A STUDY OF THE BIOCHEMICAL CHANGES WHICH OCCUR IN EXPERIMENTAL CADMIUM POISONING.

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ADRIAN CONAL GAIN, B.Sc. (Stell.)

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PART I

INTRODUCTION AND LITERATURE REVIEW.

I 1. PROPERTIES OF CADMIUM.

Cadmium (Gr. kadmia - earth) is an element with the following physical constants: atomic weight 112.41; atomic number 48; melting point 320.9°C., boiling point 767°C., specific gravity at 20°C 8.65, valency 2. It belongs to the second sub-group (Group II B) of the Periodic Table. It is a soft, bluish-white metal, which exists naturally as a mixture of eight stable isotopes[1].

Type	% Natural Abundance.
Cd^{106}	1.22
Cd^{108}	0.89
Cd^{110}	12.43
Cd^{111}	12.86
Cd^{112}	23.79
Cd^{113}	12.34
Cd^{114}	28.81
Cd^{116}	7.66

Certain of these isotopes appear suitable for activation analysis by neutron or other type of bombardment, which, in the future, could provide a much more sensitive technique for determination of trace quantities of the element than the spectrographic or colorimetric methods currently employed.

The metal tarnishes in air and burns when heated forming the oxide. It occurs naturally in small quantities associated with zinc, and was discovered by Strongmeyer in 1817 as an

impurity in zinc carbonate. Cadmium volatilises before zinc during the course of preparation of the metal, and condenses as a brown oxide, which is then reduced with carbon. It forms a number of salts, the chloride and sulphate being readily available in high degree of purity.

I 2. INDUSTRIAL USES OF CADMIUM AND ITS COMPOUNDS.

Cadmium has for many decades been used in industry, where its advantages have been numerous and divergent.

Prior to the advent of nuclear fission and subsequent nuclear reactors, cadmium was used mainly in the following industrial processes:-

(a) Cadmium carbonate was found to be an extremely efficient polishing material owing to its excellent abrasive action,

(b) Cadmium was used in the form of cadmium oxide in the manufacturing of alkaling accumulators (Friberg,L 1950)[2]. It was in these factories that cadmium-intoxication first became medically significant.

(c) Cadmium was used in an alloy with copper, in the manufacturing of copper electrical wires. It was found that cadmium in the copper wires greatly increased its wear and duration without significantly reducing its electrical conductivity. The cadmium was heated in a large open smelt and

workers were exposed to the fumes which were evolved.

(d) A rapidly expanding application is as a component of one of the lowest melting alloys and it is extensively used in bearing alloys with low coefficients of friction and great resistance to fatigue.

(e) Cadmium barriers to control atomic fission greatly facilitated manufacture of nuclear products in the United States of America.

(f) Cadmium finds application too, in standard cells for the accurate measurement of E.M.F. and as the sulphide as a yellow pigment in the paint industry.

Cadmium continues to find application in these ways, although probably its most important use is in the nuclear energy field.

I 3. EXPOSURE TO CADMIUM AND ITS COMPOUNDS AND CLINICAL EVIDENCE OF INTOXICATION.

Workers in industry have been most severely exposed to cadmium in two industrial processes, manufacture of copper-cadmium alloys or of alkaline accumulators. In the first, the men become intoxicated by inhalation of the brown fumes of cadmium oxide evolved from a molten mix, in the second they are exposed to cadmium oxide dust.

The toxicity of cadmium has been well documented during the past 20 years. Hardy and Skinner (1947)[3] investigated a group of five men who had been exposed to cadmium fumes for from 4 to 8 years. These men complained of chronic cough and gastro-intestinal symptoms and it was suggested that these symptoms were caused by cadmium. In 1950 Friberg in Sweden made important contributions through his studies on workers in the alkaline accumulator industry in that country. Kench and his colleagues (1955)[4] in a large series were able to confirm and extend Friberg's observations.

Baader (1951)[5] studied a group of 11 workers in an alkaline accumulator factory in Germany. Eight of these were found to have emphysema, proteinuria and loss of weight. He considered that a watery discharge from the nose, which he called "Cadmium snuffles" was an important early sign of toxicity. Friberg mentioned anosmia as an early sign of cadmium poisoning.

Two fatal cases reported by Campbell and Lane (1954)[6] died of emphysema. The men were workers casting copper-cadmium alloys and the emphysema developed rapidly over a period of two years without preceeding chronic bronchitis or asthma.

Bonnell (1955)[7] after obtaining evidence of intoxication by cadmium oxide dust, which had been studied by Friberg (1950)

and by Kench and his colleagues (1955)[4], decided to investigate other industries using cadmium, in order to establish whether it was, in fact, cadmium which had the toxic effect on the workers. He described five cases of chronic cadmium poisoning in men employed at two factories where an alloy of copper and cadmium was manufactured. The men presented clinically with what was becoming a clear picture of cadmium intoxication. They had emphysema in both lungs, extreme dyspnoea, loss of weight and appetite and also had increased amounts of protein and of amino acids in the urine.

In all cases described in the literature, the workers have suffered from a chronic type of poisoning, which developed after many years of exposure. The most important route of entry of the cadmium was via the lungs in the form of dust or fume. Uptake also takes place via the gastro-intestinal tract, due to contamination of the hands and thence into the mouth. Due to the inefficiency of gastro-intestinal absorption of cadmium relatively small quantities were taken in this way and intoxication was a slower process. Nevertheless, cadmium becomes highly dispersed by either route. As a result of the findings of the M.R.C. Sub-committee on Chronic Cadmium Poisoning, this type of intoxication was, in 1959, declared to be a notifiable disease in England and Wales under the provisions of the Factory Act.

Many sporadic cases of acute cadmium poisoning in man have

been described from time to time, and the course and progress of acute intoxication in experimental animals has frequently been documented, in which similar lesions, histological and biochemical, have been described by a number of investigators.

I 4. DISTRIBUTION OF CADMIUM IN THE BODY.

Cadmium on oral administration is mostly unabsorbed and excreted in the faeces. If administered parenterally it passes into the body pool and is retained in the soft organs, i.e. spleen, liver and kidneys. Excess cadmium in the pool is excreted mainly in the urine.

In contrast to radio-isotopes of biologically-active and physiologically-occurring elements which, following intravenous injection, are excreted at rates that increase upon metabolic loading with a stable carrier, Cd^{109} showed negligible total body turnover, regardless of challenges with cadmium or zinc, Cotzias et al (1961)[8]. It, therefore, does not seem to obey haemostatic control.

Nevertheless, although cadmium is in the main unabsorbed, the small amount retained in vivo, has a most significant toxicity with resultant biochemical and clinical effects.

