and quite easily quantitated by turbidimetry of antigen-antibody precipitates[82]. But the technique did not gain acceptance, probably due to the high cost of antibody and the general unavailability of commercial quantities of the reagents.

In 1960, Leone studied the reaction of a hemocyanin and specific antibody mixture by turbidimetry and emphasized the rapidity with which visual precipitates formed[83]. This information was not put to practical use until about a decade later when Hellsing showed that the addition of a polyanion such as polyethylene glycol markedly accelerated the precipitation of antigen-antibody complexes from solution[84]. In 1966, Stone and Thorp utilized a newly developed micronephelometer to estimate the concentration of triglyceride-rich lipoproteins[85]. In the same year, Kahan and Sundblad introduced a fully automated turbidimeter[86] and compared the results obtained with

more standard manual methods[87].

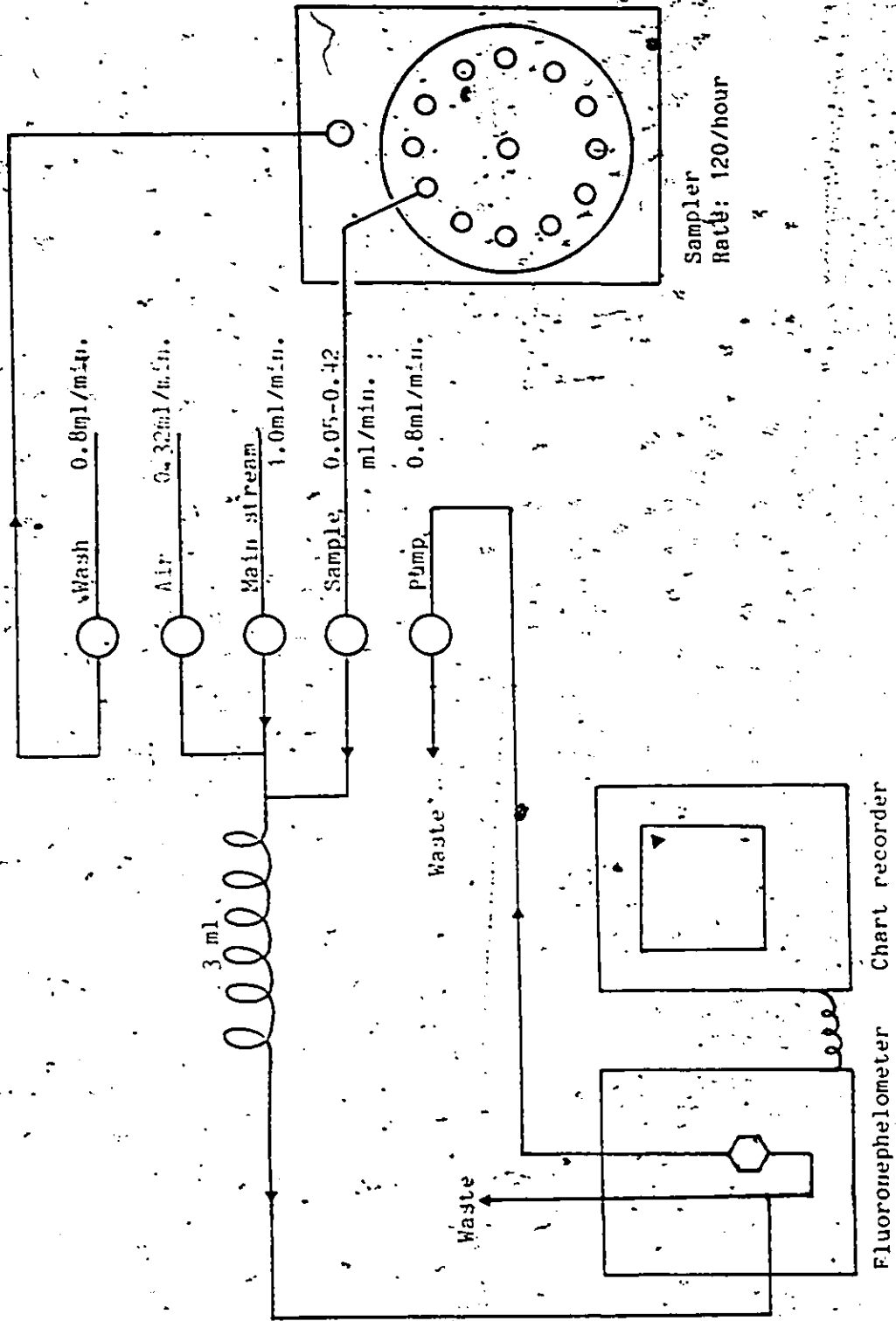
In 1968, a high quality fluorometer was modified to perform as a nephelometer by Alper and Propp to analyze for the first time, the third component of the complement system[88]. The description of a fully automated nephelometric immunoassay system, using microgram quantities of antigen and microlitre volumes of antibody, was presented in 1969 by Ritchie et al.[89]. The system has eventually become known as the automated immunoprecipitin (AIP) system (Technicon Instruments Corporation, Tarrytown, New York). Further improvement produced an instrument capable of processing a rate of over 100 samples per hour, consuming very small volumes of antigen and antibody and yielding results comparable with the conventional manual radial immunodiffusion technique[90].

### 3. The Technicon AutoAnalyzer II

The AutoAnalyzer II (Technicon Instruments Corporation, Tarrytown, New York) is the second generation of a continuous-flow system in which individual operations are performed on the flowing stream as it progresses through the system. Five modules are involved in the present investigation: (i) proportioning pump, (ii) sampler, (iii) manifold, (iv) fluoronephelometer and (v) chart recorder (Figure 5).

The proportioning pump regulates both the rate and the quantity of reagent flow in the system. The regulatory section consists of two parallel stainless steel chains with eight equally spaced rollers. This roller-head assembly is driven by a constant-speed gear head motor

Figure 5. Schematic diagram of AutoAnalyzer II continuous flow system



Poor Copy



and moves in an elliptical path pushing the rollers upward against the platen (Figure 6a). Since the manifold pump tubings are pressed between the rollers and the platen, samples and reagents are pulled from the sample cups and reagent bottles, and are pushed through the pump tubings at a constant speed. The volume of reagent being pushed through depends on the inner diameter of the pump tubing used. To ensure consistency of volume pumped, the spring tension has to be properly adjusted and the wall thickness of all pump tubings used must be the same to afford uniform spacing between the platen and the rollers (Figure 6b).

An air bar, mechanically linked to the movement of the pump rollers, regulates the spacing of the air segments that are introduced into the analytical stream. Each time a roller leaves the pump platen, the air bar is raised to let a measured quantity of air through. At other times it occludes the air tubing.

The sampler module enables measured sample volumes to be introduced into the main AutoAnalyzer stream. The sample tray, holding the samples in 2 ml polystyrene cups, rotates at a predetermined rate of speed. Its rotation is also synchronized with the movement of the sampling probe between the sample cups and the wash reservoir. The volume in each cup needs not be exactly measured as long as it is in excess of the aspirated volume. As each cup is moved to the sample pickup position, the sampling probe dips into the cup and according to the size of the pump tubing used, a fixed volume is removed and introduced into the analytical stream. As the probe alternates between

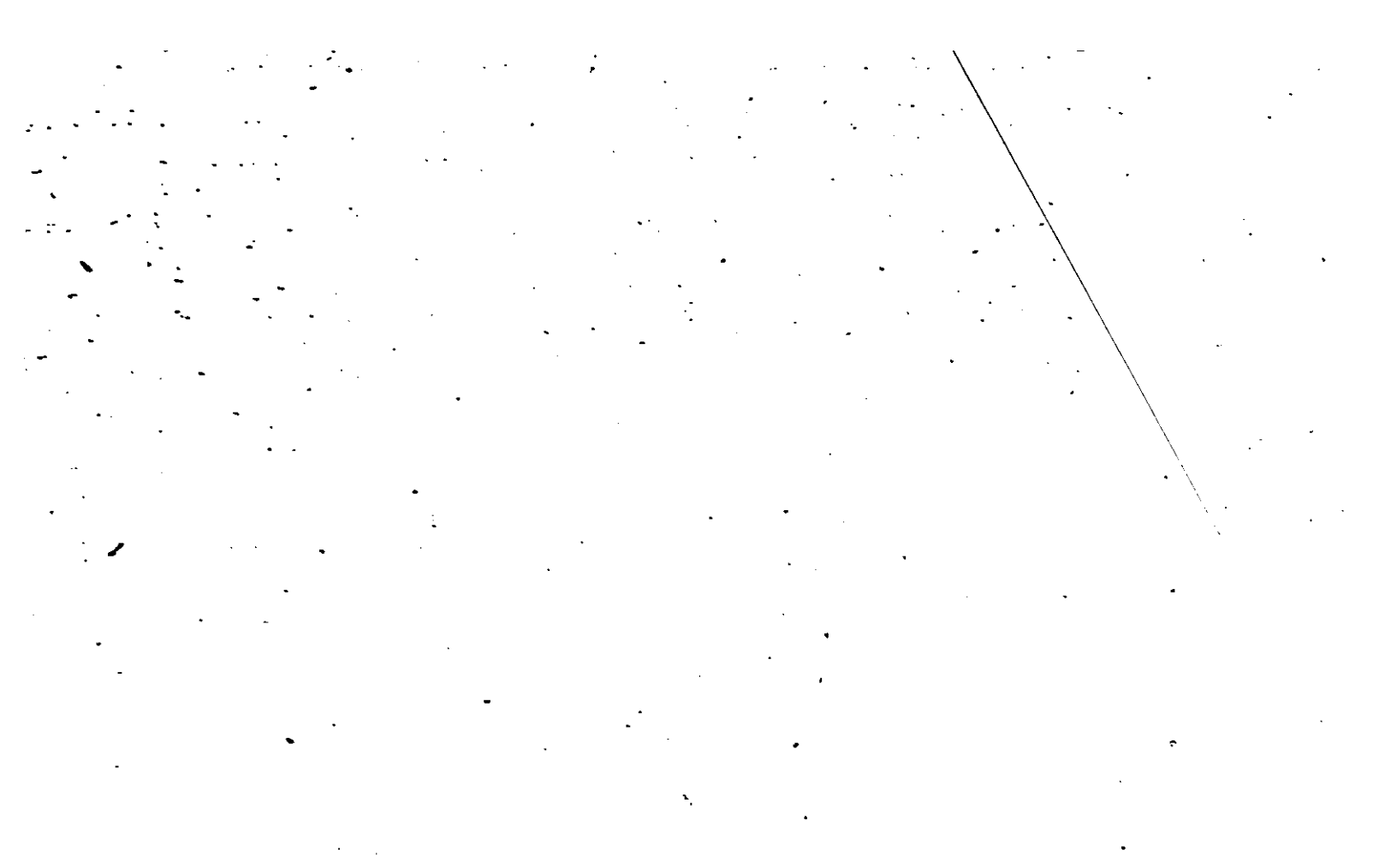
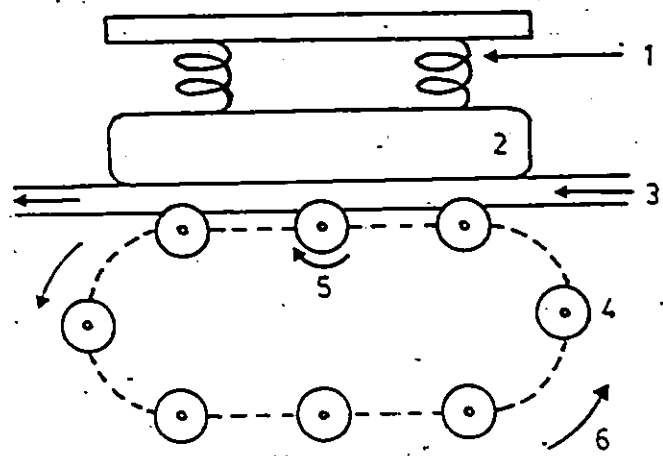
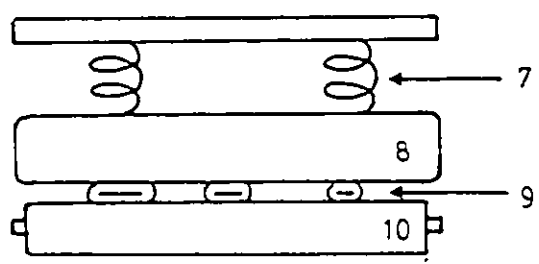


Figure 6. Diagram of regulatory section of pump



- 1. Spring
- 2. Platen
- 3. Flow of reagent through pump tubing
- 4. Roller
- 5. Rotation of roller
- 6. Motion of chain

a. Side view



- 7. Spring
- 8. Platen
- 9. Spacing equals twice tube thickness
- 10. Roller
- 11. Tubings have same wall thickness but different diameters



b. Front view

POOR COPY

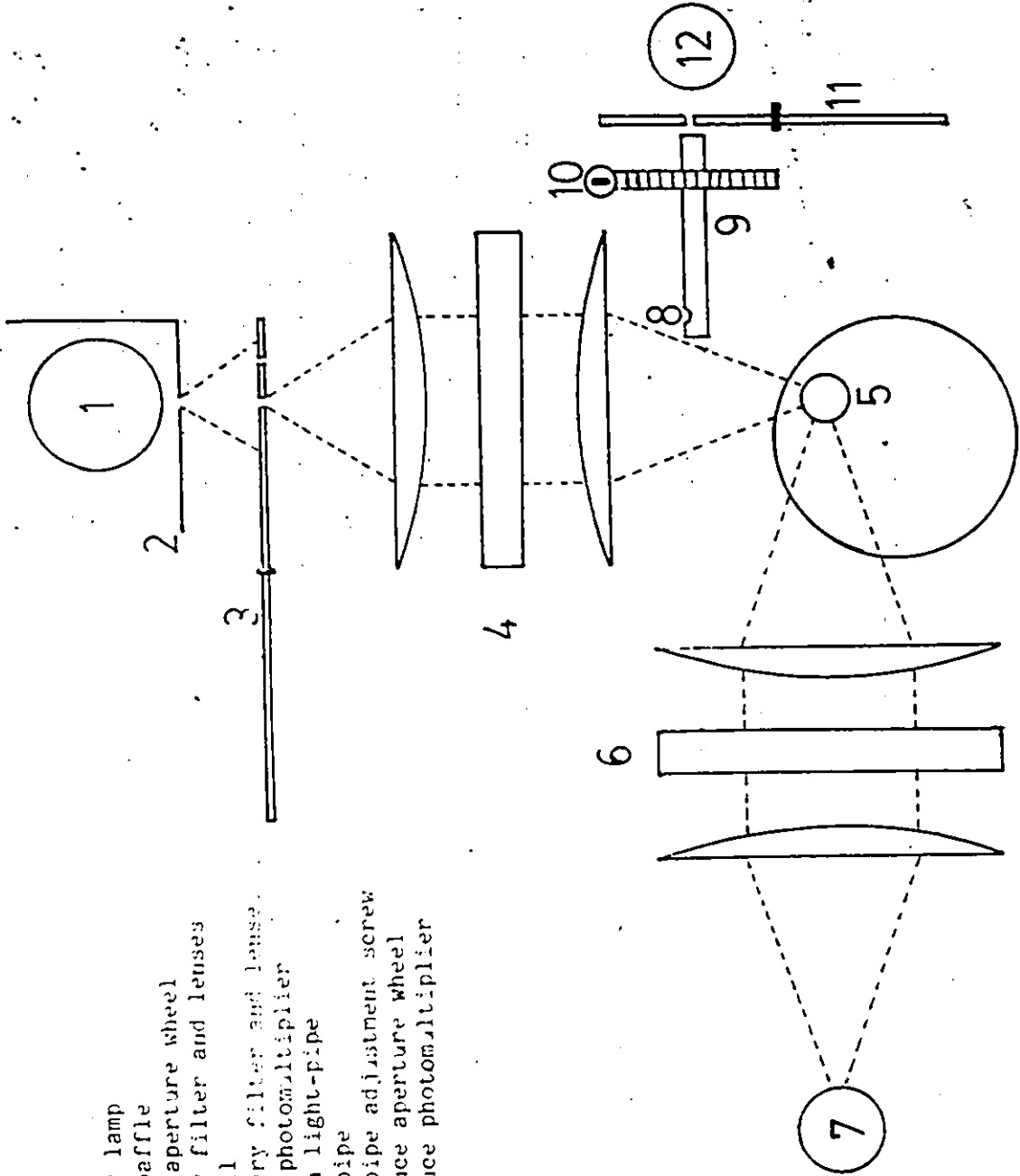
sample and wash solutions, pumping continues and air segments are introduced to keep the sample and wash segments separate. The air bubbles and wash solution together clean the input probe between samples. Sampling and washing time periods are controlled by a rotating cam.

The manifold consists of connectors, coils and tubings. The connectors direct the sequence of addition of either air bubbles, samples or other reagents into the main analytical stream. The coils cause mixing of reagents within each liquid segment in the stream. The volume contained within the coils and tubings determines the time needed before the reagents are pumped through that part of the analytical flowcell which is in the optical light path.

The Technicon fluoronephelometer can be used as a fluorometer or a nephelometer. The combination of filters and flowcell depends on usage intended. The light source is a high-pressure mercury lamp that provides energy in the spectral region from 250-600 nm. The light passes through a pre-selected aperture on the rotatory sample aperture wheel, a collimating quartz lens and then a primary filter (Figure 7). The openings of the sample aperture wheel determine the amount of light that strikes the sample flowing through the exposed portion of the flowcell.

The primary filter allows passage of a narrow band of light which is concentrated into a narrow beam by a condensing and collimating lens system onto the molecules of the sample flowing through the flowcell. Scattered light or fluorescence light (at an angle of 90 degrees from the incident beam) passes through a collecting

Figure 7. Light path diagram of fluoronephelometer



- 1. Mercury lamp
- 2. Light baffle
- 3. Sample aperture wheel
- 4. Primary filter and lenses
- 5. Flowcell
- 6. Secondary filter and lens
- 7. Sample photomultiplier
- 8. Hole in light-pipe
- 9. Light-pipe
- 10. Light-pipe adjustment screw
- 11. Reference aperture wheel
- 12. Reference photomultiplier

and collimating lens system and a secondary filter onto a photomultiplier tube. The photomultiplier tube transforms the energy into a linear photocurrent signal which is then recorded. For the nephelometry of antigen-antibody complexes, both the primary and secondary filters used are for a wavelength of 355 nm.

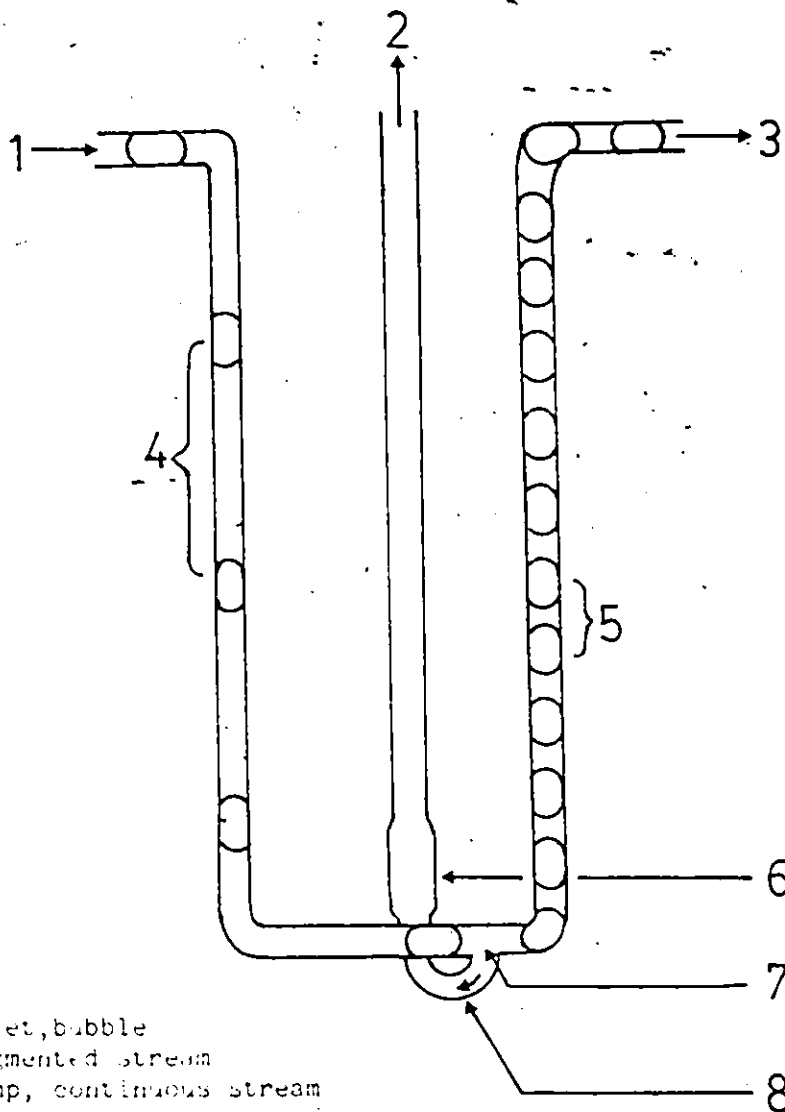
A reference beam provided by a small portion of light from the source lamp passes through a small outer aperture of the sample aperture wheel, a light pipe and the reference aperture onto a reference photomultiplier tube (Figure 7). The dual beam operation of the sample and reference photomultipliers in conjunction with the recorder provides a null-balance ratio system to resolve fluctuations in light-source intensity.

Incorporated into the flowcell (Figure 8) is the debubbling device designed to get rid of the segmenting air-bubble which would otherwise interfere with the light measurement. To minimize error due to mixing between samples, measurements are made as soon as possible after debubbling. Flowcells for use in nephelometry are entirely coated black, except for the small portion in the light path, to reduce stray scattering.

The recorder for the AutoAnalyzer system is of the null-balance potentiometric type for ratio-recording. Voltage from the photomultiplier on the sample side is compared with that on the fixed-reference side to give a balanced ratio which is indicated by the stylus on the chart paper of the recorder. Tracings are of the rectilinear type.

Figure 8. Sideview of flowcell used in fluoronephelometer  
showing debubbling mechanism





1. Inlet, bubble segmented stream
2. Pump, continuous stream
3. Waste
4. Wide separation of bubbles
5. Narrow separation of bubbles.
6. Enlarged section in light path for measurement
7. Major portion of stream withdrawn for measurement
8. Continuous path as short as possible before measurement

Poor Copy

#### 4. Automated Immunoprecipitin Technique

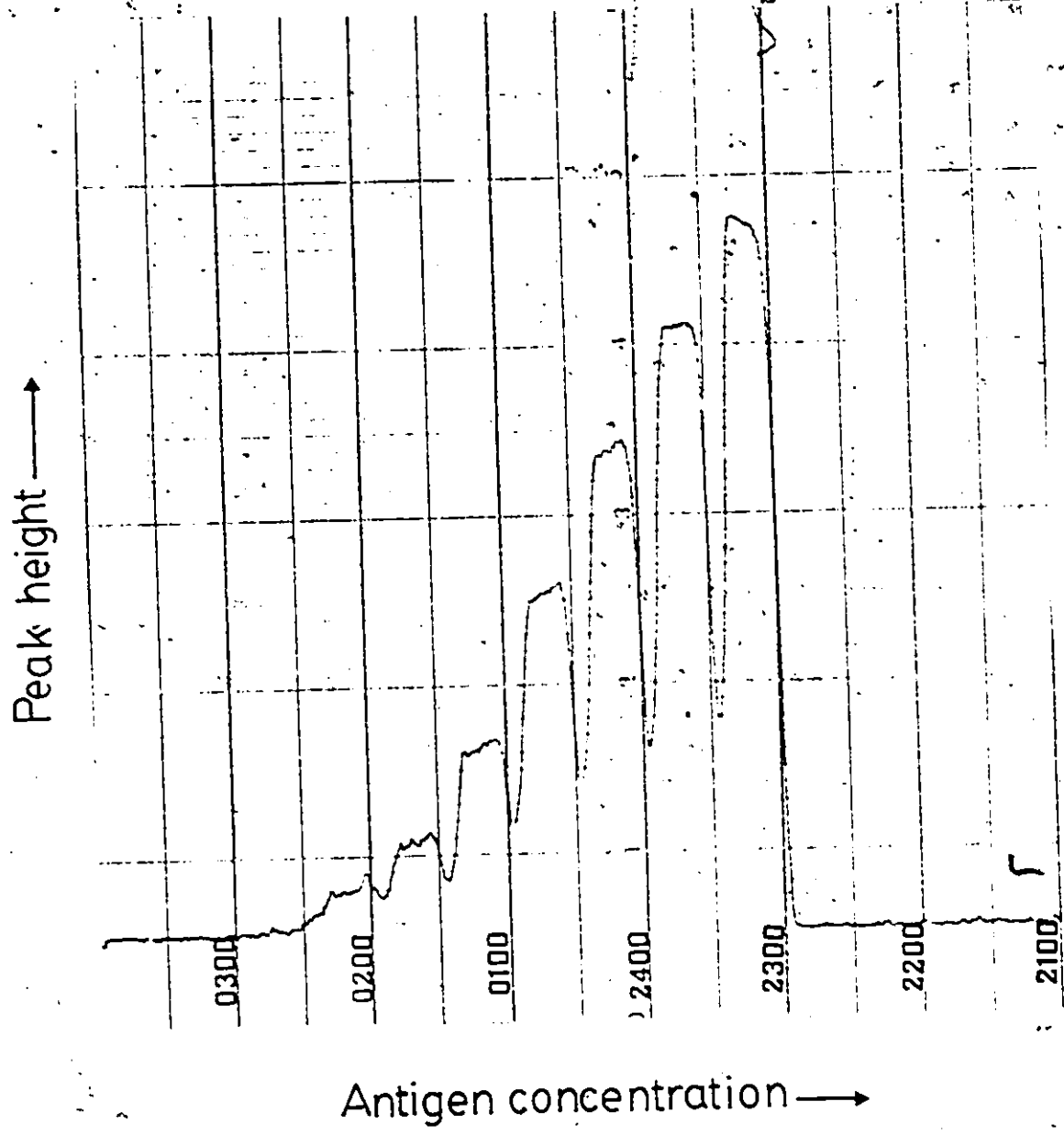
Within a few years after the introduction of the automated nephelometric immunoassay, several workers described systems for the analysis of a variety of human serum proteins including haptoglobin, C3,  $\alpha_1$ -antitrypsin, transferrin, immunoglobulins A, G and M, fibrinogen and cerebro-spinal fluid proteins[91-99]. These systems were almost identical, with various modifications of the basic analytical set-up as shown in Figure 5. In the presence of polyethylene glycol, the length of the polyethylene mixing coil[100] is adjusted to allow a reaction time of 3-5 minutes before optical analysis of the reaction mixture.

The reaction between antigen and antibody in the continuously moving fluid medium produces fairly uniform spheroidal particles that remain unaggregated for a limited length of time[90]. As these particles flow through the flowcell, light scattering increases from the baseline level until a peak level is reached. Then the intensity of the scattered light decreases as the sample passes through the optical path and is replaced by the following washing reagent. This continuous increase and decrease of scattered light intensity gives rise to the characteristic peak tracings as recorded by the recorder (Figure 9).

#### 5. Automated Nephelometric Inhibition Immunoassay

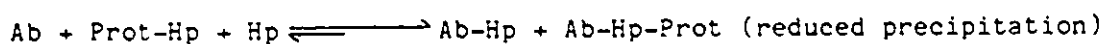
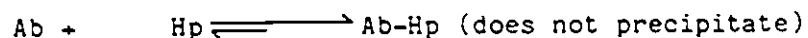
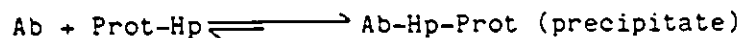
In the automated immunoprecipitin (AIP) system, the protein to be determined is reacted with a slight excess of specific antibodies. The height of the nephelometric peak depends on the concentration of

Figure 9. Recorder tracing of nephelometric peaks



antigen, of antibody and of various factors promoting the antigen-antibody precipitation. With haptens, antigen-antibody precipitation reaction does not occur. However, a protein carrying several residues of hapten molecules (the developer-antigen) can be used in the precipitin reaction. This led to the development of the nephelometric inhibition immunoassay (NINIA)[101,102] for the quantitation of haptens.

The NINIA technique makes use of the fact that antisera contain a fraction of the total immunoglobulins that represent molecules with high affinity or avidity for the corresponding antigen molecules[103-106]. A small amount of hapten can react with this highly active population and effectively remove it from participating in the precipitin reaction. This significantly reduces the overall precipitating capacity of the antiserum[107]. The reactions are summarized below:



There are two approaches by which the quantity of hapten can be assessed. In one, the optimal conditions for precipitation between the developer antigen and the antiserum is first established with the antigen introduced from the sampler. The simultaneous addition of free hapten and developer-antigen to the antiserum will result in a decrease in the nephelometric response. The free hapten competes with the developer antigen for the available antibody combining sites, preferentially interacting with the high affinity antibody molecules

and reducing the formation of precipitates.

Alternately, a nephelometric response can be obtained by pumping a solution of the developer-antigen through the main analytical stream and introducing the antiserum from the sampler. Free hapten can then be preincubated with the fixed amount of diluted antiserum in the sample cups and the residual precipitating capacity of the antiserum is back-titrated with the developer-antigen. The resulting nephelometric response is reduced as the high affinity antibody molecules have preferentially bound with the available hapten molecules.

## II.E. Radioimmunoassay

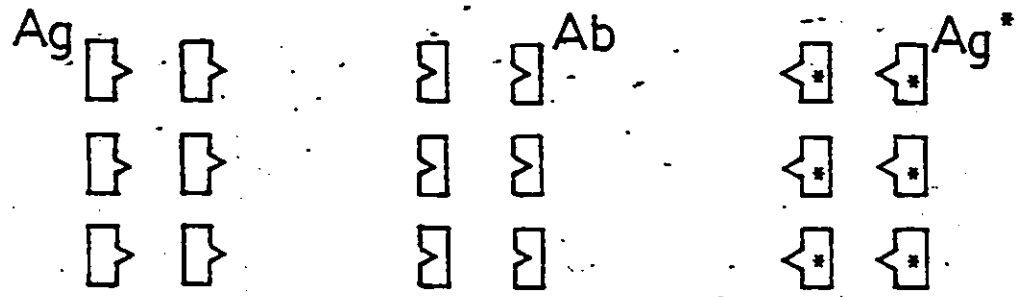
### 1. Principle of Radioimmunoassay Technique[108,109]

Radioimmunoassay (RIA) is one form of competitive binding assay. In the usual form, there are four main reacting components: the antigen, the radiolabelled antigen, the binding antibody and the separation step (Figure 10). The remaining components are buffers, protective agents and extraction chemicals.

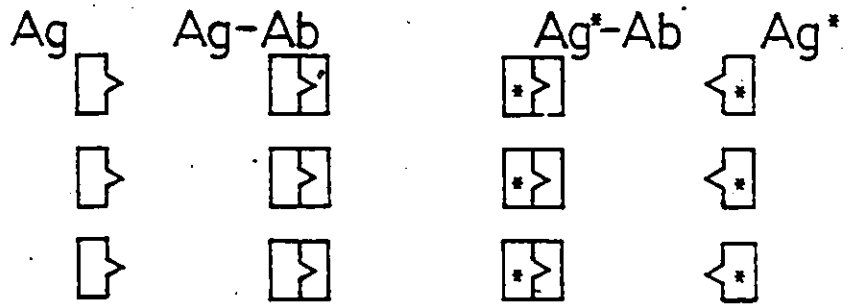
The antigen (Ag) is the subject of the assay in the standard analytical form. It is frequently a substance of relatively low molecular weight (in comparison with the antibody) and is capable of being specifically complexed to the antibody (Ab), which has a molecular weight of around 160,000. The symbol Ab-Ag is used to represent the complex formed by the binding of antigen with antibody.

The antigen is introduced in two forms into the assay, labelled ( $Ag^*$ ) and unlabelled (Ag). The labelled form includes a

Figure 10. Diagrammatic representation of the principle of radioimmunoassay



Incubation



Separation





radioactive element, commonly tritium, carbon-14 or iodine-125. In the course of the assay, both Ag and Ag\* existed in one of two states, bound (B) if they have combined with the antibody or free (F) if they have not.

In the development of the assay, a fixed amount of radioactive labelled antigen is incubated in the presence of an unknown amount of unlabelled antigen with a constant amount of antibody. Ag and Ag\* compete for the limited binding sites on the antibody, and the amount of Ag\* bound will be inversely related to the amount of unknown Ag present.

Either the bound or free or both forms of the Ag\* may be determined separately depending on the separation technique chosen. Ideally, it will be one that will not disturb the equilibrium between the bound and free portions. This separation step may involve a substance such as dextran-coated charcoal or a saturated solution of ammonium sulphate; a second antibody specific to the first one; a physical process such as electrophoresis or dialysis; or a combination of them.

The RIA methods require only that the antigen combine with the antibody (the primary reaction) and there need be no precipitation or other visual evidence (secondary reactions) of this combination. It is this fact that results in the surprising sensitivity of the method. At low levels of antigen and antibody, virtually complete combination takes place rapidly and equilibrium is established in a short period of time.

To determine the amount of unlabelled antigen in each sample, a

standard curve (Figure 11a) can be manually plotted for each assay. The amount of  $Ag^*$  used in preparation of the standard curve is identical to that used in the test. A dilution of the antibody is chosen such that 40-60% of  $Ag^*$  is converted to the bound form in the absence of  $Ag$ . Known amounts of  $Ag$  are added to successive tubes containing the same fixed amounts of  $Ag^*$  and  $Ab$ . More  $Ag^*$  is displaced from the  $Ab$  as increasing amounts of  $Ag$  are added.

Alternately, the standard curve can be plotted with  $B/F$  versus  $B$ . This is known as the Scatchard plot[110] and yields a linear relationship as from the following consideration:

Unoccupied binding sites + Free antigen  $\rightleftharpoons$  Bound antigen

$(Ab - B)$                        $(F)$                        $(B)$

$$F \cdot (Ab - B) / B = K^{-1}$$

where  $B$  is the concentration of the bound antigen,

$F$  is the concentration of the free antigen,

$Ab$  is the total concentration of antibody present, and

$K$  is the equilibrium constant of the reaction.

Rearranging,

$$(Ab - B) = (B/F) \cdot K^{-1}$$

$$B = Ab - (B/F) \cdot K^{-1}$$

which is the Scatchard equation[111]. Assuming there is no discrimination of binding between  $Ag$  and  $Ag^*$ ,

$$B/L = B^*/L^* = y$$

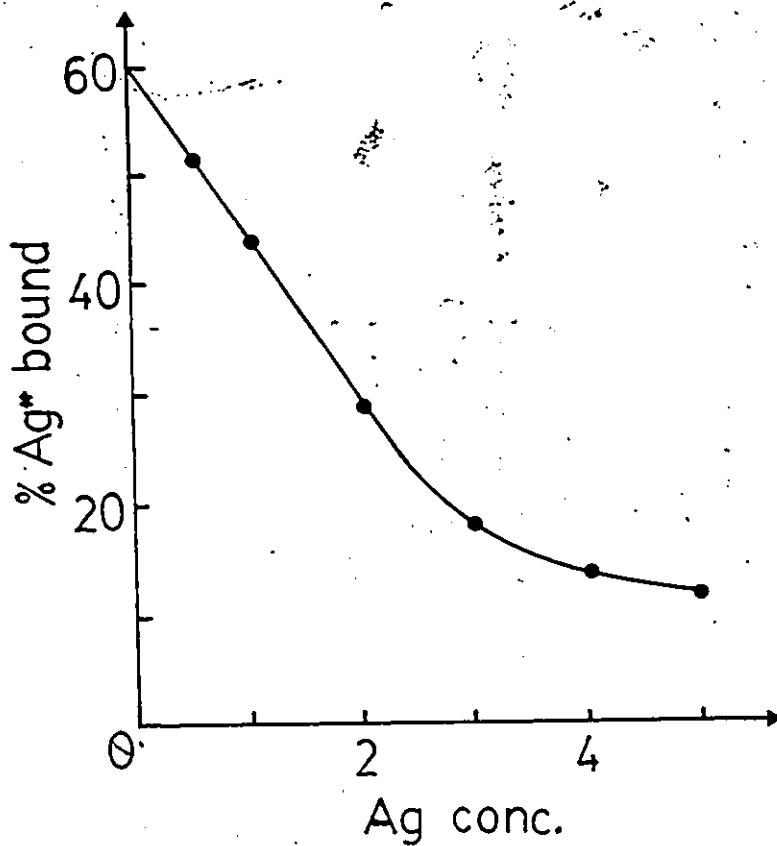
$$B = L \cdot y$$

where  $L$  is the total concentration of labelled and unlabelled antigen

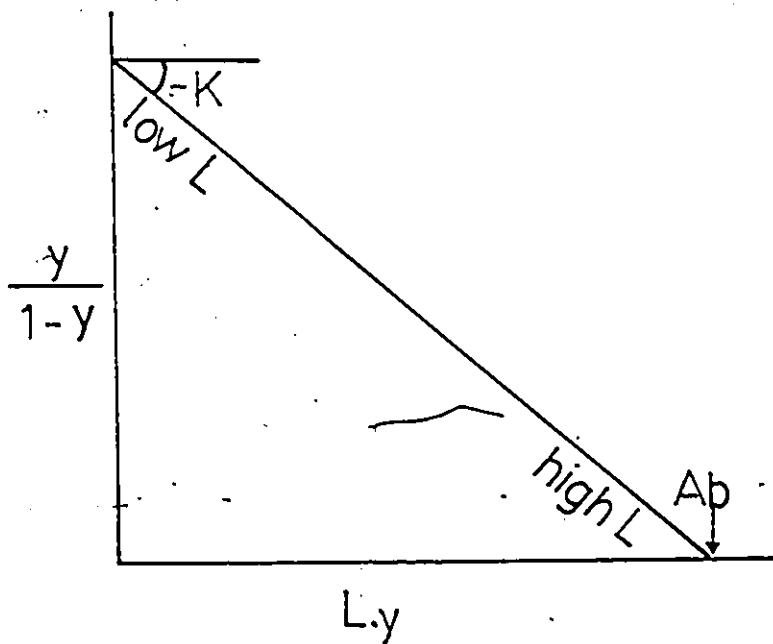
and  $L^*$  is the concentration of labelled antigen alone.

Figure 11. Plotting of the standard curve in radioimmunoassay

- a. % labelled antigen bound versus antigen concentration
- b.  $y/(1-y)$  versus  $L \cdot y$  (The modified Scatchard's plot)



a.



b.

$$B + F = L$$

$$F/L = 1 - B/L = 1 - L.y/L = 1 - y$$

$$B/F = L.y/L.(1 - y) = y/(1 - y)$$

Substituting for B and F, the Scatchard equation is rewritten as:

$$L.y = Ab - K^{-1}.y/(1 - y)$$

Since the standard amount of unlabelled antigen used is known,  $y$  is obtained from the ratio of counts bound to total counts used and  $L$  can be calculated from the specific activity or determined from a standard curve run,  $y/(1 - y)$  can be plotted against  $L.y$  (Figure 11b). With a computer program, counting results can be entered directly and the standard curve calculated and plotted by linear regression. The slope of the linear plot gives  $-K$  and the abscissa intercept gives  $Ab$ .

## 2. Disadvantages of Radioimmunoassay

Since the successful production of digoxin-specific antibodies by Butler and Chen[112], the use of radioimmunoassay technique in the determination of serum or plasma digoxin has been widely accepted. Although this method is adequately specific and sensitive, it has the disadvantages inherent to all radioisotopic methods when used in the routine clinical laboratory: specific isotopic safety considerations, decay of radiolabelled reagents, radioactive waste disposal, the need of highly purified organic solvents in the case of liquid scintillation counting, and also involvement of much manual manipulations. These considerations give rise to the current high cost per test in radioimmunoassay.

It has been recognized that radioimmunoassays are subjected to

interference by unidentified components in serum or plasma[113-115]. The presence of such substances as urea and bilirubin may alter the affinity or avidity of the antigen-antibody reaction[116]. Hemolysis presents a source of error in the RIA of digoxin though it may be corrected by treatment with a hypochlorite bleach[117]. One type of the inhibitors of the antigen-antibody reaction appears to be identical to serum complement[118]. Also, the presence of radiotopes in the plasma or serum because of various diagnostic tests seriously affects the accurate quantitation of radioactivity in either the bound or free phases in the routine RIA of digoxin[119,120].

Since very small volumes of 5-50 microlitres are often used in RIA, the accuracy of pipetting becomes extremely critical. In the methodology itself, there is the obligatory separation of antibody-bound from unbound isotope. Ideally, the separation procedure should not disturb the equilibrium established between the antibody and the antigen. Among the most commonly employed methods, however, it has been reported that the use of dextran-coated charcoal or saturated ammonium sulphate solution does disturb the equilibrium[121,122] so that the time interval from the addition of the separating agent through the physical separation step to the final counting step should be as short and consistent as possible to reduce error. This puts a limit on the maximum number of samples that can be handled at one time. High levels of human IgG in the samples have been shown to interfere in the second antibody technique[123] in which great care has to be exercised also in establishing the optimal equivalence condition for complete precipitation of the bound isotope.

## II.F. Staphylococcal Protein A

Jensen[124,125] noted that most *Staphylococcus aureus* contain an antigen that interacts specifically and strongly with human serum immunoglobulin and causes agglutination. The antigen, which is present in large amount in a Cowan type I strain of the bacteria, was found to be a protein[126] and was named protein A[127]. Lofkvist [128], using a more highly purified antigen preparation, confirmed Jensen's results. Later, protein A was found to be associated mainly with the cell wall of the bacteria[129].

Forsgren and Sjoquist[130] and Kronvall and Frommel[131] identified the corresponding reactive ligand in human serum to be on the Fc portion of IgG molecules. Phylogenetic studies[132,133] indicated that a variable proportion of the serum IgG molecules of most mammalian species exhibited reactivity with protein A. However, the ability of IgG to combine with protein A is restricted to particular subclasses, such as IgG-1, IgG-2 and IgG-4 (not IgG-3) in human[134]; IgG-2 (not IgG-1) in sheep[133]; and IgG-2a, IgG-2b and IgG-3 (not IgG-1) in mice[135]. The percentage of protein A-reactive IgG in non-immune sera ranges from well over 90% in rabbits[133,136] and human[136] to a much smaller proportion in sheep[133].

Isolated protein A was characterized as a functionally bivalent, single polypeptide chain of molecular weight of 42,000[137] containing several regions of internal homology[138]. Equilibrium constants determined for the interaction of this molecule with free human IgG and rabbit IgG ranged from  $4 \times 10^7$  to  $2 \times 10^8$  litres per ..

mole[139-141].

These and related properties have made chemically modified protein A-bearing staphylococci the ideal type of solid-phase adsorbent for antigen-antibody complexes. It has been greatly utilized in antigen isolation studies[141-144] and proven useful in the separation of bound and free portions in RIA[140].

Recent studies have also shown that 30-50% of individual human serum monoclonal IgM and IgA proteins[145,146] and 35-67% of polyclonal IgM from all individuals tested[147] do react with protein A.



### III. EXPERIMENTAL SECTION

#### III.A. Apparatus

Calibrated volumetric ware and serological pipettes were used when appropriate. For microlitre volume pipetting, a Gibson Pipetman model P200 was used with Eppendorf (100 uL) disposable pipette tips.

Weighings were made on a Mettler semi-micro, constant load analytical balance (Type H20T).

Measurements of pH were made with a Radiometer pH meter, model pH Meter 26, fitted with a Radiometer GK2322 C combined electrode.

Centrifugation at low speeds were performed either in a Sorvall Superspeed RC-2B centrifuge or an International Equipment Company model PR-J centrifuge. Ultracentrifugation was performed in an International Equipment Company model B-60 ultracentrifuge. When Brinkman 1.5 ml polypropylene micro test tubes were used in radioimmunoassay, centrifugation was performed in an Eppendorf model 3200 centrifuge.

Spectrophotometric measurements for protein determination were made with a Beckman ACTA III dual beam spectrophotometer.

Nephelometry was performed with the AutoAnalyzer II system from Technicon Instruments Corporation (Tarrytown, N.Y.). Modules include a fluoronephelometer, a proportioning pump, a sampler, a recorder and a manifold with the necessary tubings and connectors. The mixing coil was made from intramedic polyethylene tubing PE-280 (internal diameter 2.15 mm, external diameter 3.25 mm) from Clay Adams, Becton Dickinson and Company. The tubing was wound around a 15 mm diameter test-tube

and was heat-treated in boiling water for two minutes followed with rapid cooling in cold running tap water. Nalgene 0.2 um membrane filtering units were obtained from Nalge Company.

For the growth of *Staphylococcus aureus*, incubation was done in a Controlled Environment Incubation Shaker from New Brunswick Scientific Company.

Radioactive beta-counting was made with a Beckman LS-230 liquid scintillation system and gamma-counting was made with a Beckman GAMMA-300 system.

Radioimmunoassay calculations were made with a Wang 600 programmable calculator equipped with a Wang 612 plotter.

### III.B. Reagents

All common laboratory chemicals were reagent grade.