Kench et al made exhaustive tests to ascertain the distribution of absorbed cadmium in the body. In their experiments on rabbits, they produced chronic and acute cadmium intoxication using intravenous injections of cadmiumsulphate and after killing the animals, found that the cadmium did, in fact,

accumulate in all the soft organs, especially the kidneys, liver, testes, spleen and lung - Smith, Kench and Smith (1957)[9]. See Table 1. Experiments with radio-active Cd^{115} performed recently show conclusively that cadmium is selectively accumulated in the cortex of the rat kidney.

FIGURE 1.[10]

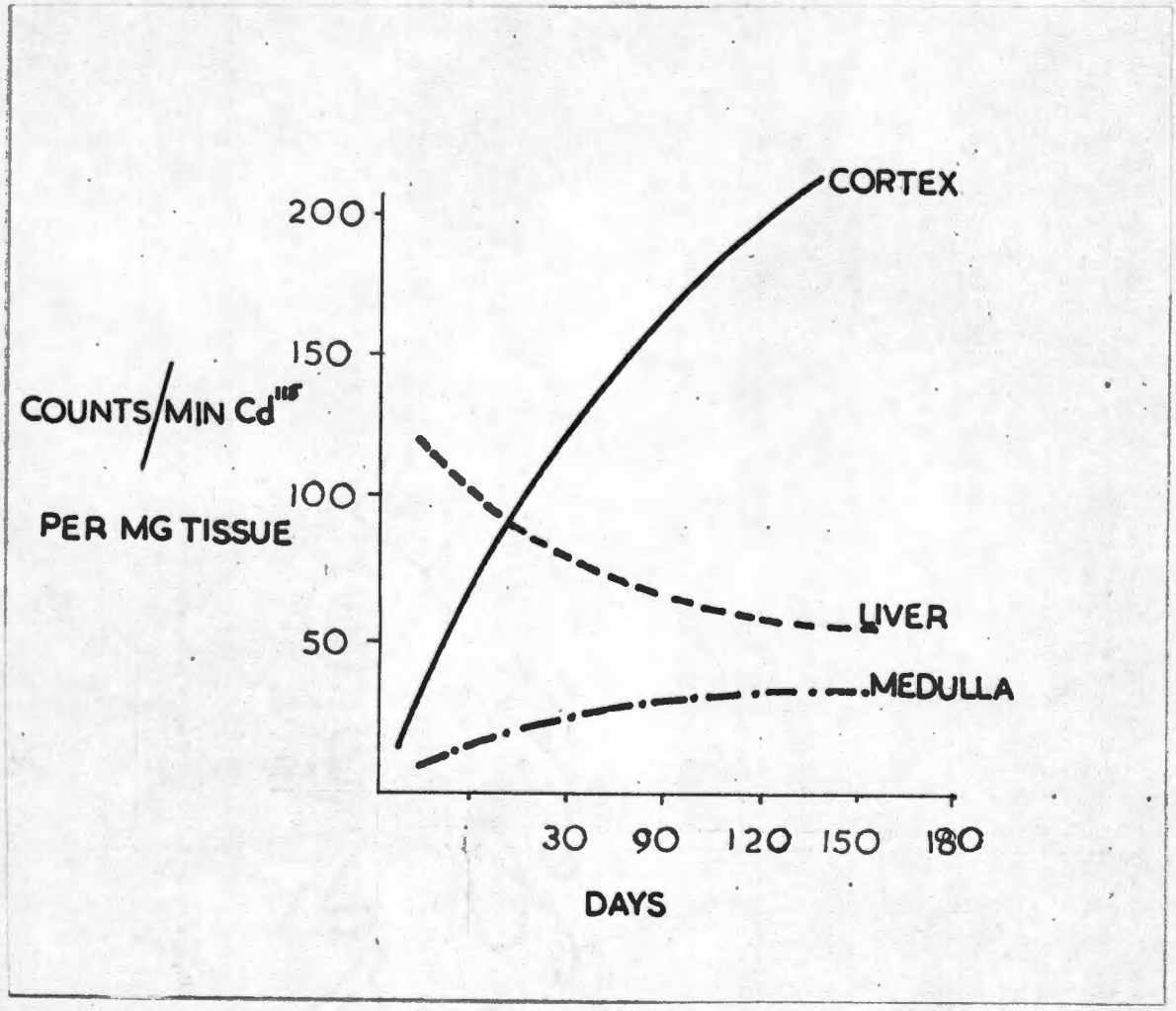


TABLE I.[9]

CADMIUM CONCENTRATION IN TISSUES OF A MAN (J.R.) EXPOSED TO CADMIUM AND OF THREE MEN WITHOUT KNOWN EXPOSURE.

Tissue	Tissue Cadmium Concentration (g./g. wet weight)			
	J.R. (46)	N.1 (56)	N.2 (56)	N.3 (49)
Adrenals	24	0.42	0.60	-
Artery (mesenteric)	100	-	-	-
Artery (aorta)	-	0.40	0.35	0.48
Brain	3.5	0.12	0.12	0.12
Bone marrow	4.1	0.31	0.30	0.24
Colon	20	0.29	-	0.24
Epididymis and vas	54	0.63	0.55	0.59
Gall bladder	21	0.62	0.53	0.65
Bile	24	-	0.088	0.071
Heart	11	-	0.18	0.18
Kidney cortex	150	14	18	12
Kidney medulla	130	12.5	15	8.5
Liver	160	2.0	2.8	2.1
Lung	13	0.62	0.73	0.79
Lung oedema fluid	5.0	-	-	-
Mesenteric fat	11.5	-	-	-
Skeletal muscle	7.1	0.095	0.11	0.13
Pancreas	84	-	0.35	0.61
Prostate	37	0.17	0.10	0.18
Rib cartilage	11.5	0.26	0.20	0.20
Seminal vesicles	24	-	-	-
Skin and panniculus	4.0	0.16	0.10	0.27
Spleen	55	0.35	0.29	0.36
Stomach	32	0.30	-	0.31
Testis	38	0.16	0.20	0.19
Testis capsule	94	0.30	0.32	0.34
Tongue	11	0.21	0.16	0.19
Urinary bladder	30	0.31	0.31	0.40
Bladder urine	0.34	-	-	-
Vein (mesenteric)	150	-	-	-
Vena cava	-	1.2	-	0.87
Cranium	0.91	0.12	0.09	0.18
Femur	1.2	0.12	0.20	0.15
Rib	0.88	0.14	0.10	0.18

I 5. HISTOLOGICAL EVIDENCE OF INJURY TO TISSUES IN CADMIUM POISONING.