Bovine serum albumin (Pentax Bovine Albumin, crystallized, 98%) was purchased from Miles Laboratories, Incorporated. Keyhole limpet hemocyanin was obtained from Pacific Bio-marine Supply Company. Ferritin and  $^{59}\text{Fe}$ -125 labelled ferritin were received as a gift from Dr. W.H.C. Walker, Department of Pathology, McMaster University.

Digoxin (crystalline), octyl phenoxy polyethoxyethanol (Triton X-100) and niacin were purchased from Sigma Chemical Company.

Lanoxin (0.25 mg digoxin/ml, 2 ml/vial) was purchased from Burroughs Wellcome and Company.

Polyethylene glycol 6000 (averaged molecular weight 6000 to 7500) was obtained from J.T. Baker Chemical Company.

Phenol reagent solution (Folin-Ciocalteu, 2N), PPO

(2,5-diphenyloxazole), POPOP (1,4-bis(2-(5-phenyloxazolyl)) benzene), Tween 20 (polyoxyethylene sorbitan monolaurate) and toluene (scintillation grade) were purchased from Fisher Scientific Company.

Digoxin, tritium labelled (general) and I-125 labelled, were obtained from New England Nuclear Company.

*Staphylococcus aureus* Cowan I strain (ATCC 12578) was obtained as a subculture from Dr. H. Richardson, Department of Pathology, McMaster University.

Complete Freund's adjuvant (Bacto), Penassay Broth (Bacto), vitamin free casamino acids (Bacto) and yeast extract (Bacto) were purchased from Difco Laboratories.

Thiamin hydrogen chloride was obtained from Merck and Company, Limited.

$\beta$ -glycerophosphate (sodium salt, pentahydrate) was obtained from Calbiochem.

1,2-dichloroethane was purchased from Eastman Chemical Company.

The Solid Phase Digoxin Reagent System for radioimmunoassay of digoxin was purchased from Beckman Instrument Incorporated.

### III.C. Protein Determination by Folin's Method

The concentration of protein solutions were determined spectrophotometrically according to the method by Folin [148]. Aqueous standards were prepared volumetrically with a weighed amount of bovine serum albumin. A sodium carbonate mixture was freshly prepared by mixing together 1 ml of 2% sodium tartrate, 1 ml of 1% cuprous sulphate and 100 ml of 2% sodium carbonate in 0.1M sodium hydroxide.

For each sample or standard, 0.05 ml of solution was mixed with 2 ml of freshly prepared sodium carbonate mixture and 0.2 ml of phenol reagent of Folin-Ciocalteu (diluted 1:1 with distilled water). Saline or distilled water (0.05 ml) was used in place of standard for blanks. After thorough mixing and standing at room temperature (22°C) for 30 minutes, absorbance was read at 750 nm.

#### III.D. Purification of Keyhole Limpet Hemocyanin

The protein keyhole limpet (*Magathura crenulata*) hemocyanin was obtained as an ammonium sulphate slurry. The purification was adapted from Herscowitz [149] for crude KLH preparation.

The ammonium sulphate slurry of KLH (11 gm) was suspended in 50 ml of phosphate buffered saline (PBS, pH 7.2, 0.15M phosphate, 0.15M NaCl) and placed in a cellophane dialysis bag. It was dialysed against two changes of PBS (3 litres each) at 4°C for 24 hours. The dialysate was centrifuged at 1400xg for 30 minutes at 4°C. The grey sedimented material was discarded and the blue supernatant was centrifuged at 78,000xg in an IEC B-60 ultracentrifuge for 4 hours at 4°C. The colourless supernatant was discarded and the blue sediment was resuspended in 20 ml of PBS. This alternating centrifugation at low and then high speed was repeated three times and the crude KLH was finally resuspended in 8 ml of PBS. The protein concentration was determined by Folin's method and the solution stored at -20°C until use.

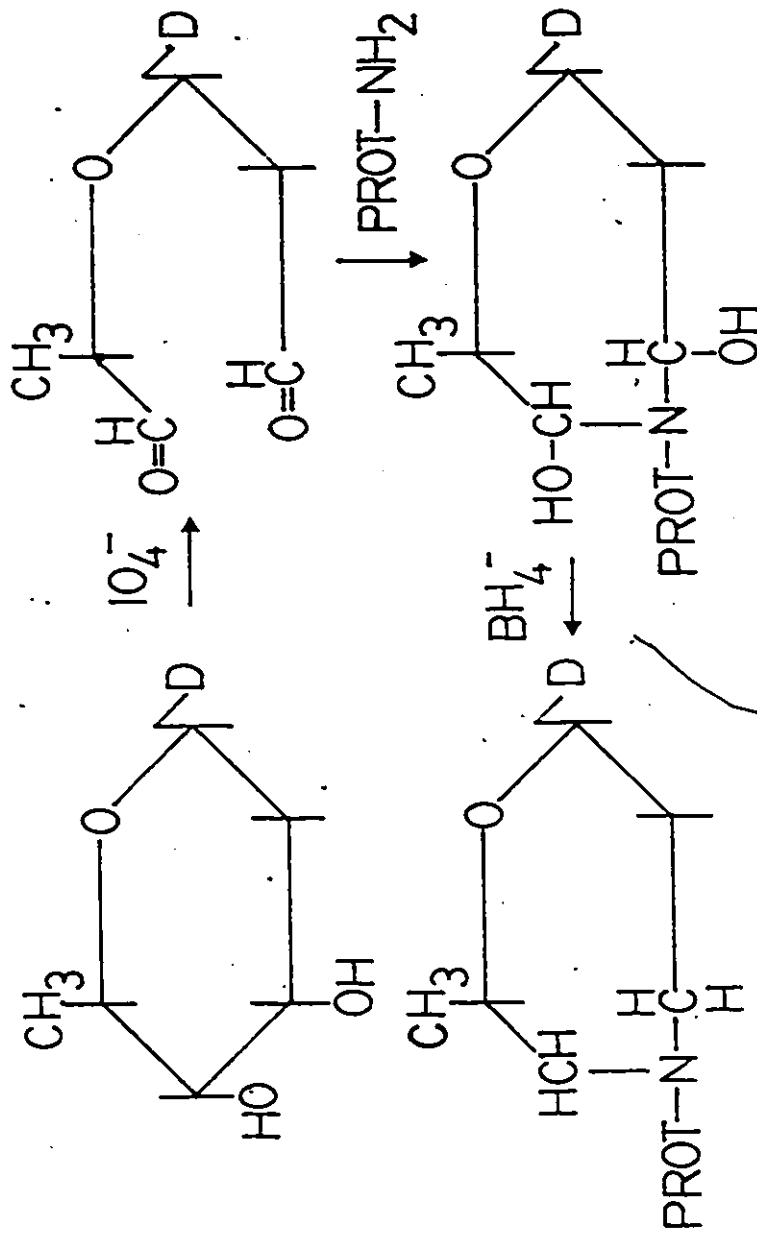
### III.E. Synthesis of Protein-Hapten Conjugates

#### 1. Bovine Serum Albumin-Digoxin (BSA-Digoxin)

The preparation of BSA-digoxin was by an adaption of the method described by Butler and Chen[122] (Figure 12).

Digoxin in crystalline form (100 mg) was dissolved in 2 ml of dimethyl sulphoxide and 2 ml of absolute ethanol at room temperature. Sodium periodate (4 ml, 0.1M) was added and the solution was gently mixed by magnetic stirring for one hour. Ethylene glycol (150  $\mu$ l, 1M) was then added and mixed. After five minutes, the reaction mixture was added dropwise with magnetic stirring to a solution of 100 mg of BSA in 5 ml of water, previously adjusted to pH 9.5 with 5% potassium carbonate. The pH was maintained at 9.0 to 9.5 by further dropwise addition of 5% potassium carbonate. After one hour, sodium borohydride (70 mg freshly dissolved in 3 ml of water) was added. Three hours later, the pH was lowered to 6.5 by dropwise addition of formic acid (1M) and after another hour, the pH was raised to 8.5 by the addition of aqueous ammonia (1M). The reaction mixture was then placed in a cellophane dialysis bag and dialysed overnight against cold running tap water. Hydrochloric acid (0.1M) was then added to the dialysate to lower the pH to 4.5 and the mixture was allowed to stand at room temperature for one hour and then at 4°C for four hours. The precipitate was collected by centrifugation at 1000xg for 30 minutes at 4°C. The precipitate was dissolved in 3 ml of sodium hydrogen carbonate (0.15M) and dialysed for two days against cold running tap water and then against four changes of saline (0.15M NaCl, 3 litres

Figure 12. Synthesis of protein-digoxin conjugate



each) at 4°C over the next two days.

The protein concentration of the final dialysate was determined by Folin's method. The solution was aliquoted (1 ml) and stored at -20°C.

## 2. Bovine Serum Albumin-Digoxin-<sup>3</sup>H (BSA-Digoxin-<sup>3</sup>H)

In order to examine the relationship between hapten to protein ratio of the developer-antigen and precipitability with antibody, digoxin was conjugated to BSA at different starting molar ratios. Tritiated digoxin (200 ng, 4.85 Ci/m-mole) giving 1.4 million cpm was added to provide an estimate of the amount of digoxin covalently bonded to each prepared conjugate.

Four BSA-digoxin-<sup>3</sup>H conjugates were prepared by reacting differing amounts of crystalline digoxin (150, 100, 50 or 25 mg), mixed with 200 ng of tritiated digoxin, with 50 mg of BSA. The other reagents (dimethyl sulphoxide/ absolute ethanol solvent, sodium periodate, ethylene glycol and sodium borohydride) were adjusted proportionally. The procedure in section 1 above was otherwise followed.

After the final dialysis against saline, the protein concentration of the dialysate was determined by Folin's method and aliquots of the dialysate were counted for radioactivity.

## 3. Keyhole Limpet Hemocyanin-Digoxin (KLH-Digoxin)

The synthesis of KLH-digoxin followed closely the procedure for BSA-digoxin but with a simplified work-up of the reaction mixture.



A solution of 50 mg of KLH in 5 ml of phosphate buffered saline (PBS, pH 7.2, 0.15M phosphate, 0.15M NaCl) was adjusted to pH 9.5 with 10% potassium carbonate. Crystalline digoxin (50 mg) was dissolved in 1 ml of dimethyl sulphoxide and 1 ml of absolute ethanol. Sodium periodate (2 ml, 0.1M) was added and the suspension was mixed gently by magnetic stirring for one hour after which ethylene glycol (75  $\mu$ L, 1M) was added and mixed for five minutes. The reaction mixture was then added dropwise to the solution of KLH with magnetic stirring and the pH was maintained at 9.0 to 9.5 with further addition of potassium carbonate solution (10%). After one hour, 35 mg of sodium borohydride (freshly dissolved in 2 ml of water) was added. Three hours later, the pH was lowered to 6.5 by dropwise addition of formic acid (1M) and after another hour, the pH was raised to 8.5 by the addition of aqueous ammonia (1M). The reaction mixture was then placed in a cellophane dialysis bag and dialysed in cold running tap water for two days and then against four changes of saline (0.15M NaCl, 3 litres each) at 4°C over the next two days.

The protein concentration of the final dialysate was determined by Folin's method. The solution was aliquotted (1 ml) and stored at -20°C.

### III.F. Immunization of Rabbits

White New Zealand rabbits (about 3 kg) were each immunized with 1 mg of purified keyhole limpet hemocyanin (1 ml) emulsified with 1 ml of complete Freund's adjuvant. Injections were made into deep intramuscular sites on the four legs. After three weeks, each rabbit

was boosted with 2.4 mg of KLH-digoxin (1 ml) without adjuvant to make use of the carrier effect[150]. Subsequent injections with KLH-digoxin were made without adjuvant. Bleeds were taken by puncture at the ear vein of the rabbits. The collected blood was clotted at 37°C and centrifuged at 500xg for 20 minutes. The sera were taken and centrifuged at 10,000xg for 15 minutes before being tested for reactivity with digoxin by radioimmunoassay.

### III.G. Radioimmunoassay of Digoxin

#### 1. Titre of Antisera

Each serum was tested for reactivity by assessing its binding capacity with tritiated digoxin (digoxin-<sup>3</sup>H), which was diluted before use in phosphate buffer (pH 7.4, 0.05M phosphate) to give a solution having about 28,000 cpm/ml.

A serial dilution was made of the serum in the digoxin-<sup>3</sup>H-phosphate buffer. Pooled normal human plasma containing no digoxin was mixed with 0.5 ml of each serum dilution. The tubes were vortexed and incubated for 45 minutes at 37°C. Saturated ammonium sulphate solution (1 ml, 4.1M) was added to each tube and vortexed, and the tubes were centrifuged for 20 minutes at 2500xg in a refrigerated centrifuge at 4°C. The supernatant (1 ml) was pipetted into 10 ml of scintillation fluid (4 gm PPO, 0.05 gm POPOP, 250 ml Triton X-100 and 750 ml toluene to give one litre) and counted. The dilution at which 40% of the amount of digoxin-<sup>3</sup>H was bound was taken to be the titre.

## 2. Radioimmunoassay Procedure

### 2.a. Digoxin-<sup>3</sup>H Tracer and Ammonium Sulphate Separation

All samples and standards were done in duplicate. Digoxin-<sup>3</sup>H was diluted in phosphate buffer (pH 7.4, 0.05M phosphate) to give a solution having about 28,000 cpm/ml. Antiserum was diluted with the digoxin-<sup>3</sup>H-phosphate buffer to the titre of that particular serum. Standards were prepared by first diluting 0.8 ml of Lanoxin (Burroughs Wellcome and Co., 0.25 mg digoxin per ml) to 200 ml with 10% ethanol in water, giving a concentration of 1 ug/ml. Then 0.5 ml of this alcohol solution was made up to 100 ml with pooled normal human plasma (containing no digoxin) to give a stock standard concentration of 5 ng/ml. The series of working standards (0-5 ng/ml) was prepared by appropriate dilution of the stock standard with the same pooled plasma.

Each standard or sample (0.5 ml) was mixed with 0.5 ml of the diluted antiserum in digoxin-<sup>3</sup>H-phosphate buffer. The tubes were vortexed and incubated for 45 minutes at 37°C. Saturated ammonium sulphate solution (1 ml, 4.1M) was added to each tube. The tubes were vortexed and centrifuged at 2500xg for 20 minutes at 4°C. The supernatant (1 ml) was pipetted into 10 ml of scintillation fluid and counted for two minutes. The value for total counts was obtained by counting 0.25 ml of the diluted antiserum in digoxin-<sup>3</sup>H-phosphate buffer.

Results for the samples were obtained by the use of a computer

program with which the standard curve was calculated by linear regression of the Scatchard's equation.

#### 2.b. Digoxin-<sup>125</sup>I Tracer and Second Antibody Separation

The Solid Phase Digoxin Reagent System (Beckman Instrument Inc.) was used and the instruction with the kit was strictly followed in its use. Each kit contains all the necessary reagents which includes the following:

- (1) I-125 labelled digoxin, lyophilized. Each vial contains digoxin labelled with less than ten microcuries of I-125. The iodine is attached to an histidine group which is covalently bonded to the terminal sugar of the digoxin molecule.
- (2) Rabbit antiserum to digoxin, lyophilized. Each vial contains antiserum to digoxin prepared in rabbits, mixed with an inert blue food dye as an indicator. Binding of labelled digoxin in the absence of unlabelled digoxin was about 60%.
- (3) Digoxin solid phase precipitating antibody (goat anti-rabbit gamma globulin). The goat anti-rabbit gamma globulin is fractionated from serum from goats immunized with rabbit gamma globulin, and is covalently bonded to microcrystalline cellulose. The solid phase precipitating antibody is suspended in 0.025M sodium citrate, 0.10M sodium chloride, 0.005M EDTA, 0.1% gelatin, 0.1% bovine serum albumin, 0.01% sodium azide and 0.01% Triton X-100.
- (4) Digoxin buffer, lyophilized. The buffer is composed of digoxin-free human defibrinated plasma.
- (5) Digoxin standards, lyophilized. The standards are prepared from

USP digoxin and are standardized against USP Reference Standard Digoxin (Lot No. L-1). The standard concentrations are 0.5, 1, 2, 3, 4 and 6 ng/ml.

(6) Digoxin control serum, lyophilized. The vial contains human serum containing a predetermined amount of digoxin specified on the vial label.

Reagents (1) and (2) were each reconstituted with 5 ml/vial of 5% methanol in sterile distilled water, and contained 0.025M sodium citrate, 0.1M sodium chloride, 0.005M EDTA, 0.1% gelatin, 0.1% bovine serum albumin and 0.01% sodium azide. Reagent (4) was reconstituted with 5 ml/vial of sterile distilled water. Reagent (5) and (6) were each reconstituted with 2 ml/vial of sterile distilled water. All reagents were stored at 4°C and before use, were swirled gently to mix and let stand at room temperature for 30 minutes.

Tests were performed in duplicate. The entire contents of the digoxin-<sup>125</sup>I vial were transferred to combine with the solid phase precipitating antibody. Continuous mixing was by magnetic stirring. To each of the respectively labelled tube was added 100 uL of either buffer, standard, control serum or patient sample. The digoxin-<sup>125</sup>I-precipitating antibody mixture (300 uL) and dilute anti-digoxin antiserum (100 uL) were then added and the content was mixed by gentle vortexing. The tubes were then incubated for at least 30 minutes at room temperature. Blank tubes were prepared by mixing 100 uL of digoxin buffer, 100 uL of saline (0.15M NaCl) and 300 uL of digoxin-<sup>125</sup>I-precipitating antibody mixture. The total count tubes contained only 300 uL of the digoxin-<sup>125</sup>I-precipitating

antibody mixture.

After incubation, 0.5 ml saline was added to each tube (except total count tubes) and the tubes were immediately centrifuged for 3 minutes at 5000xg. Immediately afterward, the supernatant was decanted and discarded, and the remaining supernatant that rimmed the top of the tube was blotted.

The tubes were then counted for 2 minutes in a well-type gamma counter, and the calculations were done using a computer program based on the linear regression of the Scatchard's equation.

#### III.H. Radioimmunoassay of Ferritin

The procedure for the radioimmunoassay of ferritin using second antibody separation was according to Luxton et al. [151]. Standards were performed in duplicate. Samples were performed at two dilutions, straight serum and serum diluted 1/2 with 1% bovine serum albumin in phosphate buffered saline (1% BSA-PBS, pH 7.8, 0.01M phosphate, 0.15M NaCl). Ferritin-<sup>125</sup>I was diluted with 1% BSA-PBS to give 100,000 cpm per ml. Rabbit anti-ferritin antiserum was diluted 1/500,000 with 2% normal rabbit serum in EDTA-phosphate buffered saline (2% NRS-EDTA-PBS, pH 7.8, 0.01M phosphate, 0.15M NaCl, 0.005M EDTA). Goat anti-rabbit gamma globulin (GARGG) was diluted 1/10 with phosphate buffered saline (PBS, pH 7.8, 0.01M phosphate, 0.15M NaCl). Polyethylene glycol (10% PEG) was prepared in phosphate buffered saline (PBS, pH 7.8, 0.01M phosphate, 0.15M NaCl).

Samples or standards (50 uL) were mixed with 100 uL of diluted rabbit anti-ferritin antiserum and 100 uL of diluted I-125 labelled

ferritin tracer. The background controls were set up with 50 uL of 1% BSA-PBS instead of serum and 100 uL of 2% NRS-EDTA-PBS in place of rabbit anti-ferritin antiserum. The tubes were vortexed and incubated at 4°C for 24 hours. GARGG (100 uL) and 10% PEG (100 uL) were added to each tube to give a final PEG concentration of 2.2%. The tubes were vortexed and further incubated at 4°C for a minimum of one hour to allow complete precipitation between the first and the second antibodies. The tubes were then centrifuged for 30 minutes at 4°C and 1500xg. The supernatant was carefully aspirated and the precipitate was counted in a well-type gamma counter for one minute.

The value for the total radioactivity used was obtained by counting 100 uL of the diluted ferritin tracer in 1% BSA-PBS. Calculations were performed using a computer program with linear regression of the Scatchard's equation.

### III.I. Growth of Staphylococcus aureus

*Staphylococcus aureus* Cowan I strain was obtained as a subculture from Dr. H. Richardson, Department of Pathology, McMaster University.

The method of growth was adapted from Kessler[143]. The growth medium was a fortified Penassay broth, supplemented with vitamin-free casamino acids (10 mg/ml),  $\beta$ -glycerophosphate (5 mg/ml, sodium salt, pentahydrate), yeast extract (2.5 mg/ml), niacin (4 ug/ml) and thiamin hydrogen chloride (2 ug/ml). Niacin and thiamin solutions were sterilized by filtration (Nalgene 0.2 um membrane unit, sterilized) and added after the medium was autoclaved and cooled. A total medium of

five litres was divided into six 2-litre conical flasks for each incubation.

Individual colony from a streaked blood agar plate was used to inoculate a small primary broth (10 ml) maintained at 37°C for eight hours with shaking. About one ml of this was inoculated into each of the six flasks together with the necessary niacin and thiamin solutions. These were then incubated overnight (18-24 hours) at 37°C in a rotary shaker.

The bacteria were collected and washed twice by centrifugation at 8000xg for 20 minutes in phosphate buffered saline (PBS, pH 7.2, 0.15M NaCl, 0.04M phosphate) containing 0.05% sodium azide (PBS-azide). They were resuspended in PBS-azide at a concentration of about 10% (w/v) and were stirred at room temperature for 90 minutes in the presence of 1.5% formaldehyde. They were then washed once in PBS-azide and resuspended at the same concentration in the buffer with formaldehyde.

The bacteria were transferred to a 500-ml round bottom flask attached to a rotary evaporator turning at maximum speed. The flask was lowered into a water bath at 80°C for four minutes for rapid heat-killing of the bacteria, followed by rapid cooling in an ice-water bath. The bacteria were washed twice in PBS-azide and resuspended at 10% (w/v) in the same buffer. The final suspension was stored at 4°C or -20°C until use.

Immediately before use, the bacteria were pelleted by centrifugation at 2000xg for 30 minutes at 4°C. They were resuspended and incubated in 0.5% Triton X-100 in NaCl-EDTA-Tris



buffer (NET buffer, pH 7.4, 0.15M NaCl, 0.005M EDTA, 0.05M Tris, 0.02% sodium azide) for 15 minutes at room temperature. The bacteria were washed once in 0.05% Triton X-100 in NET buffer and finally resuspended at 10% (w/v) in NET buffer containing 0.05% Triton X-100.

## IV. RESULTS AND DISCUSSION

### IV.A. Automated Nephelometric Inhibition Immunoassay of Digoxin

#### 1. Optimization of the Analytical System

The technique of automated immunoprecipitin (AIP) looks primarily at the small amount of high affinity antibody present in an antiserum[103,104]. Cambiaso et al[102] had inferred that it is this high affinity antibody which is most sensitive to inhibition and that the most sensitive conditions for nephelometric inhibition immunoassay (NINIA) would be to use a high titred antibody at high dilutions.