Much evidence has been accumulated over many years to show that kidney tissue is particularly susceptible to injury by heavy metals. Lead causes gross renal damage - Aub, Fairhall, Minot & Reznikoff (1926). This damage has been located especially in the tubules. Gross kidney damage in experimental uranium poisoning was detected and described by MacNiden in 1924. Evidence of similar damage by inorganic mercury was shown by Edwards (1942) and the toxicity of organomercurials was well known. Since then, blockage of Sodium reabsorption formed the basis for their use as diuretics.

Renal damage has been described in animals with experimental cadmium intoxication by Schwartze and Allberg (1923), Prodan (1932) and Pancheri (1937). Foster and Cameron (1963)[13] confirmed the accumulation of cadmium in the kidney. By giving subcutaneous injections of cadmium chloride to rabbits, they produced changes in the proximal convoluted tubules which could be regarded as being a consequence of an increase in the permeability of the glomerular complex to plasma proteins. This conclusion was drawn after noting the presence of large numbers, compared with normal animals, of periodic acid Schiff (P.A.S.) positive stainable droplets in the cells of the proximal convoluted tubules.

Similar anomalies in the proximal tubular epithelium have

been described by Millichamp, Llewellin and Roxburgh (1932) and by Marsden and Wilson (1955).

Kench et al (1962)[11] observed only minimal changes post mortem in their experimental rabbits, but noted the presence of a fine multilobular cirrhosis of the liver.

Subcutaneous injections of cadmium chloride given to rats by J. Parizek and Z. Zahor (1956) produced macroscopic lesions in the testes[12]. Even 2 - 4 hours after the injection capillary stasis and oedema of the interstitium could be observed microscopically and 8 hours after the injections extensive haemorrhage had occurred. In view of the fact that it had been possible to suppress the destructive effect of cadmium on the testis by using many times the dose of zinc, they also postulated a mechanism of competitive inhibition between these two metals, especially as similar lesions are caused by prolonged nutritional deficiency of zinc, which itself has a rapid turnover in the testes and a very high concentration in the spermatozoa.

In similar experiments on rabbits, Cameron and Foster (1963) found that subcutaneous injections of a diluted aqueous solution of cadmium chloride (9.12 mg./ml) gave variable results and that certain individuals had a much higher tolerance than others[13]. Nevertheless, testicular damage was apparent in most cases. In sensitive animals, cadmium produced marked swelling and discolouration of the testes. The colour, which was due to extravasation

of blood into the interstitium, ranged from light reddish hue, appearing on the first day after the initial injection, to a purple or dark mahogany shade on the second and third days. Histological lesions included profound changes in the spermatogenic epithelium within a few days, the Leydig cells were also affected and the interstitium was oedematous and distended. Hyperaemia was followed by profuse haemorrhage and free erythrocytes, in vast quantities, were clearly visible between the tubules.

They concluded that the destructive effect of cadmium is due to the following actions:

(a) it inhibits sulphydryl enzymes by forming mercaptides with their sulphydryl groups, an effect which can be reversed by dithiols.

(b) it combines with similar sulphydryl groups in the mitotic apparatus or its vicinity.

(c) it competes with zinc

I 6. THE BIOCHEMICAL EFFECTS OF CADMIUM IN VIVO.

I 6 (i) Binding of cadmium to proteins.

Very little is known regarding the binding sites for cadmium in cells and tissues. Some interesting observations have been made, however, on three dogs which had been exposed to radio active cadmium. The lungs were fractionated and the average concentration

of cadmium in each fraction expressed as µg.Cd/g. dry weight was

Albumin 120

Globulin 60

Lipoid 4.5

This high concentration found in the albumin fraction is rather significant in the light of results obtained from our present study, in which we find albumin metabolism to be deranged in cases of cadmium poisoning.

I 6 (ii) Amino aciduria.

Amino acids circulating through the renal glomeruli are filtered off at the rate of 65 g. in the 180 litres of filtrate during each 24 hours. Of this amount only 1 g. is excreted daily in the urine, the remaining 64 g. being reabsorbed in the proximal convoluted tubules.

Amino aciduria will arise whenever the rate at which one or more amino acids passed into the glomerular filtrate is greater than the rate at which the amino acids can be reabsorbed by the renal tubular cells. This may occur when

(a) The renal tubular cells are normal, but the blood concentration of amino acids is abnormally raised - the over-flow type.

(b) The blood concentration of amino acids is normal, but

the renal tubular cells are functionally defective - renal tubular amino aciduria.

(c) The blood concentration is raised and renal tubular cells are also subnormal - mixed type of amino aciduria (Table II). Poisoning with heavy metals gives rise to acquired renal tubular defect, without metabolic disorder. The excretion in the urine of at least some of these metal ions increases proportionally to the clearance of creatinine - Smith & Kench (1957)[15], and the evidence available supports the conclusion that most of the metals are filtered at the glomeruli. The metal causes damage to the proximal renal tubules, the extent of which depends on the nature of the metal ion, the amount and the duration of the period of intoxication. More than one function of the tubules may be affected, thus the aminoaciduria of lead poisoning is usually accompanied by glycosuria and phosphaturia.

The aminoaciduria is a generalised one and has been reported in workers exposed to either lead, cadmium, mercury, uranium or copper. Although the aminoaciduria affects all amino acids, some metals give rise to a disproportionate increase in a few amino acids; thus alanine and γ-aminoisobutyric acid predominate in lead poisoning, glycine is particularly marked in mercury poisoning, in uranium poisoning the γ-aminoisobutyric acid excretion is low when compared with that of alanine and in cadmium poisoning serine and threonine may be excreted in excess, the rest of the pattern being normal - Clarkson & Kench (1956)[11].

TABLE II

PROVISIONAL CLASSIFICATION OF THE AMINOACIDURIAS.

I Aminoaciduria without Renal Tubular Defect. With Raised Plasma Aminoacid Level		II Aminoaciduria with Renal Tubular Defect. Without a Raised Plasma Aminoacid Level				III Aminoaciduria with Renal Tubular Defect. With Raised Plasma Aminoacid Level	
		Acquired		Congenital		Acquired	
Acquired	Congenital	With Metabolic Disorder	Without Metabolic Disorder	With Metabolic Disorder	Without Metabolic Disorder	With Metabolic Disorder	Without Metabolic Disorder
Liver Disease	Phenylketonuria Tyrosinosis Alkaptonuria "Maple Syrup" Disease	Wilson's disease Galactosaemia Rickets Scurvy Nephrotic syndrome	Heavy metal poisoning lead, cadmium, mercury, uranium, copper Oxalic acid poisoning Lysol poisoning Burns	Fanconi syndrome Cystinosis Hartnup disease	Cystine-Lysinuria	Coeliac disease Adult idiopathic steatorrhoea	Phosphorous poisoning

with acknowledgment to Ivor Smith "Chromatography" Vol. I p. 125 (1963).

Investigations have revealed that cadmium and uranium produce aminoaciduria more readily than the other heavy metals mentioned. The kidneys recover their normal function when the patient is removed from contact with the metal. The observed fact[11] that serine and threonine appear together before other amino acids are excluded into the urine, suggests that these two hydroxy amino acids share a transport pathway through the proximal renal tubular cells, distinct from that followed by the other amino acids. These observations were made on large series of workers exposed in industry, but, unfortunately, no parallel measurements of blood amino acid levels could be made. It is not possible to conclude, therefore, that the amino aciduria was entirely renal in origin - there may, in fact, have been a metabolic component in it.

I 6 (iii) Proteinuria.

The early observations of Friberg (1950)[2] stimulated much of the subsequent interest in cadmium poisoning. He noted a high incidence of proteinuria in workers in the alkaline battery industry under his care. Pedersen (findings published in Friberg's monograph, page 37) examined the protein and found it to have a molecular weight of 25 - 30,000. The protein has been found consistently since then in man and animals poisoned by cadmium, by inhalation or parenteral route.

Kench and his colleagues (1955)[4] in Manchester carried out

a series of investigations on the incidence and degree of proteinuria, which have confirmed and extended the observations of Friberg. They determined the incidence and degree of proteinuria in relation to cadmium exposure and excretion. Urinary cadmium and excreted protein appear to vary independently of one another in the series of 95 workmen exposed to the hazard, but the individual who worked in a severely contaminated atmosphere consistently excreted most cadmium and protein in his urine. Currently exposed persons generally exhibited higher rates of urinary cadmium excretion than those not currently exposed, whilst 95 workers in the same factory, who had not been in contact with cadmium oxide duct, were within the normal range. The quantity of urinary protein was 1.0 - 3.2 g./l. - Smith, Wells & Kench (1961)[17], which is much in excess of the average normal value of 133 mg./24 hr. found by Webb and his co-workers in young adult males - Webb, Rose & Sehon (1958)[18].

As regards the nature of the urinary protein, Friberg described it as having the electrophoretic mobility of α_2-β globulins. The proteinuria could not unequivocally be demonstrated by the heat coagulation test - Kazantis, Bonnell[19]; Smith, Kench & Lane (1955)[4]. Complete precipitation is effected by addition of 10% w/v aqueous trichloracetic acid (T.C.A.) (1 vol. T.C.A. to 1 vol. serum or urine) in the experience of Kazantis, Bonnell, Smith et al and of the present workers.

Olhagen (page 36 of Friberg's monograph) observed that the urinary protein was precipitated when the urine was fully saturated with ammonium sulphate, but not on 50% saturation. The sequence of events as observed by the Manchester workers was the initial appearance of an α_2 globulin, followed closely by a β globulin fraction. Later, protein fractions appeared migrating as serum albumin and γ globulins, until eventually the urinary protein pattern appears similar to that of serum. Kekwick (1955) observed that even when the pattern was complex in this way, the urinary proteins all sedimented in the ultracentrifuge in the range 20 - 30,000 - Kekwick (1955)[20]. Physicochemical studies - Webb et al (1958)[18] have revealed the presence in normal urine of α_2 globulins of low molecular weight, 10,600, but failed to distinguish between the urinary albumin fraction and normal serum albumin. The α_2-β and other globulins excreted normally - Webb et al (1958) - or in conditions of renal tubular necrosis - Creeth, Kekwick, Flynn, Harris and Robson (1963)[21] - have molecular weight similar to those found in cadmium poisoning, and the latter workers are convinced that the proteinuria of cadmium poisoning is a mixture of globulins typical of any non-specific renal tubular defect or necrosis, as obtains in hepatolenticular degeneration or Fanconi syndrome. The proteinuria of cadmium poisoning is, in their view, neither specific nor unique. It appears from the publication of Creeth et al that they regard the early urinary peak also as probably an α_1 globulin. Recently

Poortmans and Fraechem (1965)[22] have shown that the mobility of serum albumin and other serum proteins is somewhat augmented in urine, an affect which they ascribe to adsorbed urinary pigments. This albumin found in normal urine - Webb et al (1958)[18] - and in the urine of nephritic and nephrotic patients - Rowe (1957)[23] Smith, Wells and Kench (1961)[17] - is indistinguishable from normal serum albumin in molecular size. The same was true in a person whose proteinuria was due to metallic mercury poisoning - Smith and Wells (1960)[24], a relatively rare manifestation of this intoxication.

On the basis of its close similarity in amino acid structure to serum albumin - Smith, Well and Kench (1961)[17], its electrophoretic mobility and low molecular weight it was postulated that the special feature of cadmium intoxication is the excretion of an albumin of low molecular weight. As later studies have tended to support this hypothesis, this urinary protein has been referred to by us as minialbumin. The cadmium-poisoned workpeople so far examined have excreted only minialbumin, unless renal disease from other cause has been present. In a study of rabbits chronically poisoned by repeated intravenous injections of cadmium sulphate - Kench, Wells and Smith (1962)[14], it was found that both albumins, of low and of normal molecular weight, appeared in the urine. The metabolic turnover of the low molecular species appeared to be more rapid than that of normal albumin, and, on intravenous injection of the C^{14}-labelled urinary proteins into normal rabbits, much more activity was recovered in the urine as protein than when C^{14}-labelled

serum proteins were similarly injected. The evidence, however, was only tentative in kind, since techniques for separating such closely related molecules (e.g. minialbumin and normalbumin, myoglobin and haemoglobin - Berman and Kench (1963)[25]) were not, at that time, available.

The present thesis is an account of biochemical studies designed to separate and characterize, with greater exactitude than was previously possible, the urinary minialbumin of cadmium poisoned animals, and to obtain evidence on its tissue source and the mechanism of its formation. In this context, the question of renal permeability to serum proteins of different molecular weights is a very important one. Hardwicke (1965)[26] has shown, both by immunological procedures and by use of gel filtration through sephadex G 200, that renal clearances of serum proteins increase steadily with diminishing molecular weight.

I 6 (iv) Other biochemical effects of cadmium in vivo.

In experiments on dogs, Vander (1962)[27,28,29] has observed that cadmium has a marked inhibitive effect on renal tubular absorption of sodium and water, more especially in the proximal convoluted tubules. Furthermore, Kägi and Vallee have isolated and characterised a cadmium- and zinc-containing protein present normally in the renal cortex of the horse - Kägi and Vallee (1960)[30,31]. This equine protein, which they have named metallothionem, contains

2.9% cadmium, 10.6% zinc and 4.1% sulphur per gram dry weight of protein. The protein has a number of unusual features. The nitrogen content 13.2%, is low compared with that of most proteins. Aromatic amino acids are almost completely absent, with lack of spectral absorption at 280 mμ. It is, however, very rich in cysteine residues[32], which, of course, bind the zinc and cadmium atoms. The sedimentation constant S_{20} is 1.8 - 2.18, which for a spherical molecule gives molecular weight of 17 - 18,000. On dialysis cadmium will displace zinc from the protein, but once cadmium is bound to the protein it cannot in turn be displaced by zinc.