Another theoretical consideration is to use low concentrations of developer antigen having a low hapten to protein ratio in the conjugate. Complexes formed from such a conjugate would be more prone to disruption in the presence of small amounts of free hapten. Thus the intention was to optimize the assay conditions to approach the theoretical system exhibiting high sensitivity.

#### 1.a. Hapten-Protein Ratio of Developer Antigens

To examine the relationship between the hapten to protein ratio of the developer antigen and precipitability or sensitivity to inhibition, digoxin was conjugated to bovine serum albumin (BSA) at different starting molar ratios. Four different conjugates were prepared by the method described in Section III.D.2 (Table I). It was noted that at a lower starting digoxin/BSA ratio than 19.2 to one, the

Table I. Estimation of digoxin/BSA ratios of various conjugates

Molar ratio of dig./BSA used in synthesis	% recovery of BSA in conjugates	% recovery of <sup>3</sup> H-digoxin in conjugates	Digoxin/BSA ratios in conjugates
115.2:1	68.7	—	7.3(*)
76.8:1	44.4	3.60	6.23
38.4:1	68.5	7.94	4.45
19.2:1	29.6	5.04	3.27

(\*) Estimated from precipitin data of Table II and Figure 13

#### Sample calculation of digoxin/BSA ratios in conjugates

Starting digoxin/BSA ratio in synthesis = 76.8:1

For 1 mole of BSA used, recovery =  $1 \times 44.4\% = 0.444$  mole

For 76.8 moles of digoxin used, recovery =  $76.8 \times 3.6\%$   
= 2.765 moles

Digoxin/BSA ratio in conjugate =  $2.765/0.444$   
= 6.23

conjugate precipitated from the reaction mixture only with great difficulty, and the amount recovered was so little that preparation was not practical.

Recovery estimation and calculation of the digoxin/BSA ratio of each conjugate prepared was done using tritiated digoxin in the synthesis. Protein concentration of each conjugate was determined by the Folin's method. Table I shows the recovery of the various preparations and the resulting molar ratios of digoxin/BSA in the conjugates. A sample calculation of the digoxin/BSA molar ratio in the conjugate is also included.

The precipitability of each developer antigen was tested at 10 ug/ml against a 1/30 dilution of two antisera from different rabbits. Due to the physical nature of digoxin, conjugates containing more digoxin per mole BSA exhibited a distinct hydrophobic character. In dilute solutions in the presence of polyethylene glycol (4% PEG, 0.1% Tween 20, 0.15M NaCl) it would rapidly precipitate. Since this would interfere with the methodology, the experiments were performed in the presence of BSA (2.5 mg/litre). This protein matrix altered the solubility characteristics of the conjugates. Table II and Figure 13 show the peak heights obtained with the various conjugates indicating that the precipitability of the conjugates was directly proportional to the substitution with digoxin.

To examine the sensitivity to inhibition of the various conjugates, 1 ml of each conjugate at 10 ug/ml concentration was mixed with varying amounts of free digoxin (5-250 ng) in the sample cups. These were run against antiserum diluted 1/30. From Table III and

Table II. Precipitability of various BSA-digoxin conjugates

Digoxin BSA ratio of conjugates	Peak heights with 10 ug/ml concentration	
	Antiserum 1 (1/30)	Antiserum 2 (1/30)
7.3	36	24
6.23	28	18.5
4.45	14	9
3.27	4	3

Table III. Sensitivity to inhibition of various BSA-digoxin conjugates

Digoxin/BSA ratio of conjugates	Uninhibited peak height (chart units)	% uninhibited peak height with free digoxin added (ng)				
		5	10	50	100	250
7.3	37	100	100	100	95.9	78.4
6.23	28	100	101.8	<del>96.4</del>	89.3	78.6
4.45	15.5	100	100	100	90.3	71.0

Figure 13. Precipitability versus moles digoxin per mole BSA

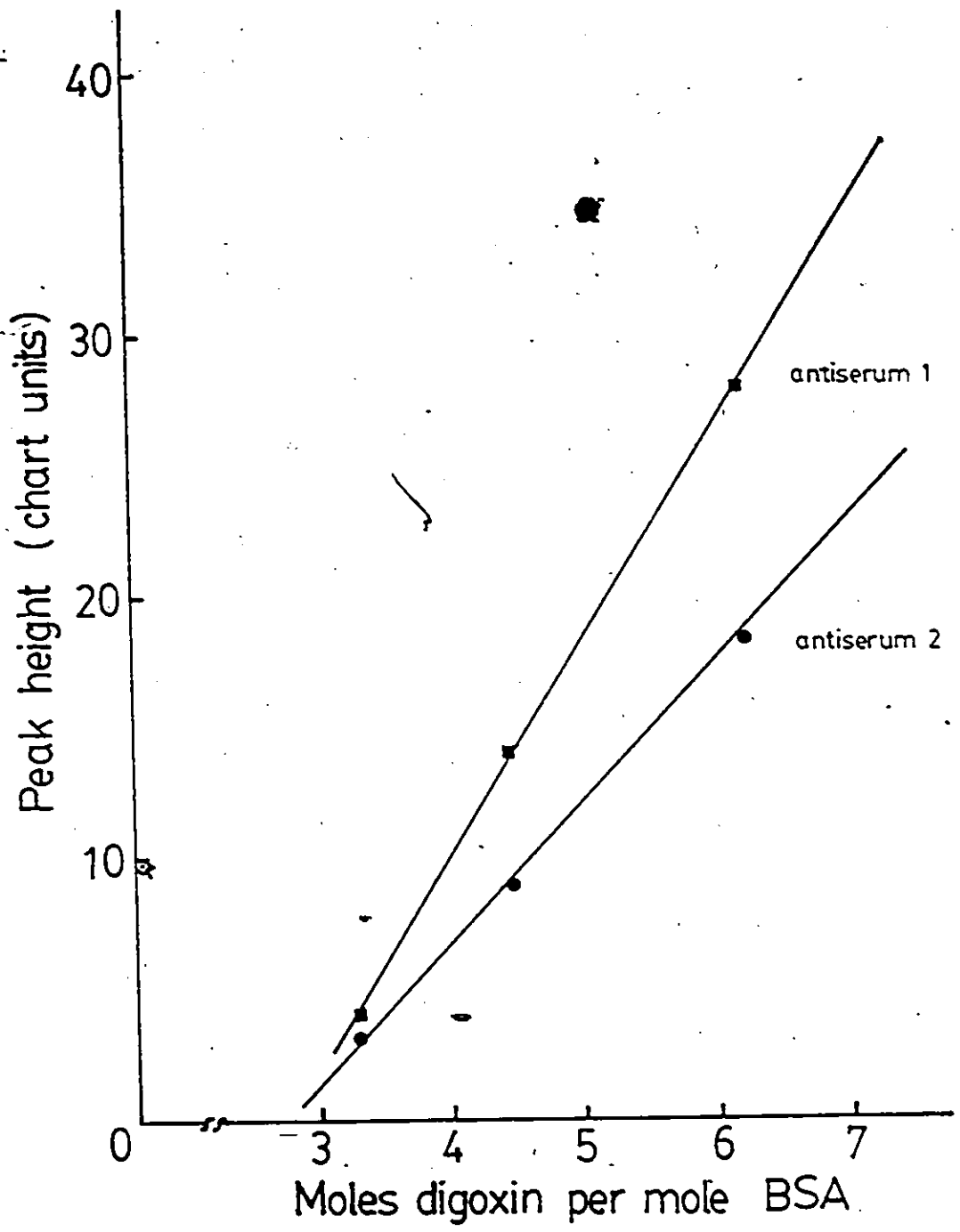


Figure 14. Sensitivity to inhibition of various BSA-digoxin conjugates



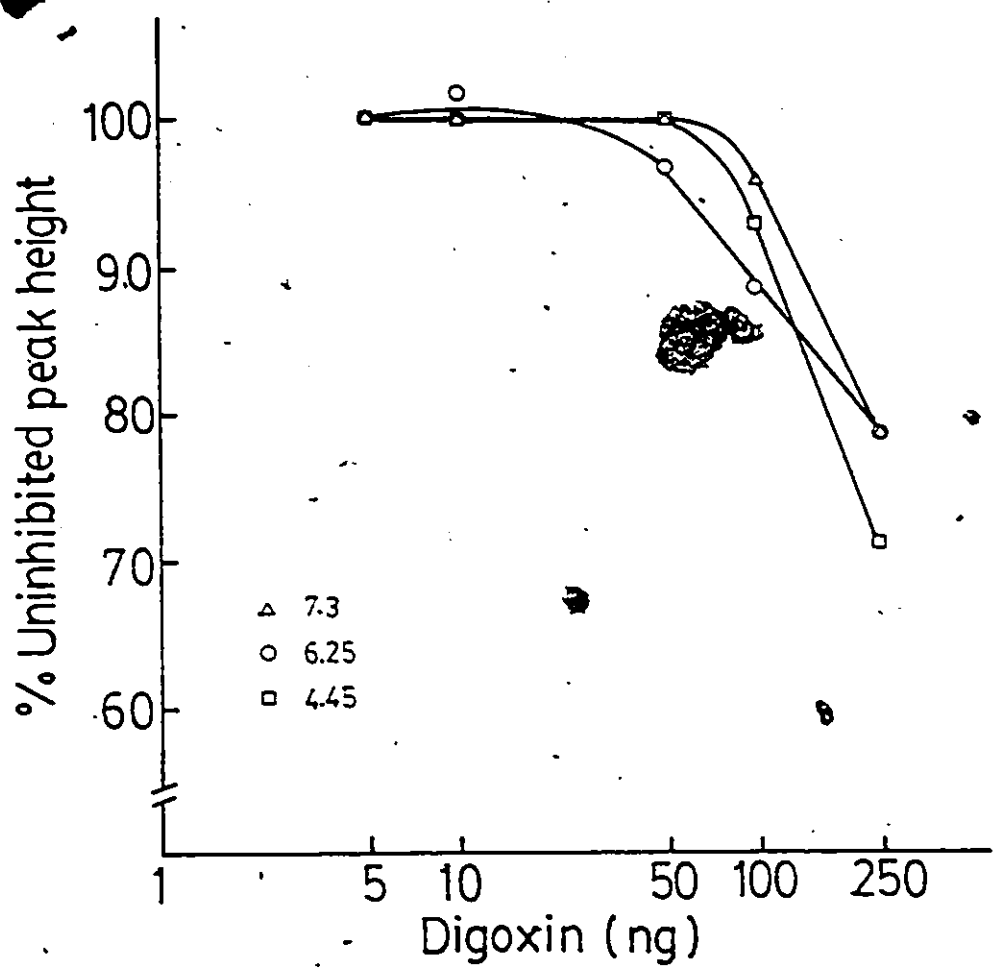


Figure 14, the conjugate having 6.23 residues of digoxin bound per mole of BSA exhibited the optimum characteristics of good measurable peak heights while being most sensitive to inhibition by added free digoxin. Thus it does not appear to be either the conjugate with the highest or the lowest digoxin/BSA ratio which exhibits the greatest sensitivity to inhibition but rather an intermediate one.

#### 1.b. Temperature of Incubation

Previously it has been noted that a change in the temperature of incubation could affect the results in radioimmunoassay [152]. To assess the effect of a change in temperature on the sensitivity to inhibition in our assay the reaction was carried out at three temperatures (4, 22 and 37 degrees centigrade) both for simultaneous addition of free digoxin and for preincubation of free digoxin with antiserum.

In the simultaneous addition method, free digoxin (5-250 ng) was added to 1 ml of the developer antigen (10 ug/ml, digoxin/BSA ratio=6.23). The various temperatures were attained by submerging the reaction coil in waterbaths at the respective temperatures. Table IV and Figure 15 show that there was a general increase in the precipitin peak height as the temperature was increased. However, at 37°C, the sensitivity to inhibition was notably lowered. Between 22°C and 4°C, the difference in sensitivity was small, with a slightly better sensitivity exhibited at 22°C. As a result, all further experiments were performed with the coil at room temperature.

Table IV. Variation of temperature on sensitivity to inhibition  
(Simultaneous addition)

Temperature of coil	Uninhibited peak height (chart units)	% uninhibited peak height with free digoxin added (ng)				
		5	10	50	100	250
37°C	31.5	100	100	98.4	98.4	82.5
22°C	28	100	101.8	96.4	89.3	78.6
4°C	25	100	98	96	88	72

Table V. Variation of temperature on sensitivity of inhibition  
(Preincubation)

Temperature of incubation	Uninhibited peak height (chart units)	% uninhibited peak height with free digoxin added (ng)				
		5	10	50	100	250
37°C	96	100	99	--	82.3	65.6
22°C	95	100	100.5	90.5	76.3	68.9
4°C	90	100	98.9	86.1	77.2	72.2

Figure 15. Variation of temperature on sensitivity to inhibition  
(Simultaneous addition)

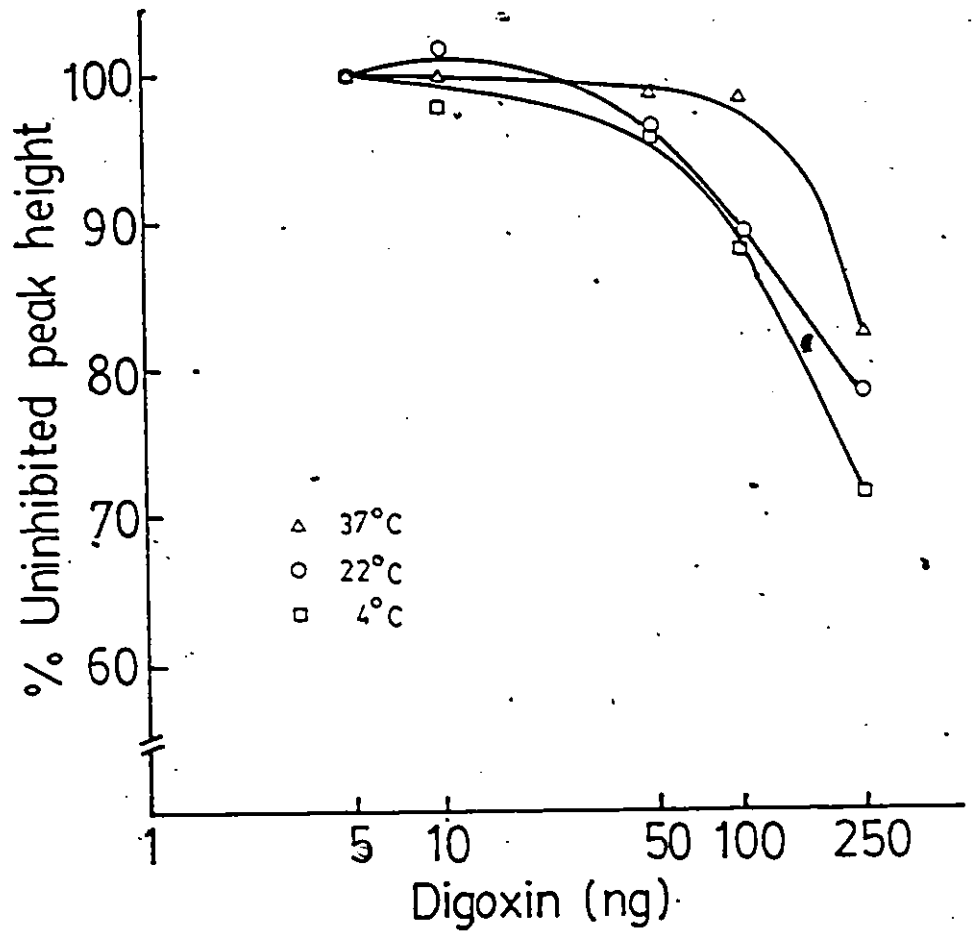
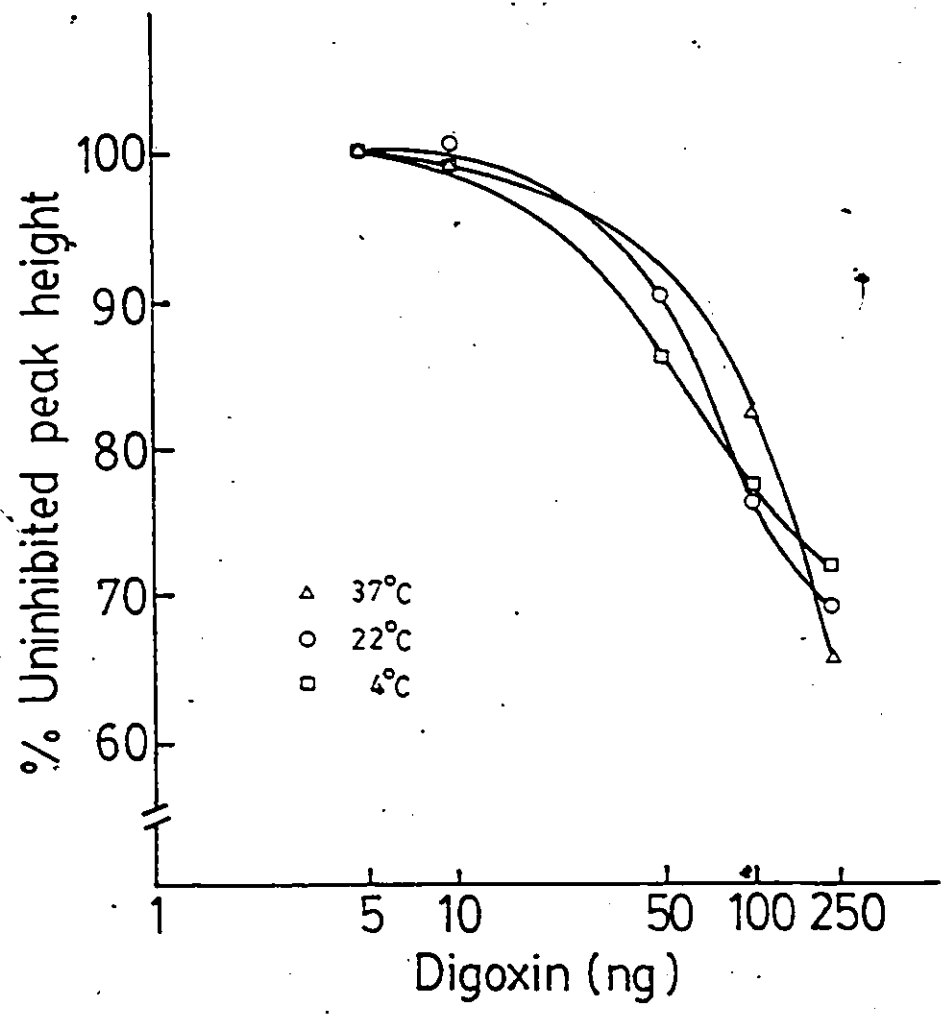


Figure 16. Variation of temperature on sensitivity to inhibition  
(Preincubation)



With preincubation, free digoxin (5-250 ng) was added to 1 ml of antiserum (dilution 1/10) which was incubated at 4, 22 and 37 degrees centigrade. The residual antiserum activity was then back-titrated with developer antigen (2 ug/ml, digoxin/BSA ratio=6.23). Table V and Figure 16 show that the change in temperature caused essentially no difference in inhibition sensitivity. As such, further experimentation was carried out at room temperature (22°C) also.

#### 1.c. Immune Response and Antiserum Dilution

In order to characterize the antiserum obtained, the time course of the immune response was examined both by radioimmunoassay (RIA) and by the automated immunoprecipitin (AIP) technique. Serum samples were taken 1, 4, 8, 13 and 21 days after a booster injection. RIA showed that the respective titres of the bleeds were 1/3000, 1/3300, 1/3050, 1/2950 and 1/2500 (Table VI).