Evidence of human renal damage has been brought forward by Friberg and by Kazantis et al (1963)[19].

I 7. BIOCHEMICAL EFFECTS OF CADMIUM IN VITRO.

I 7 (i) Cellular changes caused by cadmium.

Jacobs and his co-workers (1956)[33] found that cadmium appeared to uncouple oxidative phosphorylation in tissues. The uncoupling was reversible, and by adding E.D.T.A., oxidative phosphorylation returned to normal, i.e. the P/O ratio became approximately 3/1. These investigators reported that there is a strong binding of cadmium to the mitochondrial proteins, even beyond the point of complete uncoupling, which was achieved at a cadmium concentration of 5×10^{-6}M. The reversibility of uncoupling has also beendescribed by Fletcher et al (1962)[34], who

suggested that the inhibitive effect of cadmium was due to a reaction with essential thiol-groups in the system.

These cellular disturbances are also interesting in the light of the present study and of the important observations of Lehninger (1961)[35] on the role of bovine serum albumin in the maintenance of coupled oxidation of isolated rat liver mitochondria. He demonstrated an uncoupling of oxidation and respiration, with consequent swelling, in such organelles following addition of Ca^{2+} or thyroxin, due apparently to the formation of an endogenous uncoupling factor ("factor" presumed to be non-esterified fatty acids) - Wojtzak and Lehninger (1961). When bovine serum albumin was added to the system, the free fatty acid became bound to the protein and could no longer act as an uncoupling agent.

F.P. Simon and his colleagues (1947)[36] have reported metabolic inhibitions by cadmium, to a greater or lesser extent, on many tissue slices and enzyme preparations. Kidney brei was more sensitive to cadmium than were liver or lung brei or slices. Added thiols were protective against the metal.

I 7 (ii) Action of cadmium on isolated enzymes.

Succinic dehydrogenase prepared from lung tissue is virtually completely inhibited by cadmium in concentrations as low as 10^{-4}M. The sulphydryl group is an essential requirement

of the succinic enzyme and it is believed - Simon et al - that cadmium combines with and inactivates the free -SH group.

In relation to the yellowish bands which accrue at the base of teeth of persons chronically exposed to cadmium compounds, Burnstone (1957)[37] presented evidence that genuine esterase activity occurs in bone matrix and dental pulp and that the esterase activity would be markedly depressed by 0.001 molar concentrations of cadmium ions. Similar inactivation would arise from equivalent concentrations of the heavy metal ions Cu^{2+} and Zn^{2+}. Cadmium is inhibitory, also, to the enzyme prolidase, which hydrolyses substrates in which the susceptible linkage lacks a peptide hydrogen, as e.g. glycyl-L-proline where linkage occurs to the tertiary nitrogen atom of the pyrrole ring - Adams and Smith (1952)[38].

In contrast to the above, certain enzymes are known to be activated by cadmium. This Sano et al[39] have shown that cadmium ions are essential for the action of histidase on histidine and arginase is activated by cadmium - Edelbacher and Baur (1938)[40]. The metal also activates oxaloacetic carboxylase, Herbert (1951)[41] found in the bacterium micrococcus lysodeikticus, and at 0.001 M activates the specific iminodipeptidase which hydrolyses dipeptides containing N-terminal L proline or hydroxy L proline - Neuman and Smith (1951)[42], and, in addition, a number of plant carboxylases.

At present, it does not seem possible to evaluate these facts

from the point of view of either the possible role of cadmium as an essential trace element in metabolism or of biochemical changes evoked in cadmium poisoning.

I 8. CONSIDERATIONS RELATIVE TO THE NATURE, ORIGIN AND FORMATION OF THE URINARY PROTEIN OF CADMIUM POISONING.

The present thesis is the product of a continuing interest in the metabolic effects of cadmium ions, and, more especially in the nature, origin and formation of the urinary protein which arises in cadmium poisoning. Amino acid analyses based on 8 accurately measurable amino acids have shown identity in composition between normal human serum albumin and the low molecular protein, found in the urine of cadmium poisoned workmen - Smith et al[9], which has an electrophoretic mobility similar to that of normal serum albumin. Later experiments with other animals (rabbits, dogs and monkeys) have revealed the fact that similar low molecular proteins are excreted in the urine, when the animals are chronically poisoned with the metal. The delay before the appearance of the protein may be as long as 3 months after the commencement of treatment.

These observed facts lend themselves to a number of interpretations as to causative mechanisms, assuming that the protein is, indeed, a true minialbumin.

1. Cadmium causes a partial block in the synthesis of the long single polypeptide chain which constitutes the normal serum albumin

molecule. The target would be the liver microsomes, and the minialbumin so formed would be rapidly filtered by the renal glomeruli into the urine. Delay in its appearance in the urine could reasonably be ascribed to the time needed for cadmium to bring about damage to the proximal renal convoluted tubules, which would otherwise reabsorb the minialbumin filtered by the glomeruli. This is the working hypothesis favoured by Kench and his colleagues (1962)[14], and the springboard for earlier and also the present investigation.

2. The low molecular albumin might originate in the renal tubular cells as a consequence of degradation into large fragments of serum albumin normally reabsorbed into them. Any non-specific tubular lesion could then release the protein into the urine. This appears to be the view of Creeth et al[21], since they report that the urinary protein pattern in cadmium-poisoned persons is identical with that of patients suffering from Fanconi syndrome, hepatolenticular degeneration, galactosaemia - in short all non-specific renal tubular lesions. A variant on this renal tubular hypothesis could envisage that cadmium within the tubular cell gave rise to the formation of the low molecular albumin from its normal serum prototype, reabsorbed in the normal way, and that subsequently, membrane permeability defects allowed the release of the protein into the urine.

3. A third hypothesis which may merit consideration is that a certain proportion of small albumin molecules are normally formed,

but are effectively reabsorbed by the renal tubular cells. Only when these latter are damaged by cadmium or other noxious agent will the small molecules escape into the urine. In a preliminary paper Merler et al (1962)[43] report that normal human urinary albumin has, in fact, smaller molecular weight S_{20}, W of 2.6 S when compared to 4.2 S for crystalline serum albumin. Kench et al (1962)[14] have tentatively demonstrated in the rabbit that the small albumin has a high turnover rate, on the basis of which, and on the known excretion in cadmium workers, it was computed that approximately 20% of all albumin molecules, formed in the livers of poisoned workmen, would be of this small species. Some other experiments with rabbits seemed to indicate that the minialbumin was rapidly cleared through a normal kidney. If the small albumin molecules have a short half-life normally, their concentration might well be beyond the limits of detection by present techniques. Heterogeneity in the albumin fraction is an established fact, as seen in the hereditary condition in man known as bisalbuminaemia - Knedel (1957)[44] - an exceedingly rare trait. Otherwise, serum albumin once freed of α_1 glycoproteins is one of the more homogeneous proteins available, if its aggregation behaviour at low pH is excluded. The variable sulphydryl content originally described by Hughes (1947)[45], has been classified by King (1961)[46]. The free sulphydryl group of serum albumin is readily masked by combination with cysteine or glutathione: King explained his results by masking of the sulphydryl-free albumin monomer by an additional half-cysteine residue (or to a lesser

extent by glutathione) bound via a disulphide linkage.

Microheterogeneity of albumin as revealed by chromatography on DEAE cellulose has been described - Rejnek, Koci and Bednarik (1963)[47]. Two distinct fractions were obtained, one of which probably could be further subdivided. There were differences in antigenicity amongst the components. No significant difference in molecular size has been detected in macroglobulinic serum albumin given as 66,400 by Caputo and Galcotti (1963)[48].

In this department we have been unable to detect any significant differences from normal in the amino acid composition of the serum albumin of children suffering from grave protein depletion in kwashiorkor, Potgieter, Hines and Kench[49], and adjustment to amino acid deprivation appears to be contrived mainly by a considerable retardation in metabolic turnover - Purves and Hansen (1962)[50]. This latter mechanism may be associated with the marked quantitative and qualitative changes in urinary peptide pattern which we have followed during the periods of protein deprivation and repletion, Berman and Kench (1963)[51]. Whether peptides as such can exert a metabolic influence on protein turnover, or if abortive synthetic fragments are merely discarded, are questions being further explored.

The sedimentation value reported by Merler et al (1962)[43] for normal human urinary albumin is at variance with that reported earlier by Rowe (1957)[23], who observed similar values for serum and urinary human albumins. Likewise, the molecular weights

of urinary albumin of nephrotic and nephritic patients or persons poisoned with metallic mercury were all close to that of normal human serum albumin[9,23,24].

The isolation and characterisation of fragments of serum albumin retaining antigenic properties is being pursued actively along the lines similar to those so successful in the elucidation of the antigenic structure of γ-globulins, Small, Kehn and Samm (1963)[52]. Press and Porter (1962)[53] have studied the degradation of human serum albumin with α-chymotrypsin. Four serologically active components were produced, ranging in molecular weight from 7100 to 23,400. The smallest component did not precipitate antibody but partially inhibited precipitation. Its amino acid composition differed greatly from that of the whole molecule : for every 100 amino acid residues, there was only one residue each of arginine, proline, glycine, methionine, and isoleucine, 1.5 residues of histidine and serine and tyrosine was absent. The overall figures for the 611 amino acid residues of the complete albumin molecule are arginine (25), proline (31), glycine (15), methionine (6), isoleucine (9), histidine (16), serine (25), tyrosine (18), which gives per 100 residues arginine 4, proline 5, glycine 2.5, methionine 1, isoleucine 1.5, histidine 2.5, serine 4, tyrosine 3 - values calculated from Brand (1946)[54]. Altogether the serum albumin molecule is a single folded polypeptide chain containing 35 - 37 half-cystine residues, only one free sulphydryl group, and therefore 17 - 18

intramolecular disulphide linkages. The molecular weights for human, rat, rabbit and guinea pig serum albumins are all close to 65,000 - Charlwood (1961)[55].

I 9. OBJECTIVES OF THE PRESENT STUDY.

From consideration of these and other data, it became clear that further understanding of the nature, origin and formation of the urinary protein of animals poisoned with cadmium required, at least the following:-

1. A procedure which would allow rapid and complete separation of normal and low molecular albumins in the urine, when they are present together.

2. Preparation of the albumins free of other proteins, particularly α_1 and α_2 globulins: by techniques specific for serum albumin which would enable one to establish firmly that the low molecular protein did indeed in this and other respects behave consistently as an albumin.

3. Comparison of the two types of albumin as to quantity present, molecular weight, amino acid composition, electrophoretic mobility and antigenic behaviour.

4. Timing of the appearance of normal and low molecular albumins in the urine of poisoned animals.

5. Operative procedures which would help to localise the site of origin of the small albumin molecule.

Most of these requirements have been successfully met in the present study, as will be explained in the following section.

I 10. OUTLINE OF THE WORK AND THE RESULTS ACHIEVED.

It was decided to produce experimental cadmium intoxication and resultant proteinuria in three types of animals, i.e. two monkeys, two dogs and two rabbits. Previous experiments on rabbits had been carried out by J.E. Kench et al, during which he conducted analysis which included electrophoresis, ultra-centrifugation and amino acid composition, on the urinary proteins of rabbits. These rabbits had received intravenous cadmium sulphate to bring them to a state of chronic cadmium intoxication. Thus we had a reference animal with which to correlate our results.

The animals were all injected twice weekly intravenously with cadmium chloride ($CdCl_2\ 2\frac{1}{2}H_2O$) made up to an isotonic solution with sodium chloride. After each animal had received a certain critical dose, characteristic clinical signs became obvious just prior to the onset of gross proteinuria. The animals all became extremely morose and bad tempered, later very weak and lethargic, then finally moribund.

The urines were collected daily and the protein content determined by the modified biuret method of Hiller, McIntosh and van Slyke (1927)[56,57]. The digestion method of Kjeldahl, adapted by McKenzie and Wallace (1954)[58], to determine total nitrogen was used as a reference method.

The urinary albumin fraction was in earlier trials separated from other urinary proteins by electrophoresis in columns of acetylated cellulose according to the method of Porath and Flodin (1954)[59], as modified by Campbell and Stone[60]. Subsequently the trichloracetic acid procedure was employed as described by Vallence-Owen and co-workers (1958)[61], since it gives a good yield of serum albumin, and it exploits the characteristic stability of serum albumin in trichloracetic acid - acetone medium, apparently unique amongst serum proteins. Thus all traces of α globulins are excluded from the final preparation. Serum albumin which passes through the preparative process does so unscathed, as judged by the criteria of molecular weight, amino acid composition, antigenicity and other properties, Levine (1954)[62]. The method was preferred to the C.M. cellulose process perfected by Potgieter and Hines (1963)[63], since the latter also involves more steps with columns of resins and fraction collection. Both analytical procedures provide an albumin preparation in good yield and homogenous with respect to electrophoretic mobility on paper zone electrophoresis according to Flodin and Porath and on sedimentation in the analytical ultracentrifuge.