The nephelometric study was carried out using 1, 2, 5 and 10 ug/ml of developer antigen and antiserum dilutions of 1/20, 1/30, 1/40 and 1/50. Table VII tabulates the results of the various experiments. Figure 17 shows the precipitin curve at an antiserum dilution of 1/30. The peak response in this rabbit was at day 4 and had returned to base level by day 21. The slight shift of the equivalence zone to a higher concentration of developer antigen used at day 4 was probably due to the increase of high affinity precipitating antibody detectable in nephelometry. This is also evident from Figure 18 which is reverse precipitin curve (constant antigen concentration and varying antibody dilutions). This shift correlated well with the RIA results in



Table VI. Correlation of RIA and AIP function of antisera

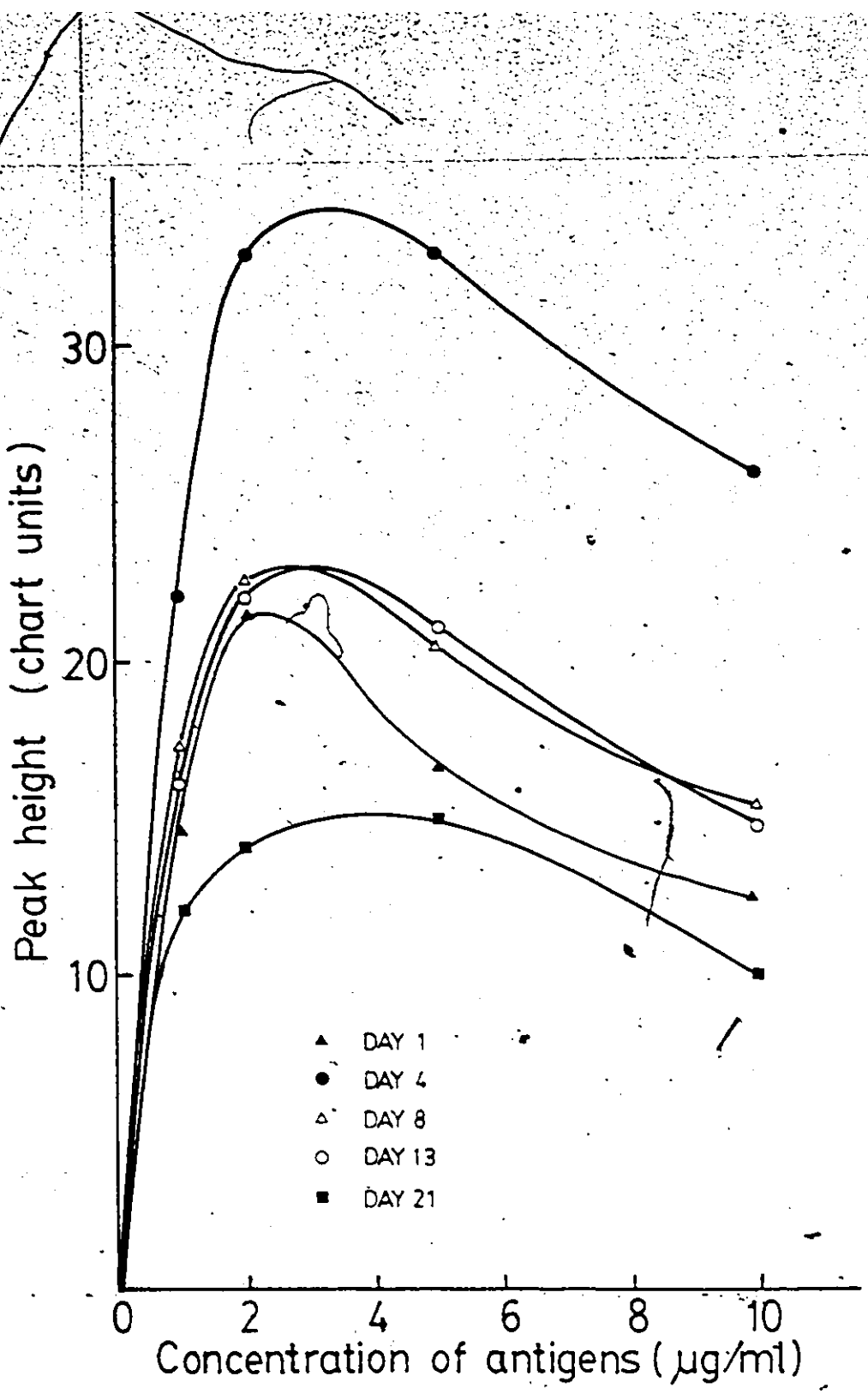
Days after booster	RIA titre (40% binding)	AIP peak height 1/30 Ab vs. 2.0 µg/ml Ag
1	1/3000	21.5 (chart units)
4	1/3300	33
8	1/3050	22
13	1/2950	21
21	1/2500	14

Table VII. AIP response of bleeds with various antigen concentrations

Days after booster	Antiserum dilutions	Peak height (chart units) for various antigen concentrations			
		1ug/ml	2ug/ml	5ug/ml	10ug/ml
1	1/20	18.5	28	26	21
	1/30	14.5	21.5	16.5	12.5
	1/40	12	13	12	8
	1/50	5.5	6.5	7.5	4.5
4	1/20	25	37	48	39
	1/30	22	33	33	26
	1/40	18	26	23	19
	<del>1/50</del>	16	22	19	14
8	1/20	21.5	31.5	30.5	24.5
	1/30	17.5	22.5	20.5	15.5
	1/40	14	16.5	14.5	10.5
	1/50	8	8	9	6
13	1/20	20	29	27	21
	1/30	16	21	21	15
	1/40	10	10	12	8
	1/50	6.5	6.5	8.5	4.5
21	1/20	17.5	23	21	18
	1/30	12	14	15	11
	1/40	7	8	10	6.5
	1/50	3	3.5	7	3

Figure 17. Precipitin curves by AIP technique

(Antibody dilution 1/30)



---

Figure 18. Reverse precipitin curves

(Antigen concentration 2 ug/ml)

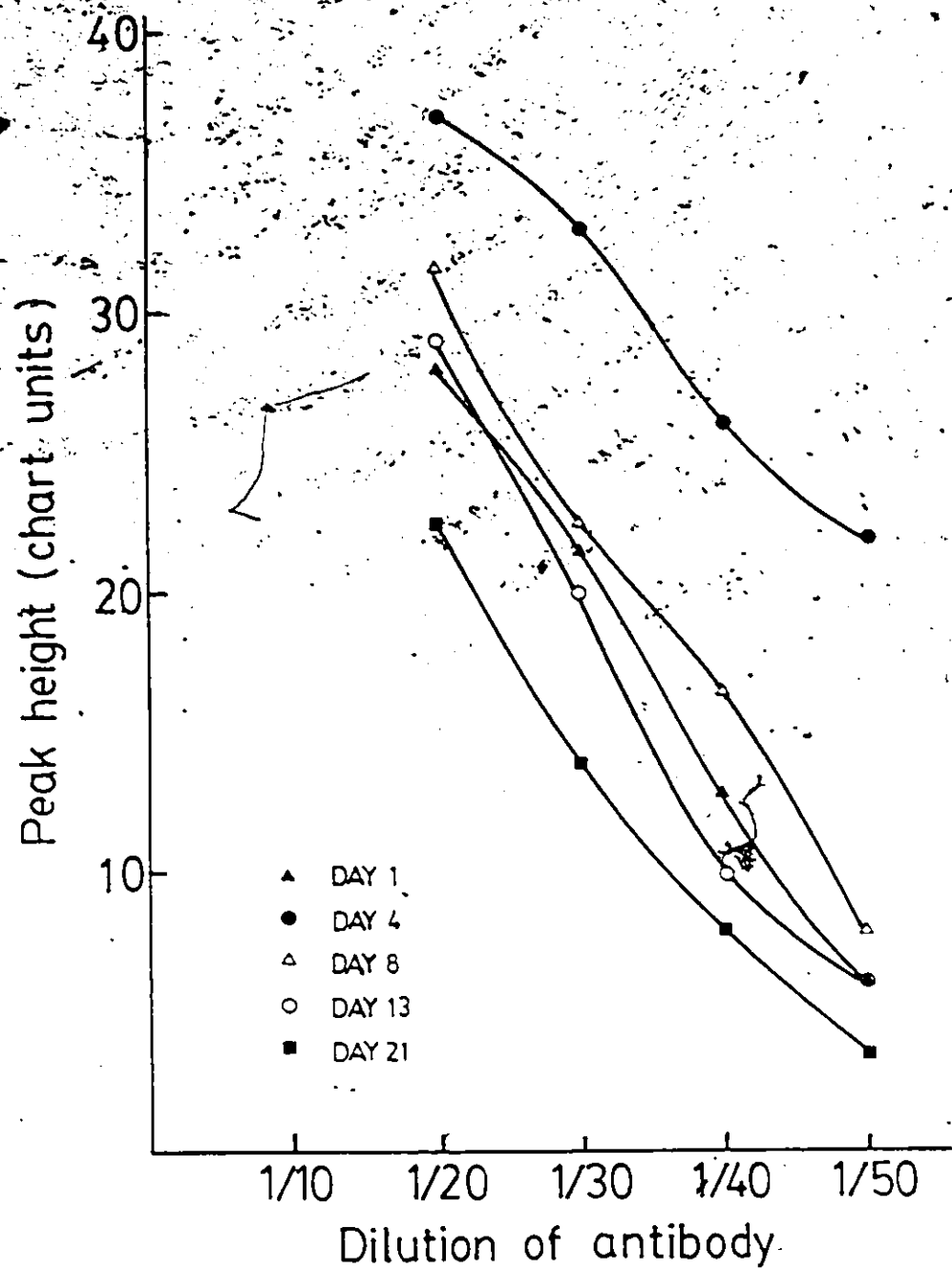


Table VI showing a similar pattern of antiserum titres for the same time frame. It would appear that antibody possessing the characteristic that allows its use in AIP (i.e. a good amount of high affinity antibody) behaves in a quantitatively and qualitatively similar manner in RIA, and vice versa. Figure 19a, the precipitin curves of a particular bleed (day 4) at various dilutions, shows that an antiserum dilution between 1/30 and 1/40 and an antigen concentration between 2 and 3 ug/ml gave a reasonable combination for inhibition studies, without undue rapid consumption of antiserum and developer antigen. However, since the titre is different for each individual bleed, the optimum combination of antiserum dilution and developer antigen concentration has to be determined each time a different antiserum is used. This is illustrated by Figures 19a and 19b, the precipitin curves of bleeds from day 4 (titre 1/3300) and day 8 (titre 1/3050). The latter antiserum had to be used at 1/20 to give a comparable peak height as that from day 4.

#### 1.d. Preincubation versus Simultaneous Addition of Hapten

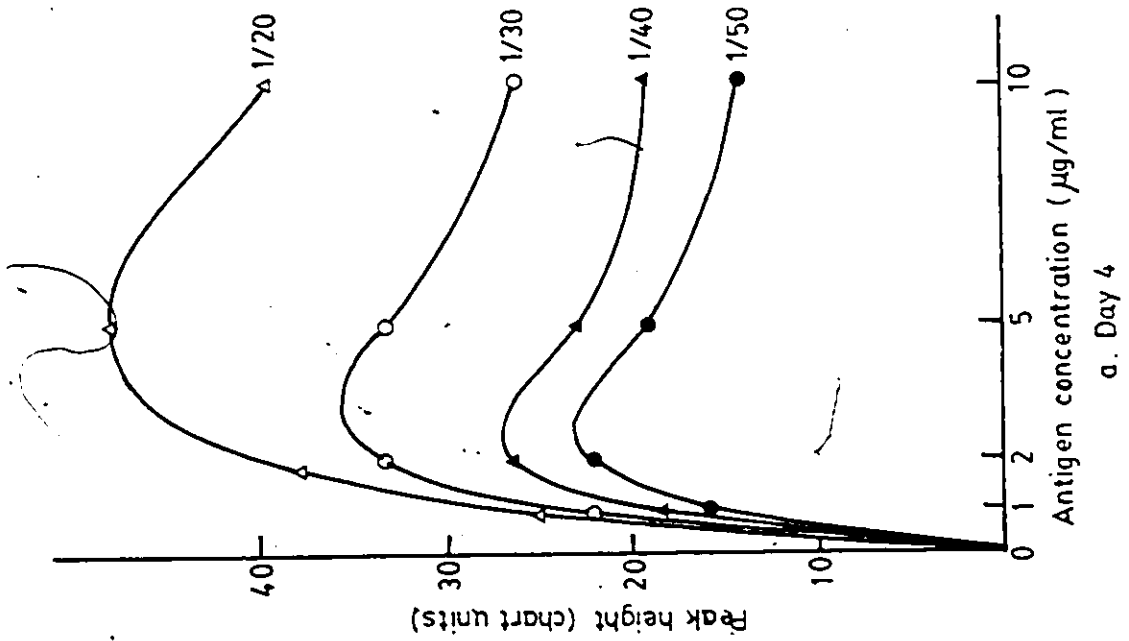
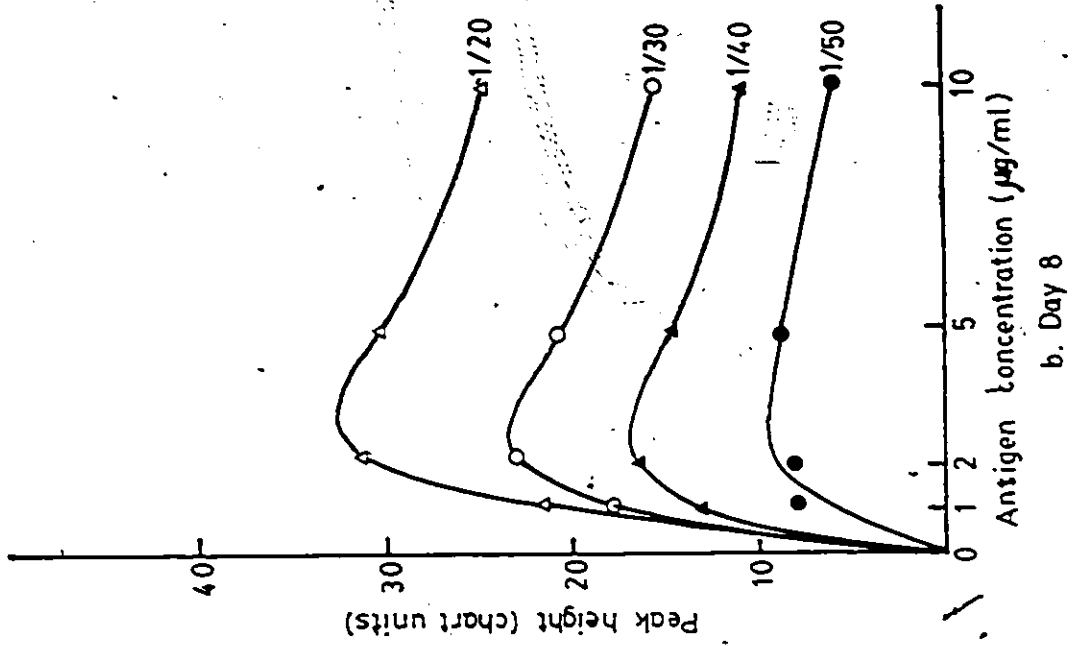
The two approaches, preincubation and simultaneous addition, to inhibition immunoassay were compared to determine whether there was any difference in sensitivity to inhibition between them. A high titred antiserum (titre 1/3000) was used at a dilution of 1/40 with the simultaneous addition approach with developer antigen (digoxin/BSA ratio=6.23) concentration of 4 ug/ml in the sample cups. Since the main stream was pumping at a rate of 1 ml/minute and the sample stream at 0.42 ml/minute, both the antiserum dilution and developer antigen

Figure 19. Precipitin curves for various dilutions of antiserum

(a)-Bleed from day 4

(b) Bleed from day 8





concentration were adjusted for the preincubation approach. An antiserum dilution of 1/15 in the sample cups and a developer antigen concentration of 2 ug/ml through the main stream were adopted. The resulting concentrations of antiserum and antigen in the reaction mixture were than comparable.

Table VIII and Figure 20 compare the results obtained with the two approaches. With preincubation, inhibition was in the range of 10 to 50 ng of digoxin whereas with simultaneous addition, a more sensitive range of 2-10 ng of digoxin was achieved. The fact that simultaneous addition was more sensitive than preincubation was a surprising but consistent finding. Even varying the dilution of the antiserum (from 1/20 to 1/50) and measuring the inhibition against various developer antigen concentrations (0.5-10 ug/ml) the inhibition sensitivity of the preincubation system did not drop below 10 ng of digoxin.

## 2. Optimum Conditions for Automated Inhibition Immunoassay of Digoxin

Combining all the findings in the optimization of the analytical system, an automated nephelometric inhibition immunoassay of digoxin was defined.

A high titred antiserum (titre 1/3000) was used at a dilution of 1/40 in simultaneous addition mode and at room temperature. The antiserum was pumped through the main stream at a rate of 1 ml/minute and samples were introduced at a rate of 0.42 ml/minute. Sampling rate was set a 120 sample cups per hour with sample to wash aspiration time ratio of 2:1. The antiserum was tested against a series of BSA-digoxin

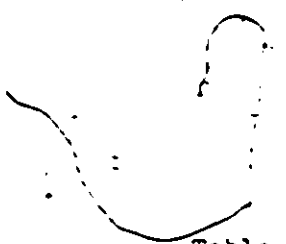
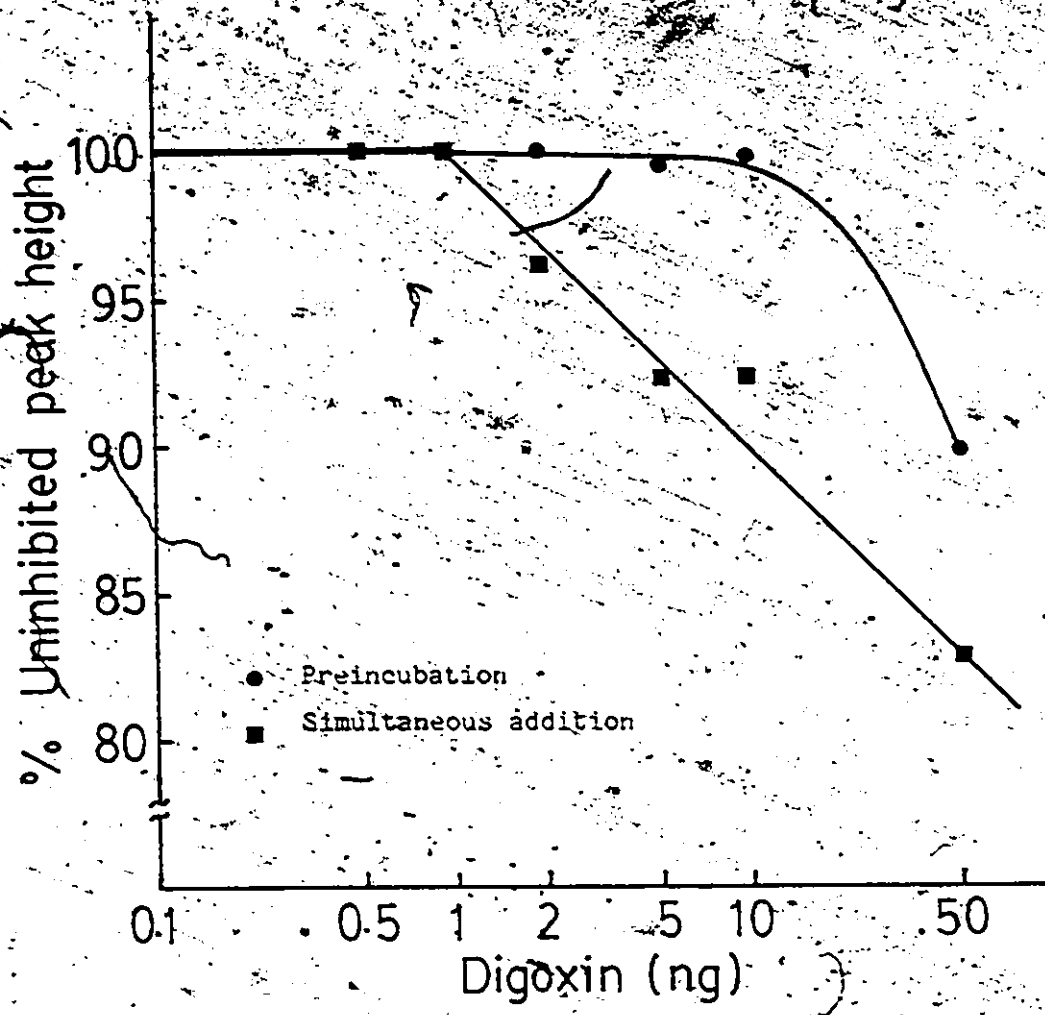


Table VIII. Preincubation versus simultaneous addition on sensitivity to inhibition

Incubation approach	Uninhibited peak height (chart units)	% uninhibited peak height with free digoxin added (ng)					
		0.5	1	2	5	10	50
Preincubation	96	100	100	100	99	99.5	89.6
Simultaneous addition	52	100	100	96.2	92.3	92.3	82.7

Figure 20. Preincubation versus simultaneous addition of hapten



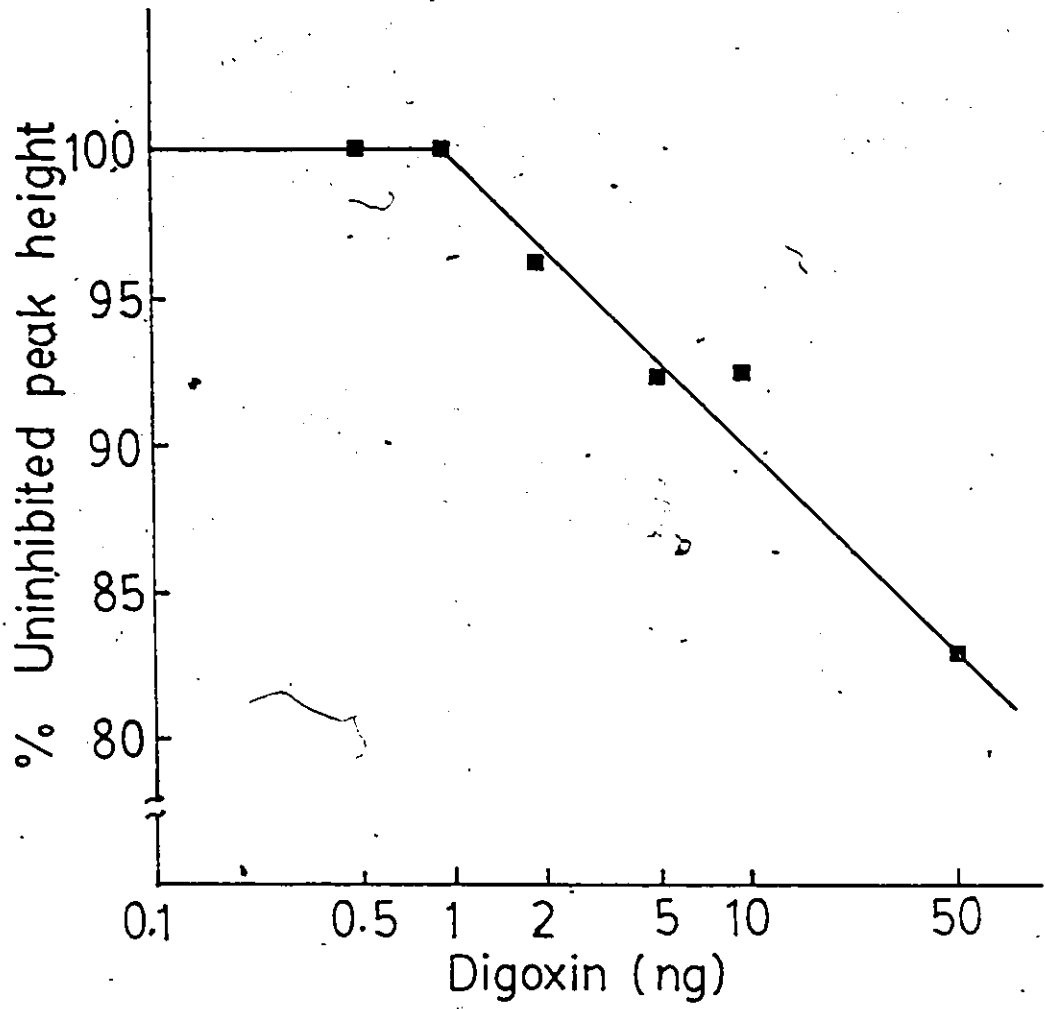
concentrations (digoxin/BSA ratio=6.23) and the optimum nephelometric response was given with a developer antigen concentration of 4 ug/ml. BSA-digoxin (1 ml, 4 ug/ml) was mixed with various amounts of free digoxin (0-50 ng) in the sample cups and both were simultaneously introduced into the antiserum main stream. The standard curve for this reaction, with a working range from 2 to 50 ng digoxin, is shown in Figure 21.

### 3. Extraction of Digoxin from Plasma

The preliminary experiments were carried out using soluble purified digoxin as inhibitor. In vivo, digoxin is present in the range of 1-2 ng/ml of plasma and may be non-covalently bound to plasma proteins. If plasma were to be used directly in the determination of digoxin, the extremely high blank background obtained would pose a definite problem. To overcome this, extraction of digoxin from plasma was attempted.

Previous workers have shown that digoxin may be extracted from aqueous solutions by various organic solvents [153,154], such as chloroform, carbon tetrachloride, methylene chloride, ethanol or a combination of these. Methylene chloride with 5% isoamyl alcohol was best to extract from protein-containing body fluid as it does not form an emulsion. However, methylene chloride (b.p. 40°C) is volatile and in an open extraction system, the volume of solvent may not be consistent due to evaporation. A substitute, 1,2-dichloroethane, was used as it is less volatile and yet possesses a boiling point (83.5°C) low enough for ease of evaporation to dryness over a

Figure 21. Standard curve for automated inhibition immunoassay  
of digoxin





stream of air.