The purified albumin was then made into a solution of adequate concentration and passed through a column of cross-linked dextran gel, which acts as a molecular sieve, separating molecules of different molecular weight. The eluate was passed through an ultraviolet absorptometer and two protein peaks were

identified, one being in the position of normal serum albumin and the other emerging later, which gave evidence of its low-molecular nature.

This low-molecular weight "albumin" was now subjected to various characterisation procedures. The amino acid composition was determined following the ion-exchange method of Moore, Spackman and Stein (1958)[64]. Titration curves were carried out, carbohydrate content and diffusion constants worked out and the sedimentation constant deduced from ultra-centrifugation studies. Its antigenic behaviour was examined by immunoelectrophoresis and by Ouchterlony plate techniques employing polyvalent Coombs anti-human serum and specific rabbit anti-monkey normal serum albumin. In all these, its antigenic behaviour was indistinguishable from normal monkey serum albumin, with possible proviso that its rate of diffusion through the agar gel was slightly quicker than that of normal serum albumin.

In all aspects the low molecular weight protein behaved and reacted like an albumin, the only difference being that its molecular weight was 18,000.

In order now to gain more facts as to the in vivo metabolism of this "minialbumin" it was decided firstly to produce chronic cadmium intoxication in a monkey and during the stage of gross proteinuria to inject a radio-active amino acid and to "follow"

its path of incorporation into normal serum albumin and into the minialbumin. These albumins would then be separated from the serum and the urine and their specific activity measured in a liquid scintillation counter.

The minialbumin in the urine was found to have a much faster turnover rate than that of normal serum albumin; in fact it was excreted at such a rate from the serum that it was impossible to detect it in the vascular pool.

Having measured the turnover rates, we then decided to collect together as much C^{14} labelled low molecular weight albumin as possible and to re-inject this into a normal monkey. In the hope of confirming its turnover rate and also to determine its fate, we thought perhaps it would be incorporated into a normal albumin and taking hourly blood samples we could measure the radio-active increase of the normal serum albumin.

Enzymatic studies were carried out on the same monkey, giving it regular cadmium chloride injections until it became chronically ill. This was in order to ascertain any specific lesion which could be caused by excess cadmium ions in the body.

Finally, bilateral nephrectomy was performed, and the experimental animal was supported for 6 post-operative days by peritoneal dialysis on alternate days. Examination of the peritoneal dialysate by the above procedures demonstrated unequivocally the presence of minialbumin on all three occasions.

PART II.

EXPERIMENTAL AND RESULTS.

II 1. ANIMALS AND THEIR MAINTENANCE.

II 1 (i) Experimental animals.

As mentioned earlier, it was decided in the initial experiments to study three species of animals, namely, rabbits, monkeys and dogs. The rabbits were a mixed breed, with many characteristics of the Flemish giant. Vervet monkeys (Cercopithicus aethiops) were captured in the wild state. The dogs were a rather impure Cairn variety. All the animals were males.

Two individuals of each species were chosen and placed in metabolic cages, in order to ascertain their normal biochemical parameters. Young full-grown specimens of steady weight and, therefore, in nitrogen balance were chosen.

II 1 (ii) Maintenance of Animals.

The metabolic cages were all of similar dimensions, i.e. 18" x 24" and 18" high. These cages, each holding a single individual were suspended by means of galvanised iron strips to angle-iron supports in an enclosure. The bottoms of the cages were fitted with $\frac{1}{2}$" mesh 14 gauge wire meshing. Each bottom was hinged to facilitate cleaning. This mesh bottom acted as a primary, rough separating layer, which allowed the urine to pass through, keeping back most of the stool and debris.

The suspended cages were surrounded by a wire framework, 8" from the sides of the cage. This framework was covered

with polythene sheeting which tapered to a point under the cage to form a funnel.

During later experiments on the monkeys, they were placed on stainless steel trays, these being covered with mosquito netting in order to prevent any contamination of the urine. This method of collection was possible at this stage because the monkeys, having become less active due to cadmium intoxication, did not urinate out of the sides of the cage but only through the bottom.

The animals were all fed daily between 11 a.m. and 3 p.m. Their diets consisted of the following:

Monkeys: Carrots, cabbage, sweet potatoes, oranges and dried maize. They do not drink water and none was provided.

Dogs: Raw meat (beef), cooked mealie meal and dog biscuits. ½ pint of milk was given daily, plus water every morning after collecting their urines.

Rabbits: Rabbit biscuits containing an adequate healthy mixture of foods in the form of an enriched protein concentrate. Water was always available in bottles fitted with glass straws, through which the animals sucked at will.

VERVET MONKEY

EXPERIMENTAL MONKEY

II 2. COLLECTION AND PRESERVATION OF SPECIMENS.

II 2 (i) Blood.

Specimens of blood were collected twice weekly from all animals throughout the early observation period before poisoning with cadmium, but subsequently less frequently as required for the experiment. Volumes of blood taken from animals were usually about 8 ml. at each time, without anticoagulant. Blood was taken from the rabbits by nicking the marginal ear vein and allowing the blood to drip into a beaker. Syringes were used for the other animals, bleeding the monkey from the saphenous vein in the lower back leg and the dog from the radial vein in the lower forearm. In all cases, the blood was left to clot at room temperature and then centrifuged: the pale straw-coloured serum was then analysed.

Sera that had to be kept was desalted by dialysing in visking tubing against distilled water which was thrice changed over a period of eight hours. The desalted sera were then slightly ($\frac{1}{2}$) concentrated by pervaporation in the visking tubing and finally they were lyophilized on a freeze drying apparatus, which could hold up to six vials each containing from 2 - 20 ml.

Whole blood was employed for determination of glucose by adding 0.5 ml. blood directly to specially prepared tubes containing sodium fluoride and sodium oxalate.* For other constituents of the blood the specimens were allowed to clot at room temperature

* 4 mg. and 12 mg. respectively.

for 2 - 3 hours and the serum then separated with care not to cause haemolysis of the red blood cells. The serum was used immediately for the purposes for which it was prepared.

II 2 (ii) Urine.

Urine was collected in the plastic cover and ran down into a polythene container, covered with a fine mesh top to separate small pieces of food and stool. In this way the urine could be collected quantitatively without contact or contamination from stools or foodstuffs. Urine was collected daily, and if not analysed immediately, were stored in the deep-freeze at -15°C.

II 3. GENERAL BIOCHEMICAL INVESTIGATIONS ON THE ANIMALS.

II 3 (i) Range of investigations and methods employed.