Various plasmas to which had been added known amounts of digoxin, were extracted with 5% isoamyl alcohol in 1,2-dichloroethane. Tritiated digoxin (0.1 ng) was added to 1.5 ml of each plasma preparation. This was vortexed vigorously for thirty seconds with an equal volume of the extracting solvent in a test-tube. The tubes were then centrifuged for 15 minutes at 700xg and at room temperature. The upper aqueous phase and the middle layer of precipitated protein were removed by aspiration. A portion of the solvent (0.5 ml) was pipetted into 10 ml of scintillation fluid (4 gm PPO, 0.05 gm POPOP, 250 ml Triton X-100, made up to one litre with toluene) and counted. The correction factor for quenching was estimated by following the same extraction procedure with blank plasma. The counts obtained in the presence of 0.5 ml of solvent after blank extraction was expressed as a percentage of the original counts.

Table IX shows the extraction recovery from three different pooled plasmas. Plasma I and plasma II were normal human pools whereas plasma III was highly lipemic. After correction for quenching, a consistent 84% recovery (83.4-85.6%) was obtained.

As the extracted material can be taken to dryness over a stream of air at room temperature, it is possible to add digoxin, extracted from 10 ml of plasma, to the assay system without the high blank interference from plasma. Although this approach would allow the determination of digoxin in the therapeutic range in plasma, the need for extraction which to date is not capable of automation, precludes this as a possible approach for routine diagnostic purposes.

Table IX. Recovery of digoxin by extraction

Plasma I (normal pooled plasma)

Correction factor =  
 $\frac{\text{cpm after solvent addition}}{\text{cpm before solvent addition}}$   
 = 73.0%

Concentration of digoxin (ng/ml)	Recovery (%)	Averaged recovery
0.2	83.70	85.6%
0.35	85.34	
0.6	83.97	
1.1	87.94	
2.1	87.94	
5.1	86.85	
7.6	86.85	
10.1	82.19	

Plasma II (normal pooled plasma)

Correction factor = 77.25%

Concentration of digoxin (ng/ml)	Recovery (%)	Averaged recovery
0.2	81.81	84.8%
0.35	86.34	
0.6	88.28	
1.1	84.66	
2.1	82.33	
5.1	82.98	
7.6	85.95	
10.1	85.95	

Table IX. (continuel

Plasma III (lipemic pooled plasma)

Correction factor = 80.4%

Concentration of digoxin (ng/ml)	Recovery (%)	Averaged recovery
0.2	85.70	83.4%
0.35	82.21	
0.6	82.71	
1.1	84.83	
2.1	83.21	
5.1	82.96	
7.6	83.46	
10.1	82.09	

#### IV.B. Solid Phase Radioimmunoassay System

##### 1. Characterization of *S. aureus*-IgG Binding

###### 1.a. Acid-Precipitable Protein Associated I-125

Immunoglobulin G (IgG) from normal human, rabbit and sheep sera was isolated by a combination of ammonium sulphate fractionation, ion-exchange chromatography on DEAE-cellulose and molecular-seive chromatography on Sephadex G-200[155]. The concentration of each purified protein solution was determined by Folin's method and was found to be 123 ug/ml, 117 ug/ml and 90 ug/ml, respectively, for the rabbit, human sheep IgG solutions. Standards for comparison were prepared from a solution of bovine serum albumin.

The purified rabbit, human and sheep IgG's were labelled with I-125 by the chloramine T method[156] and the protein-associated I-125 was determined by precipitation of the protein with trichloroacetic acid. I-125 labelled IgG from rabbit, human and sheep (25 uL) was mixed with 0.5 ml of the respective non-labelled IgG solution and 0.5 ml of trichloroacetic acid (200 gm/litre). The mixture was vortexed and centrifuged in an Eppendorf centrifuge. Table X shows that 100%, 86% and 99% of the counts were associated with protein in the rabbit, human and sheep preparations, respectively.

###### 1.b. Binding Capacity of *S. aureus* for Rabbit IgG

Varying amounts of I-125 labelled rabbit IgG in a final volume

0.5 ml of NET buffer (section III.I.) containing 0.05% Triton X-100 were mixed with 50  $\mu$ L of *S. aureus* suspension (10% w/v). After incubation at room temperature for 30 minutes with mixing, the suspension was centrifuged in an Eppendorf centrifuge. The bacteria portion was counted after being washed twice with the same buffer. The tabulated results in Table XI were plotted in Figure 22, showing that 50  $\mu$ L of *S. aureus* suspension (10% w/v) will optimally bind up to 60  $\mu$ g of rabbit IgG.

#### 1.c. Reactivity of *S. aureus* for IgG of Various Species

I-125 labelled rabbit, human and sheep IgG at two different quantities in a final volume of one ml were mixed for 30 minutes at room temperature with 50  $\mu$ L of *S. aureus* suspension (10% w/v). The bacteria were then removed by centrifugation in an Eppendorf centrifuge and on additional 50  $\mu$ L of the same *S. aureus* suspension was added to the supernatant. After 30 minutes at room temperature with mixing, the bacteria were collected by centrifugation. Before counting, the bacteria pellets were washed twice with NET buffer containing 0.05% Triton X-100.

Table XII demonstrates that *S. aureus* will bind only 90%, 64% and 56% of rabbit, human and sheep IgG under the experimental conditions described despite the use of an excess amount of *S. aureus* cells.

#### 1.d. Reactivity of *S. aureus* Harvested at Different Phase of Growth

To determine whether there is any difference in the binding

Table X. Acid-precipitable protein-associated I-125

Species	Total cpm	Precipitated cpm	% total	Average
Rabbit	1252	1246	99.5	100.3%
	1213	1226	101.1	
Human	1066	925	86.7	86.0%
	1043	891	85.4	
Sheep	1281	1264	98.7	99.0%
	1141	1133	99.3	

Table XI. Binding capacity of *S. aureus* for rabbit IgG

Total rabbit IgG (ug)	% counts bound by <i>S. aureus</i>
0	85.8
10	80.2
20	92.5
50	90.9
100	85.0
150	70.2
200	58.8
250	56.8
300	45.2

Figure 22. Binding capacity of *S. aureus* for rabbit IgG

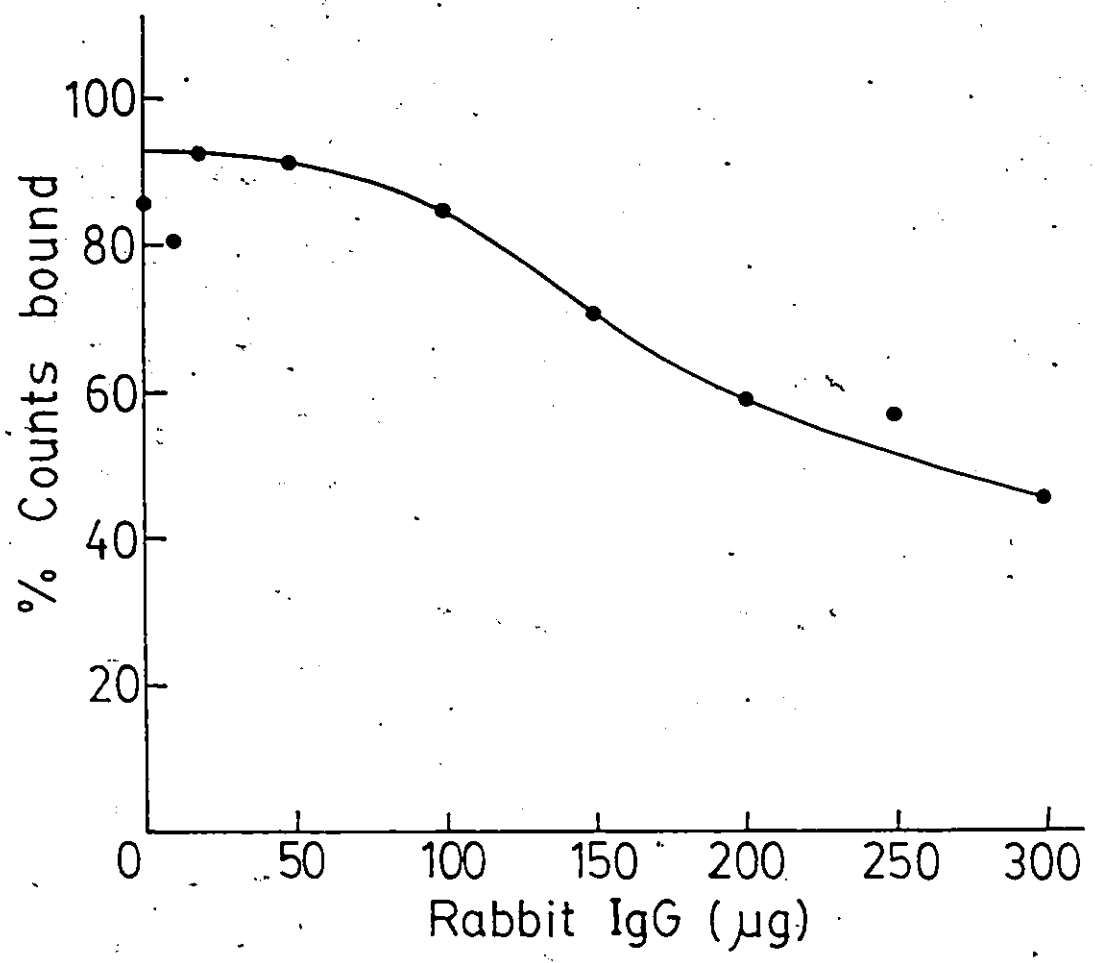




Table XII. Binding of IgG of various species by S. aureus

Species	IgG (ug)	% IgG bound			Average
		1st 50uL	2nd 50 uL	Total	
Rabbit	24.6	87.8	0.9	88.7	90.5
		90.6	1.9	92.5	
	123.0	60.4	24.6	85.0	88.5
Human	23.4	66.3	25.6	91.9	62.9
		61.3	0	61.3	
	116.8	60.6	3.9	64.5	65.9
		63.7	0.8	64.5	
Sheep	18.0	64.0	3.2	67.2	55.9
		46.8	11.0	57.8	
	90.0	43.3	10.7	54.0	55.2
		42.8	13.2	56.0	
		43.0	11.4	54.4	

capacities between *S. aureus* which is actively growing and that which is not, two cultures were started at the same time using the same medium and inoculate. After 6 hours, when the bacteria were in the exponential phase of growth, one of the cultures was terminated and the bacteria collected, chemically treated and heat-killed as described in section III.I. of the Experimental Section. The other culture was terminated at the end of 24 hours (stationary phase of growth) and collected and treated by the same method.

The binding capacity of the bacteria from each culture was assessed by incubating for 30 minutes at room temperature, 50  $\mu$ L of *S. aureus* suspension (10% w/v) from each culture with I-125 labelled rabbit, human and sheep IgG's. Although *S. aureus* from the 6-hour culture showed less clumping and was easier to resuspend during washing, Table XIII shows that there was not much difference in the binding capacities between the bacteria harvested at the two different phases of growth. In all subsequent experiments, the *S. aureus* preparations used were grown for 18-24 hours (overnight) as this yields a greater quantity of bacteria per volume medium used.

#### 1.e. Binding Competition between Rabbit and Human IgG

To assess the binding competition between rabbit and human IgG's, 500  $\mu$ g of human IgG and 20  $\mu$ g of I-125 labelled rabbit IgG were mixed with 50  $\mu$ L of *S. aureus* suspension (10% w/v). The bacteria were separated by centrifugation after 30 minutes at room temperature. The bacteria were counted after being washed twice with NET buffer containing 0.05% Triton X-100. The result showed a binding of 90% of

Table XIII. Binding of IgG by *S. aureus* from different phase of growth

Species	IgG(ug)	50uL(6-hr.)	% IgG bound		Average
			Average	50uL(24-hr.)	
Rabbit	24.6	86.5	89.0	87.8	89.2
		91.4		90.6	
	123.0	67.7	67.7	60.4	63.4
Human	23.4	67.7		66.3	
		61.3	61.5	61.3	61.0
	116.8	61.7		60.6	
Sheep	18.0	60.4	60.5	63.7	63.9
		60.6		64.0	
	90.0	38.8	37.6	46.8	45.1
		36.4		43.3	
		36.7	36.1	42.8	42.9
		35.5		43.0	

the rabbit IgG to the *S. aureus*. Assuming an average rabbit and human serum IgG concentration of 10 gm/litre, 50  $\mu$ L of *S. aureus* suspension (10% w/v) could be used to bind up to 50  $\mu$ L of rabbit antiserum (diluted 1/50) in the presence of up to 50  $\mu$ L of undiluted human serum. To achieve a margin of safety, all subsequent radioimmunoassay experiments used 200  $\mu$ L of *S. aureus* suspension (10% w/v) and 25  $\mu$ L of undiluted human serum or plasma with rabbit antiserum at dilutions from 1/100,000 to 1/500,000.

## 2. Radioimmunoassay Comparisons

### 2.a. Ferritin

Radioimmunoassay of ferritin by the second antibody separation method was as described in the Experimental Section (III.G.).

With *S. aureus* separation, the reagents were prepared as in the following. Rabbit anti-ferritin antiserum was diluted 1/500,000 in EDTA-phosphate buffered saline (EDTA-PBS, pH 7.8, 0.01M phosphate, 0.15M NaCl, 0.005M EDTA). I-125 labelled ferritin was diluted with 1% bovine serum albumin in phosphate buffered saline (1% BSA-PBS, pH 7.8, 0.01M phosphate, 0.15M NaCl) to give 100,000 cpm in one ml. *S. aureus* suspension was adjusted to 10% w/v in NET buffer (section III.I.) containing 0.05% Triton X-100. The same standards used in the second antibody separation radioimmunoassay were employed.

Standards (50  $\mu$ L) or samples (25  $\mu$ L, made up to a final volume of 50  $\mu$ L with 1% BSA-PBS) and I-125 labelled ferritin tracer (100  $\mu$ L, diluted) were incubated with diluted rabbit anti-ferritin antiserum

(100  $\mu$ L) for 18 hours (overnight) at 4°C. *S. aureus* suspension (200  $\mu$ L, 10% w/v) was added and the mixture was left at room temperature for 30 minutes with mixing. The bacteria were pelleted by centrifugation in an Eppendorf centrifuge and the supernatant was aspirated. After being washed twice, the bacteria were counted in a well-type gamma counter.

Seventy samples submitted for routine determination of ferritin were analysed by both radioimmunoassay methods. The results were plotted in Figure 23 using a correlation program with a Wang 600 programmable calculator (equipped with a Wang 612 plotter). Figure 23 shows excellent correlation (correlation coefficient  $r=0.968$ ) over the entire range of values.

It has been reported that the second antibody method for the determination of ferritin does not introduce plasma matrix effects[55].

The correlation coefficient, slope ( $b_1=0.993$ ) and intercept ( $b_0=2.32$ ) (Figure 23) indicate that the use of *S. aureus* does not introduce a matrix effect. This was confirmed by analysis of sera at two volumes (15 and 25  $\mu$ L). The data are represented by Table XIV and Figure 24, with a correlation coefficient of 0.987.

#### 2.b. Digoxin

Digoxin was measured by three methods: (i) tritiated digoxin tracer and ammonium sulphate separation (section III.G.2.a.); (ii) I-125 labelled digoxin tracer and second antibody separation (section III.G.2.b.); and (iii) I-125 labelled digoxin tracer and *S. aureus* separation as described in this section.

Figure 23. Correlation between second antibody and S. aureus.  
separation methods for assay of ferritin

Intercept  $b_0=2.32$        $S(b_0)=1.87$

Slope  $b_1=0.993$        $S(b_1)=0.029$

$y=b_0+b_1x$      $r=0.968$        $S_{y,x} = 12.14$

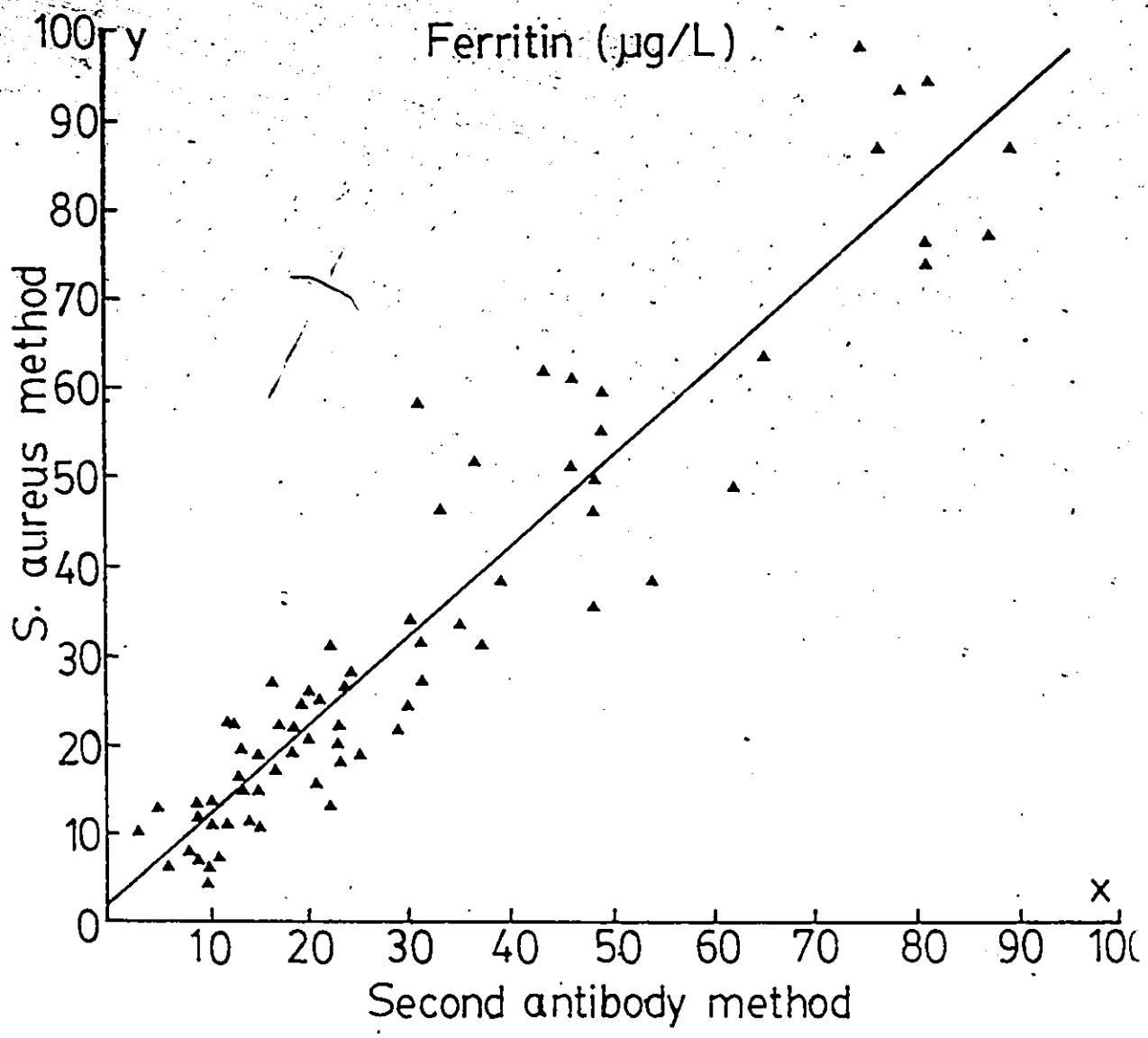


Table XIV. Determination of ferritin (ug/litre) at two volumes

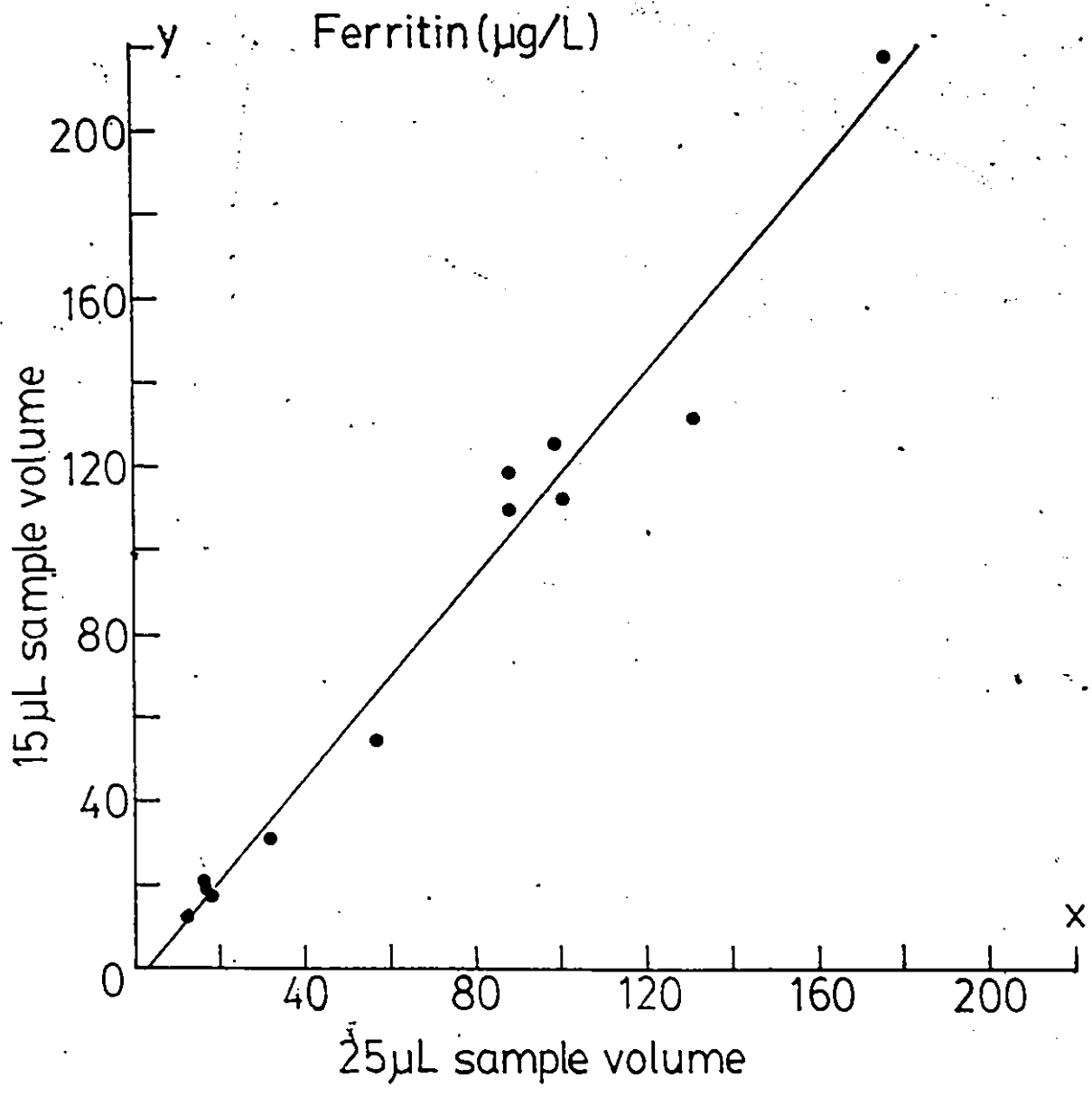
Second antibody	S. aureus	
	25 uL	15 uL
88.9	87.3	109.6
103.2	131.2	132.3
165.6	175.4	218.2
17.0	16.8	20.4
81.0	100.8	110.8
45.7	56.3	54.1
120.4	98.6	125.4
14.9	18.5	18.8
18.5	16.4	21.0
31.0	32.1	31.1
76.0	87.3	117.3
9.0	12.9	12.5



Figure 24. Determination of ferritin (ug/litre) at two volumes

Intercept  $b_0 = -2.81$        $y = b_0 + b_1 x$

Slope  $b_1 = 1.21$        $r = 0.987$



Rabbit anti-digoxin antiserum was diluted to 1/100,000 with 0.5% bovine serum albumin in phosphate buffer (0.5% BSA-PB, pH 7.4, 0.02M phosphate). I-125 labelled digoxin was diluted with 0.5% BSA-PB to give about 60,000 cpm per ml. Standards were prepared as described in section III.G.2.a. *S. aureus* suspension was adjusted to 10% w/v with NET buffer containing 0.05% Triton X-100.

Standards, samples or 0.5% BSA-phosphate buffer (25  $\mu$ L) and I-125 labelled digoxin tracer (200  $\mu$ L, diluted) were incubated with 100  $\mu$ L of diluted rabbit anti-digoxin antiserum for 30 minutes at room temperature. *S. aureus* suspension (200  $\mu$ L, 10% w/v) was added and mixed. After mixing for 30 minutes at room temperature, the mixture was centrifuged and the supernatant removed by aspiration. The bacteria pellet was washed twice before being counted in a gamma counter.

To confirm the lack of plasma matrix effects, twenty-four plasmas submitted for routine digoxin determination (tritiated digoxin tracer with ammonium sulphate separation) were analysed at two plasma volumes (15 and 25  $\mu$ L). The data are presented in Table XV and are plotted in Figure 25, which shows a slope of 1.12, an intercept of -0.2 ng/ml and a correlation coefficient of 0.942, validating the absence of plasma matrix effects.

Twenty-three samples submitted for routine digoxin measurements were analysed by all three methods (Table XV). Figures 26, 27 and 28 demonstrate that the three methods correlate well (*S. aureus*-ammonium sulphate,  $r=0.902$ ; ammonium sulphate-second antibody,  $r=0.933$ ; *S. aureus*-second antibody,  $r=0.899$ ).

Table XV. Determination of digoxin (ng/ml) by three methods

<sup>3</sup> H-digoxin/ ammonium sulphate	<sup>125</sup> I-digoxin/ S.aureus		<sup>125</sup> I-digoxin/ second antibody
	25uL	15uL	
1.3	1.3	1.5	1.1
0.7	1.1	1.1	0.7
1.1	1.8	1.7	1.2
3.8	3.9	3.5	4.5
1.6	2.4	2.0	1.5
2.1	2.7	2.5	1.8
2.3	2.6	3.3	2.0
0.9	1.3	1.7	0.8
1.0	1.3	1.1	0.9
1.8	3.3	3.6	1.9
1.2	1.6	1.2	1.0
0.5	1.2	1.0	0.8
3.1	3.6	4.4	3.6
2.5	3.1	2.9	2.0
2.0	3.0	3.4	2.7
1.4	1.9	2.0	1.3
0.8	1.0	0.8	0.8
1.9	2.2	2.7	1.8
0.9	1.6	1.4	1.1
0.8	1.6	1.5	1.0
1.4	2.5	2.7	2.0
1.8	2.0	1.7	1.3
1.5	2.2	2.0	1.4
2.0	2.2	2.0	--

Figure 25. Determination of digoxin (ng/ml) at two volumes

Intercept  $b_0 = -0.20$        $y = b_0 + b_1 x$

Slope  $b_1 = 1.12$        $r = 0.942$

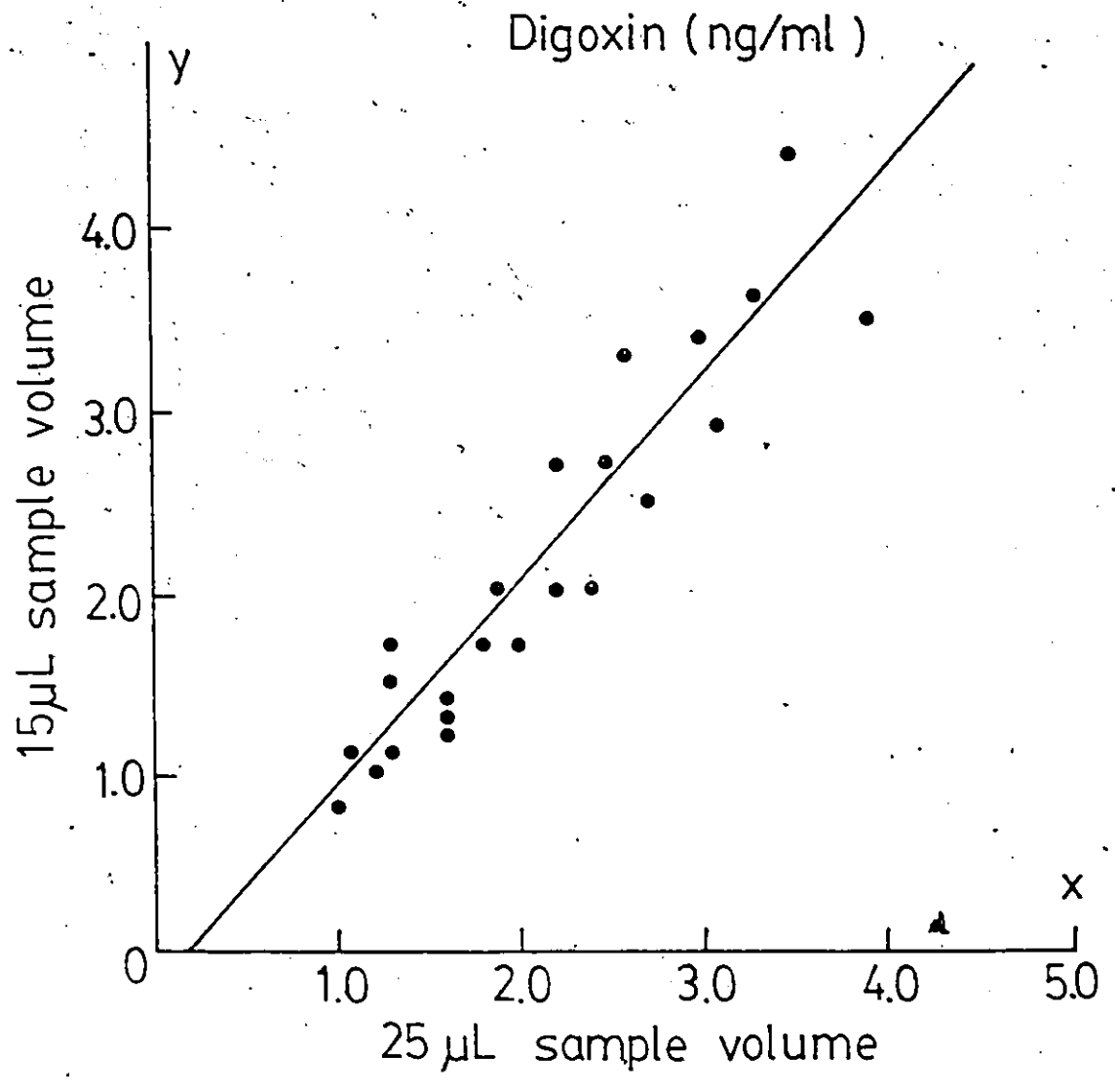


Figure 26. Correlation between S. aureus and ammonium sulphate  
methods for assay of digoxin

Intercept  $b_0=0.579$        $S(b_0)=0.352$

Slope  $b_1=0.911$        $S(b_1)=0.093$

$y=b_0+b_1x$      $r=0.902$        $S_{y,x}=0.352$

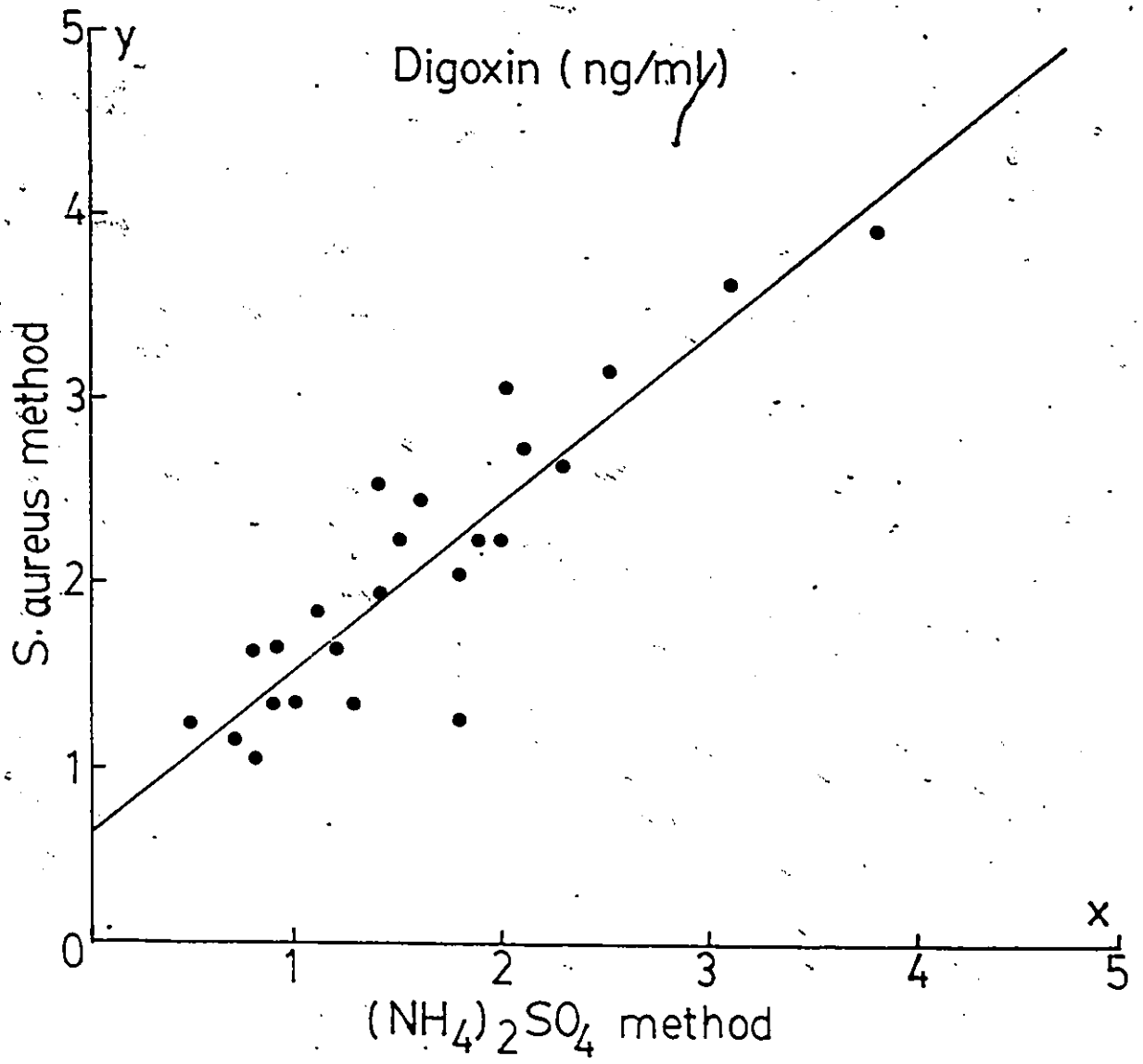




Figure 27. Correlation between ammonium sulphate and  
second antibody methods for assay of digoxin

Intercept  $b_0 = 0.282$        $S(b_0) = 0.125$

Slope  $b_1 = 0.804$        $S(b_1) = 0.068$

$y = b_0 + b_1 x$        $r = 0.933$        $S_{y,x} = 0.296$

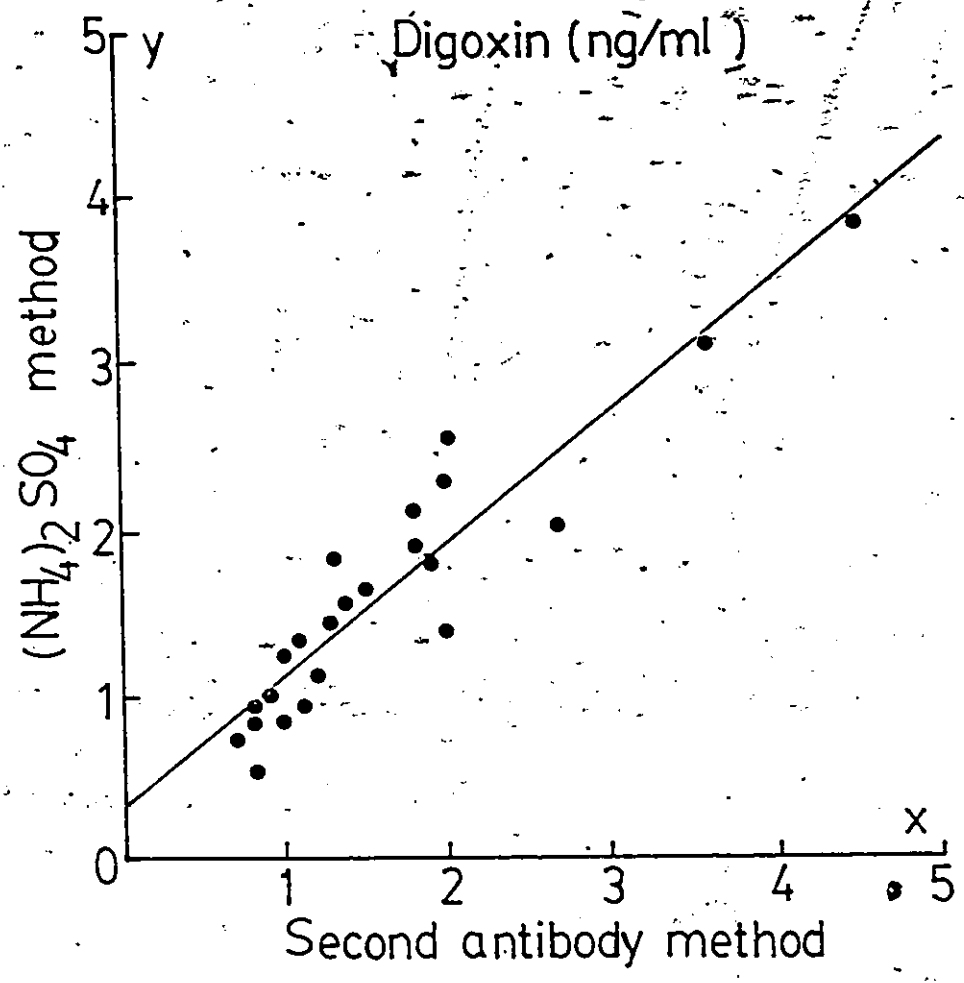
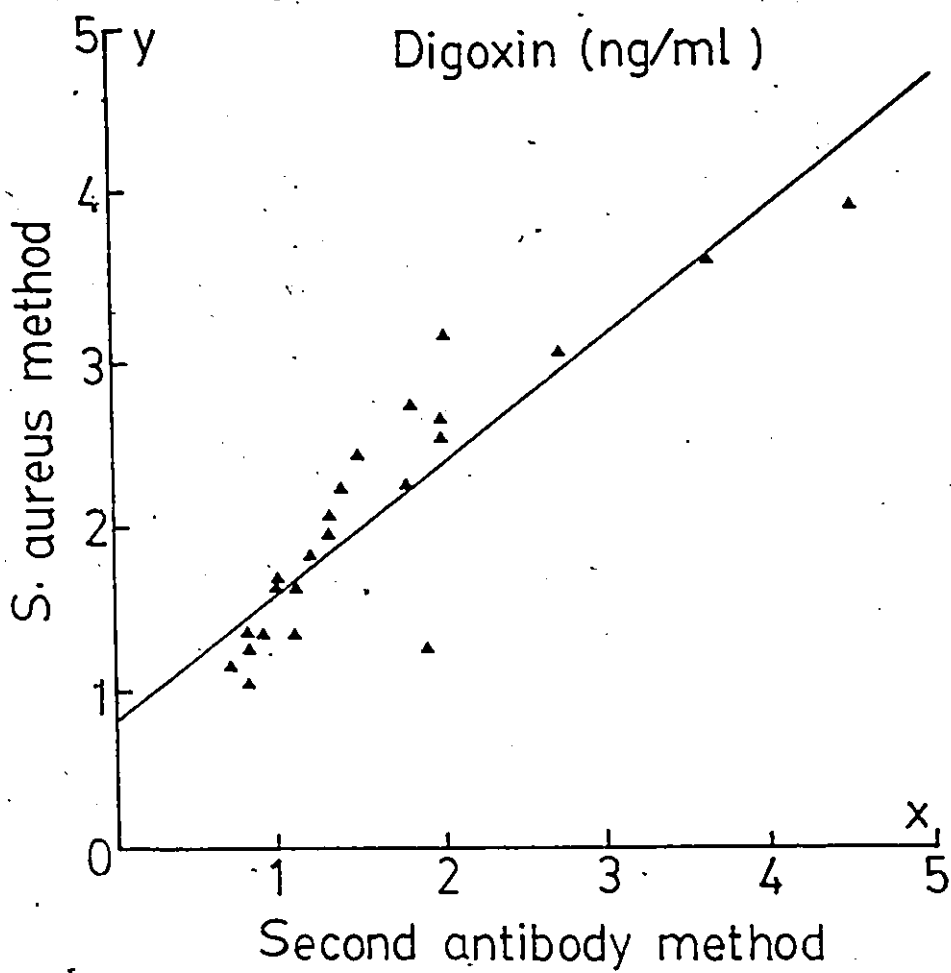


Figure 28. Correlation between *S. aureus* and second antibody methods for assay of digoxin

Intercept  $b_0=0.758$        $S(b_0)=0.156$

Slope  $b_1=0.786$        $S(b_1)=0.084$

$y=b_0+b_1x$      $r=0.899$        $S_{y,x}=0.366$



## V. CONCLUSIONS

1. Using digoxin as a model of a low molecular-weight compound of therapeutic interest, the principle of an automated nephelometric inhibition immunoassay has been demonstrated.
2. The selection of an appropriate developer protein-hapten conjugate depends on its precipitability by antibody as well as its sensitivity to inhibition by free hapten.
3. The temperature (4-37 degrees centigrade) does not significantly affect the sensitivity of the method.
4. The affinity of the antibody for the hapten affects the sensitivity of the method. An antibody with a higher affinity yields a higher sensitivity.
5. Simultaneous addition of free hapten and developer-antigen to the antibody yields a higher sensitivity than does preincubation of hapten and antibody with back titration of residual antibody activity.
6. With the available instrumentation and under optimal condition, free digoxin can be determined in the range of 2-50 ng. This range would require the extraction of digoxin from 10 ml of human plasma for diagnostic purposes.
7. The requirement of an extraction step imposes a limitation on automation and makes this assay approach not competitive with the currently available assay methods for digoxin.
8. The use of heat-killed and chemically treated protein A-bearing *Staphylococcus aureus* as a solid-phase separation step in radio-

immunoassay gives comparable results with currently applied techniques. Its use is cheaper than that of a second antibody and is superior than ammonium sulphate precipitation in that it does not suffer from plasma matrix effects.

## VI. SUGGESTIONS FOR FURTHER WORK

1. In the experiments performed, the developer-antigen used was BSA-digoxin with an hapten-protein ratio of 6.23. This gave a good measurable uninhibited nephelometric peak while being sensitive to inhibition. A better sensitivity can be reached with a lower hapten-carrier ratio if the developer-antigen can give better precipitability. This can be achieved by the use of larger protein-carriers in conjugation with digoxin.
2. With the Technicon fluoronephelometer, the angle at which light scattering can be measured is limited to 90 degrees by the design and construction of the instrument. The relationships between the intensity of the scattered light, the sensitivity to inhibition and the angle of measurement was not determined. Better sensitivity can possibly be reached by measuring light scattering at an angle smaller than 90 degrees.
3. With the Technicon AutoAnalyzer II system, light scattering from the samples (the background level) posed a problem. Undiluted samples cannot be used, necessitating an extra extraction step. In recent years, an instrument was made available (Immunochemistry Analyzer, Beckman Instruments, Inc.) that measures the rate at which light scattering is increasing. The peak rate value is a measure of concentration of the antigen. Since the background level is constant, it does not affect rate measurements. The principles of nephelometric inhibition immunoassay can be tested with this alternate kinetic approach.

## VII. APPENDIX

### Glossary of Terms

Adjuvant-- A compound capable of potentiating an immune response.

Antibody-- A protein that is produced as a result of the introduction of an antigen and which has the ability to combine with the antigen that stimulated its production.

Antibody affinity-- Antibody affinity refers to the energy of a single antibody site binding to a single antigenic determinant. It is best measured by equilibrium dialysis using small, homogenous haptens and the corresponding anti-hapten antibodies. Affinity thus measured can be expressed as a precise equilibrium constant.

Antibody avidity-- Antibody avidity refers to the relative tendency for a heterogeneous population of antibodies to react with multivalent antigens. It is a less precise term than affinity because different antisera do recognize different numbers and different kinds of determinants on the same antigen. For most antibodies, avidity is a relative measurement that is affected by the antigen used to measure it. Such measurements will have meaning only to the degree that the test antigens mimic those actually important in a patient.

Antibody combining site-- That configuration present on an antibody molecule which links with a corresponding antigenic determinant.

Antigen-- A substance which can induce a detectable immune response when introduced into an animal.

Antigen binding site-- The part of an immunoglobulin which binds antigen.



**Antigenic determinant--** That area of an antigen which determines the specificity of the antigen-antibody reaction.

**Carrier--** An immunogenic substance which, when coupled to a hapten, renders the hapten immunogenic.

**Determinant groups--** Individual chemical structures present of macromolecular antigens which determine antigenic specificity.

**Dextrans--** Polysaccharides composed of a single sugar.

**Enzyme--** Enzymes are biocatalysts produced by living tissue that increases the rate of reactions that may occur in the tissue. All enzymes are proteins. In the absence of enzymes the same or comparable reactions occur at too slow a rate to support life or require unphysiologic conditions.

**Equivalence--** A ratio of antigen-antibody concentration where maximal precipitation occurs.

**Fc fragment--** Crystallizable fragment obtained by papain (an enzyme) digestion of IgG molecules. Fc fragment consists of the carboxyl-terminal half of two heavy chains linked by disulphide bonds. It contains no antigen binding capacity but determines important biologic characteristics of the intact molecule.

**Freund's complete adjuvant--** An oil-water emulsion which contains killed mycobacteria and enhances immune responses when mixed in an emulsion with antigen.

**Gamma globulins--** Serum proteins with gamma mobility in electrophoresis which comprise the majority of immunoglobulins and antibodies.

**Hapten--** A substance which is not immunogenic but can react with an

antibody of appropriate specificity.

Heavy chain-- One pair of identical polypeptide chains making up an immunoglobulin molecule. The heavy chain contains approximately twice the number of amino acids and is twice the molecular weight of the light chain.

IgG-- Predominant immunoglobulin class in human serum (Figure 29).

Immunogen-- A substance which, when introduced into an animal, stimulates the immune response. The term immunogen may also denote an antigen which is capable of stimulating an immune response, in contrast to an hapten which can only combine with antibody.

Immunogenicity-- Property of a substance making it capable of inducing a detectable immune response.

Immunoglobulin-- A glycoprotein composed of heavy and light chains which functions as an antibody. All antibodies are immunoglobulins but it is not certain that all immunoglobulins have antibody function.

Immunoglobulin class-- A subdivision of immunoglobulin molecules based on unique antigenic determinants in the Fc region of the heavy chains. In man there are 5 classes of immunoglobulins designated IgG, IgA, IgM, IgD and IgE.

Immunoglobulin subclass-- A subdivision of the classes of immunoglobulins based on structural and antigenic differences in the heavy chains. For human IgG there are 4 subclasses: IgG-1, IgG-2, IgG-3 and IgG-4.

Immunopotency-- Capacity of a region of an antigen molecule to serve as an antigenic determinant and thereby induce the formation of

specific antibody.

**Immunoradiometric assay (one-site)**-- In this assay method, soluble and radioactive-labelled antibody is reacted with free antigen. After incubation, the excess labelled antibody is removed by a second reaction with solid-phase antigen with subsequent centrifugation. The amount of radioactivity remaining in solution is a direct function of the antigen concentration.

**Immunoradiometric assay (two-site)**-- In this assay the multivalent antigen is first insolubilized by reaction with solid-phase antibody. Purified and radioactive-labelled antibody against another antigenic determinant is then added. The resulting labelled complex is insoluble and excess labelled antibody can be washed away. The amount of radioactivity in the solid phase is directly proportional to the antigen concentration.

**Ligand**-- Any molecule that forms a complex with another molecule, such as an antigen used in a precipitin reaction or radio-immunoassay.

**Light chain**-- Polypeptide chain present in all immunoglobulin molecules. Two types exist in most species and are termed kappa and lambda.

**Matrix effects**-- This is a term that applies to the observed discrepancies in radioimmunoassay results because of the presence in serum or plasma certain unknown factors. These factors affect the reproducibility of the assay methodology by interfering with the antigen-antibody reaction. It is usually detected by assaying the sample at two volumes so that it is diluted to different extents in the final reaction matrix. The results obtained will not correlate

if matrix effects are present.

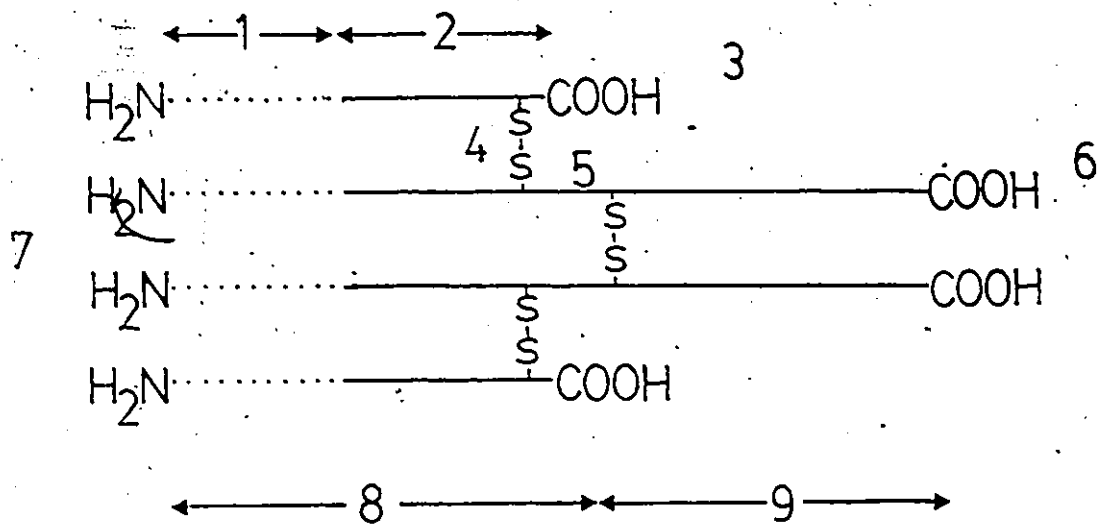
**Nephelometry--** The measurement of turbidity or cloudiness in a suspension or a solution by the scattering of an incident light source.

**Precipitation--** A reaction between a soluble antigen and soluble antibody in which a complex lattice of interlocking aggregates forms.

**Sodium/potassium dependent adenosine triphosphatase--** This is an enzyme bound to the plasmic membrane of the cell. It hydrolyses adenosine triphosphate at a rate that depends on the cation concentrations of sodium and potassium. The purpose is to maintain a homeostatic concentration of potassium within the cell and to keep sodium out of the cell.

**Titre--** This is a measure of the quantity of antibody in an antiserum. It is arbitrarily defined as the dilution of the antiserum required to bind a certain percentage (40-60%) of a fixed mass of labelled antigen under defined incubation and separation conditions.

Figure 29. Diagrammatic representation of IgG molecule



1. Variable region
2. Constant region
3. Light chain
4. Disulphide bond
5. Site where papain splits
6. Heavy chain
7. Antigen binding site
8. Fab fragment
9. Fc fragment

### VIII. BIBLIOGRAPHY

1. D.G.Friend, N.Engl.J.Med., 266 , 88,187,300,402 (1962).
2. T.W.Smith, *ibid.*, 286 , 719,942 (1973).
3. T.W.Smith and E.Haber, *ibid.*, 289 , 945,1010,1063,1125 (1973).
4. E.Watson and S.M.Kalman, J.Chromatogr., 56 , 209 (1971).
5. E.Watson, P.Tramell and S.M.Kalman, *ibid.*, 69 , 157 (1972).
6. A.Bertler and A.Redfors, Clin.Pharmacol.Ther., 11 , 665 (1970).
7. J.M.Lowenstein, Circulation, 31 , 228 (1965).
8. J.M.Lowenstein and E.M.Corrill, J.Lab.Clin.Med., 67 , 1048 (1966).
9. H.Matsui and A.Schwartz, Biochem.Biophys.Acta, 151 , 655 (1968).
10. P.F.Binnion, L.M.Morgan, H.M.Stevenson, et al., Br.Heart J., 31 , 636 (1969).
11. D.G.Grahame-Smith and M.S.Everest, Brit.Med.J., 1 , 286 (1969).
12. G.H.Burnett and R.L.Conklin, J.Lab.Clin.Med., 78 , 779 (1971).
13. D.A.Chamberlain, R.J.White, M.R.Howard, et al., Br.Med.J., 3 , 429 (1970).
14. R.J.Hoeschen and V.Proveda, Can.Med.Assoc.J., 105 , 170 (1971).
15. D.C.Evered and C.Chapman, Br.Heart J., 33 , 540 (1971).
16. T.W.Smith, V.P.Butler, Jr. and E.Haber, N.Engl.J.Med., 281 , 1212 (1969).
17. T.W.Smith, Am.Heart J., 82 , 833 (1971).
18. G.C.Oliver, B.M.Parker and C.W.Parker, Am.J.Med., 51 , 186 (1971).

19. T.W.Smith, V.P.Butler, Jr. and E.Haber, *Biochem.*, 9 , 331 (1970).
20. E.D.Horgan and W.J.Riley, *Clin.Chem.*; 19 , 187 (1973).
21. H.R.Besch, Jr. and A.M.Watanabe, *ibid.*, 21 , 1815 (1975).
22. G.Brooker and R.W.Jelliffe, *Circulation*, 45 , 20 (1972).
23. R.R.Crichton, *N.Engl.J.Med.*, 284 , 1413 (1971).
24. P.M.Harrison, *Seminars in Haematology*, 14 , 55 (1977).
25. S.Granick, *J.Biol.Chem.*, 149 , 157 (1943).
26. G.M.Addison, M.R.Beamish, C.N.Hales, et al., *J.Clin.Pathol.*, 25 , 326 (1972).
27. J.W.Halliday, K.L.Gera and L.W.Powell, *Clin.Chim.Acta*, 58 , 207 (1975).
28. L.Michaelis, C.D.Charles and S.Granick, *J.Biol.Chem.*, 148 , 463 (1943).
29. S.Granick and P.F.Hahn, *ibid.*, 155 , 661 (1944).
30. J.L.Farrant, *Biochim.Biophys.Acta*, 13 , 569 (1954).
31. P.M.Harrison, *J.Mol.Biol.*, 6 , 404 (1963).
32. H.J.Bielig, O.Kratky, G.Rohns, et al., *Biochim.Biophys.Acta*, 112 , 110 (1966).
33. V.Kleinwachter, *Arch.Biochem.Biophys.*, 105 , 352 (1964).
34. F.A.Fischbach and J.W.Anderegg, *J.Mol.Biol.*, 14 , 458 (1965).
35. C.F.A.Bryce and R.R.Crichton, *J.Biol.Chem.*, 246 , 4198 (1971).
36. R.R.Crichton and C.F.A.Bryce, *FEBS Lett.*, 6 , 121 (1970).
37. G.W.Richter and G.F.Walker, *Biochemistry*, 6 , 2871 (1967).
38. M.A.Williams and P.M.Harrison, *Biochem.J.*, 110 , 265 (1968).
39. C.P.Alfrey, Jr., E.C.Lynch and C.E.Whitley, *J.Lab.Clin.Med.*, 70 , 419 (1967).



40. R.R.Crichton, J.A.Millar and R.L.C.Cumming, *Biochem.J.*, 117 , 35P (1970).
41. J.W.Drysdale, *Biochim.Biophys.Acta*, 207 , 256 (1970).
42. J.W.Drysdale, *Biochem.J.*, 141 , 627 (1974).
43. M.Worwood, W.Aherne, S.Dawkins, et al., *Clin.Sci.Mol.Med.*, 48 , 441 (1975).
44. G.O.Walters, F.M.Miller and M.Worwood, *J.Clin.Pathol.*, 26 , 770 (1973).
45. A.Jacobs, F.Miller, M.Worwood, et al., *Br.Med.J.*, 4 , 206 (1972).
46. D.A.Lipschitz, J.D.Cook and C.A.Finch, *N.Engl.J.Med.*, 290 , 1213 (1974).
47. A.Jacobs and M.Worwood, *ibid.*, 292 , 951 (1975).
48. J.R.Wands, J.A.Rowe, S.E.Mezey, et al., *ibid.*, 294 , 302 (1976).
49. L.W.Powell and J.W.Halliday, *ibid.*, 294 , 1185 (1976).
50. M.A.Siimes, J.E.Addiego, Jr. and P.R.Dallman, *Blood*, 43 , 581, (1974).
51. E.A.Letsky, F.Miller, M.Worwood, et al., *J.Clin.Pathol.*, 27 , 652 (1974).
52. L.E.M.Miles, D.A.Lipschitz, C.P.Bieber, et al., *Anal.Biochem.*, 61 , 209 (1974).
53. D.M.Marcus and N.Zinberg, *J.Nat.Cancer Inst.*, 55 , 791 (1975).
54. Y.Niitsu, Y.Kohgo, M.Yokota, et al., *Ann.N.Y.Acad.Sci.*, 259 , 450 (1974).
55. A.W.Luxton, W.H.C.Walker, J.Gauldie, et al., *Clin.Chem.*, 23 , 683 (1977).

56. W.O.Weigle, *Immunochem.*, 1, 295 (1964).
57. A.A.Amkraut, A.Malley and D.Begley, *J.Immunol.*, 103, 1301 (1969).
58. C.Larson, *Advan.Automat.Anal.*, 4, 67 (1972).
59. C.Larson, B.Orenstein and R.F.Ritchie, *ibid.*, 1, 101 (1971).
60. R.F.Ritchie, *J.Lab.Clin.Med.*, 70, 512 (1967).
61. R.F.Ritchie, In "The Plasma Proteins: Structure, Function and Genetic Control", vol.II, p.375, F.W.Putnam (ed.), Academic Press, N.Y. (1975).
62. G.H.F.Nuttall, In "Blood Immunity and Blood Relationship", p.5 and p.96, Cambridge Univ.Press, London and New York (1904).
63. M.Heidelberger and F.E.Kendal, *J.Exp.Med.*, 55, 555 (1932).
64. M.Heidelberger and F.E.Kendal, *ibid.*, 62, 697 (1935).
65. P.A.Kober, *J.Biol.Chem.*, 8, 485 (1912-1913).
66. W.R.Bloor, *ibid.*, 7, 377 (1914).
67. W.R.Bloor, *ibid.*, 22, 145 (1915).
68. P.A.Kober, *ibid.*, 29, 155 (1917).
69. P.A.Kober and R.E.Klett, *ibid.*, 47, 19 (1921).
70. W.Denis, *ibid.*, 47, 27 (1921).
71. R.L.Libby, *J.Immunol.*, 34, 71, 269 (1938).
72. A.Boyden, *Physiol.Zool.*, 15, 109 (1942).
73. A.Boyden and R.J.DeFalco, *ibid.*, 16, 229 (1943).
74. E.T.Bolton, *J.Immunol.*, 57, 391 (1947).
75. J.G.Baier, *Physiol.Zool.*, 20, 172 (1947).
76. A.Boyden, E.Bolton and D.Gemeroy, *J.Immunol.*, 57, 211 (1947).
77. E.T.Bolton, A.Leone and A.Boyden, *ibid.*, 58, 169 (1948).

78. C.G.Pope and M.Healey, Brit.J.Exp.Pathol., 19 , 397 (1938).
79. D.Gitlin and H.Edelhoch, J.Immunol., 66 ; 67 (1951).
80. P.Johnon and R.H.Ottewill, Discuss.Faraday Soc., 18 , 327 (1954).
81. J.R.Marrack and R.A.Grant, Brit.J.Exp.Pathol., 34 , 263 (1953).
82. H.E.Schultze and G.Schwick, Clin.Chim.Acta, 4 , 15 (1959).
83. C.A.Leone, Trans.Kans.Acad.Sci., 63 , 147 (1960).
84. K.Hellsing; Colloq.A.I.P.,Brussels, 17 (1972).
85. M.C.Stone and J.M.Thorp, Clin.Chim.Acta; 14 , 812 (1966).
86. J.Kahan and L.Sundblad, Eur.Technicon Symp., Paris, 361 (1966).
87. J.Kahan and L.Sundblad, Scand.J.Clin.Lab.Invest., 24 , 61 (1969).
88. C.A.Alper and R.P.Propp, J.Clin.Invest., 47 , 2181 (1968).
89. R.F.Ritchie, C.A.Alper and J.A.Graves, Arthritis Rheum., 12 ,  
693 (1969).
90. R.F.Ritchie, C.A.Alper, J.Graves, et al., Amer.J.Clin.Pathol.,  
59 , 151 (1973).
91. C.A.Alper, Advan.Automat.Anal., 1 , 105 (1970).
92. I.Eckman, J.B.Robbins, C.J.Van der Hamer, et al., Clin.Chem.,  
16 , 558 (1970).
93. R.F.Ritchie and J.A.Graves, Technicon Int.Congr., New York,  
147 (1972).
94. L.M.Killingsworth and J.Savory, Clin.Chem., 17 , 936 (1971).
95. L.M.Killingsworth, J.Savory and P.O.Teague, ibid., 17 , 374  
(1971).
96. J.Aitken, Colloq.A.I.P., Brussels, 33 (1972).
97. D.Halberstan and G.Derrico, ibid., 43 (1972).
98. H.Markowitz and A.R.Tschida, Clin.Chem., 18 , 1364 (1972).

99. A.P.Fletcher, N.Alkjaersig, L.Roy, et al., Colloq.A.I.P., Brussels, 1 (1972).
100. C.C.Hall, Clin.Chim.Acta, 46, 21 (1973).
101. H.Riccomi, P.L.Mason, J.P.Vaerman, et al., Colloq.A.I.P., Brussels, 9 (1972).
102. C.L.Cambiaso, P.L.Masson, J.P.Vaerman, et al., J.Immunol.Methods, 5, 293 (1974).
103. C.L.Cambiaso, P.L.Masson, J.P.Vaerman, et al., ibid., 5, 153 (1974).
104. R.F.Ritchie, In "Automated Immuno Precipitin Reactions", p.59, J.D.Hamm (ed.), Technicon Instrument Corp., N.Y. (1972).
105. D.Pressman and M.Seigel, Arch.Biochem.Biophys., 45, 41 (1953).
106. L.Pauling, D.Pressman and D.H.Campbell, J.Amer.Chem.Soc., 66, 330 (1944).
107. L.Pauling, D.Pressman and A.Grossberg, ibid., 66, 784 (1944).
108. D.S.Skelly, L.P.Brown and P.K.Besch, Clin.Chem., 19, 146 (1973).
109. A.Zettner, ibid., 19, 699 (1973).
110. W.H.C.Walker, ibid., 23, 384 (1977).
111. G.Scatchard, Ann.N.Y.Acad.Sci., 51, 660 (1949).
112. V.P.Butler,Jr. and J.P.Chen, Proc.N.A.S., 57, 71 (1967).
113. C.R.Morgan, R.L.Sorenson and A.Lazarow, Diabetes, 13, 1 (1964).
114. I.M.Burr, D.B.Grant, P.C.Sizonenko, et al., J.Clin.Endocrinol., 29, 948 (1969).
115. L.B.Page, E.Desaulles, S.Lagg, et al., Clin.Chim.Acta, 34, 55 (1971).
116. A.Galskor, Acta Endocrinol., 69, Suppl.162, 3 (1972).

117. A.P.Phillips, *Lancet*, 1 , 1183 (1972).
118. C.R.Morgan; R.L.Sorenson and A.Lazarow, *Diabetes*, 13 , 579 (1964).
119. E.Cerceo and C.Elloso, *Clin.Chem.*, 18 , 539 (1972).
120. H.M.Slomon and S.D.Reich, *Lancet*, 2 , 1038 (1970).
121. T.W.Smith and E.Kaplan, In "Diagnostic Nuclear Cardiology (John Hopkins Symposium in Nuclear Cardiology)", St.Louis, C.V.Mosby Company (1973).
122. I.J.Seppala, *J.Immunol.Methods*, 9 , 135 (1975).
123. C.Chapman, W.N.Hutton and C.J.Hayter, *Clin.Sci.Mol.Med.*, 46 , 651 (1974).
124. K.Jensen, *Acta Path.Microbiol.Scand.*, 44 , 421 (1958).
125. K.Jensen, Thesis, Munksgaard, Coperhagen (1959).
126. T.Lofkvist and J.Sjoquist, *Acta Path.Microbiol.Scand.*, 56 , 295 (1962).
127. A.Grov, B.Myklestad and P.Oeding, *ibid.*, 61 , 588 (1964).
128. T.Lofkvist, *Int.Arch.Allerg.*, 29 , 240 (1966).
129. J.Sjoquist, J.Movitz, I.Johansson, et al., *Eur.J.Biochem.*, 30 , 490 (1972).
130. A.Forsgren and J.Sjoquist, *J.Immunol.*, 97 , 822 (1966).
131. G.Kronvall and D.Frommel, *Immunochem.*, 7 , 124 (1970).
132. G.Kronvall, U.S.Seal, J.Finstad, et al., *J.Immunol.*, 104 , 140 (1970).
133. I.Lind, I.Live and B.Mansa, *Acta Path.Microbiol.Scand.*, B, 78 , 673 (1970).
134. G.Kronvall and R.C.Williams, Jr., *J.Immunol.*, 103 , 828 (1969).
135. G.Kronvall, H.M.Grey and R.C.Williams, Jr., *ibid.*, 105 , 1116

(1970).

136. I.Lind and B.Mansa, Acta Path.Microbiol.Scand., 73 , 637 (1968).

137. J.Sjoquist, B.Meloun and H.Hjelm, Eur.J.Biochem., 29 , 572

(1972).

138. H.Hjelm, J.Sjodahl and J.Sjoquist, ibid., 57 , 395 (1975).

139. G.Kronvall, P.G.Quie and R.C.Williams,Jr., J.Immunol., 104 ..

273 (1970).

140. S.Jonsson and G.Kronvall, Eur.J.Immunol., 4 , 29.(1974).

141. S.W.Kessler, J.Immunol., 115 , 1617 (1975).

142. S.W.Kessler, Fed.Proc., 34 , 946 (1975).

143. S.W.Kessler, J.Immunol., 117 , 1482 (1976).

144. S.E.Cullen and B.D.Schwartz, ibid., 117 , 136 (1976).

145. M.Harboe and I.Folling, Scand.J.Immunol., 3 , 51,471 (1974).

146. I.Lind, M.Harboe and I.Folling, ibid., 4 , 843 (1975).

147. A.Grov, Acta Path.Microbiol.Scand., C, 84 , 333 (1976).

148. O.Folin and V.Ciocalteau, J.Biol.Chem., 73 , 627 (1927).

149. H.B.Herscowitz, W.W.Harold and A.B.Stavitsky, Immunol., 22 ,

51 (1972).

150. D.H.Katz, W.E.Paul, E.A.Goidl, et al., J.Exp.Med., 132 , 261

(1970).

151. Same as 55. above.

152. P.M.Keane, W.H.C.Walker, J.Gauldie, et al., ibid., 22 , 70

(1978).

153. B.Beerman, K.Hellstrom and A.Rosen, Clin.Sci., 43 , 507 (1972).

154. M.H.Gault, M.Ahmed, A.L.Symes, et al., Clin.Biochem., 9 ,

46 (1976).

155. J.R.Can, H.F.Deutsch, J.L.Fahey, et al., In "Methods in Immunology and Immunochemistry", chapter 3, vol. I, C.A.Williams and M.W.Chase (ed.), Academic Press, N.Y. (1967).
156. W.M.Hunter and F.C.Greenwood, Nature, 194 , 495 (1962).