A number of chemical constituents were determined in the blood, serum and urine of all animals during a control period and following administration of cadmium. Normal parameters were established over a period of approximately 6 weeks, with a view to following any general biochemical disturbances caused by the intoxication: in particular, to be able to recognise the onset of disturbances in general metabolism associated with acute or chronic cellular damage in the liver, pancreas, Islets of Langerhans, kidney, osteoblasts of bone and so on.

The constituents, and methods used for their measurement are

summarised below. With the exception of glucose, all other constituents of the blood were measured in the serum.

Glucose: determined in whole blood by the method of Hagedorn and Jensen (1923)[65].

Proteins: determined using biuret reagent following the method of Wolfson et al (1948)[65(a)].

Urea: measured colorimetrically with diacetyl monoxime reagent in the Technicon auto-analyzer following the method of Skeggs (1957)[66] with certain modifications by Marsh et al (1957)[67].

Bilirubin: determined as total and conjugated bilirubin by the method of Michaelsson (1961)[68], with a modification by Nosslin (1960)[69].

Serum glutamic oxaloacetic transaminase (S.G.O.T.): measured colorimetrically in a Beckman U.V. spectrophotometer, following the decrease in optical density as NAD forms from NADH during the enzymic reaction. This method was originated by Karmen (1955)[70].

NAD - oxidised nicotinamide adenine dinucleotide.

NADH - reduced nicotinamide adenine dinucleotide.

Cholesterol: measured using a Liebemann-Buchard reaction and determining the colour development in a colorimeter, using the method of Pearson et al (1953)[71].

Alkaline Phosphatase: measured using its action on a substrate phenyldisodium orthophosphate. This methos is adapted for use on the auto-analyzer, by Marsh (1959)[72] from a modification of

the King-Armstrong procedure[73].

Calcium: determined using corinth calcium, a method adapted in laboratories from one by Robnett and Kingsley (1957)[74], in which instead of a colorimetric determination, we titrate with E.D.T.A.

Inorganic Phosphorus: was measured using the method of Folin Ciocalteau (1927)[75], where the molybdenum-blue colour is measured colorimetrically.

Amylase: determined using the procedure described by Pimstone (1964)[76] based on the optical density of the starch iodine complex formed by residual substrate after the action of the enzyme.

Creatinine: determined following the method of Bonsner and Taussky (1945)[77].

Na^+ and K^+: measured in the usual manner on a Baird flame photometer - Spencer (1950)[78] and Brealey and Ross (1951)[79], the serum being diluted 1 in 200 prior to estimation.

Cl^-: determined by a method adapted by our laboratories, where use is made of the fall in electrical conductivity of the solution containing the chloride ions when silver nitrate solution is added - Glasstone (1955)[80].

Other enzymes assayed on poisoned animals.

Isocitric dehydrogenase: measured spectrophotometrically using

the increase in optical absorbance as NADPH forms from NADP during enzymatic oxidation.

NADP - oxidized nicotinamide adenine dinucleotide phosphate.

NADPH - reduced nicotinamide adenine dinucleotide phosphate.

Lactic dehydrogenase
Hydroxybutyric dehydrogenase
Aldolase

} These enzymes were measured spectrophotometrically using the decrease in optical absorbance as NAD forms from NADH.

All procedures for enzymatic estimation were adapted from methods described by Wilkinson (1962)[81] and Bergmeyer (1963)[82].

In the urine, tests were carried out for the following compounds:

Serum Proteins: by heat coagulation, trichloroacetic acid (10% w/v, 1 vol. per 1 vol. urine) and salicyl sulphonic acid (0.2% w/v) in the usual way. Special care was taken to filter the urine specimens until clear, before carrying out the quantitative tests. The following tests were performed daily:

Urinary volumes, pH and specific gravity.

Glucose: qualitative and, if necessary, quantitative Benedicts determinations described by Varley (1963)[83].

Titratable acidity: carried out on freshly collected urine using 0.2 ml. of a 2% aqueous neutral red per 10 ml. urine as an indicator - Henderson and Palmer (1914)[84].

TABLE III.

Concentrations of chemical constituents in the sera of normal monkeys, dogs and rabbits.

MONKEY.

Meq./litre		mg. / 100 ml.		UNITS	
Na^+	146	Urea	10	Alkaline phosphatase	5
K^+	4.8	Glucose	110	Acid phosphatase	-
Cl^-	96	Total Bilirubin	0.5	Thymol turbidity	2
HCO^-_3	-	Conjugated Bilirubin	-	Zinc turbidity	2
Protein G. / 100 ml.		Ca^{++}	8	Amylase	510
Albumin	3.6	HPO_4^{2-}	3		
Globulin	2.2	Cholesterol	120		
		Uric acid	-		

DOG.

Meq./litre		mg. / 100 ml.		UNITS	
Na^+	137	Urea	40	Alkaline phosphatase	8
K^+	4.3	Glucose	80	Acid phosphatase	-
Cl^-	102	Total Bilirubin	0.5	Thymol turbidity	3
HCO^-_3	-	Conjugated Bilirubin	-	Zinc turbidity	2
Protein G. / 100 ml.		Ca^{++}	10	Amylase	600
Albumin	3.6	HPO_4^{2-}	7		
Globulin	1.8	Cholesterol	112		
		Uric acid	-		

RABBIT.

Meq./litre		mg./100 ml.		UNITS	
Na^+	132	Urea	32	Alkaline phosphatase	10
K^+	4.4	Glucose	98	Acid phosphatase	-
Cl^-	9	Total Bilirubin	0.5	Thymol turbidity	2
HCO^-_3		Conjugated Bilirubin	-	Zinc turbidity	3
Protein G./100 ml.		Ca^{++}	7	Amylase	200
Albumin	3.8	HPO_4^{2-}	4		
Globulin	1.7	Cholesterol	51		
		Uric acid	-		

TABLE IV.

ENZYME CONCENTRATIONS IN AN EXPERIMENTAL MONKEY, IN UNITS/ml/min.

ENZYME	NORMAL MONKEY	CADMIUM POISONED MONKEY
Lactic dehydrogenase	60 - 420	904
Isocitric dehydrogenase	50 - 240	1,350
Hydroxybutyric dehydrogenase	40 - 300	653
Aldolase.	1.7 - 5.9	7.5
S.G.O.T.*	25 - 40	60

Na^+ and K^+: determined on a Baird flame photometer with lithium internal standard: urinary specimens were diluted 1/500 prior to the estimation.

Cl^-: determined as in serum.

Catalase: determined in a Warburg apparatus - Dounce (1949)[85].

II 3 (ii) Observed values for general biochemical constituents in normal experimental animals.

See Table III.

II 4. METHODS EMPLOYED IN POISONING ANIMALS WITH CADMIUM.

Intoxication: After nearly three months, we estimated enough data had been collected to be reasonably sure as to the biochemical baseline of each animal. It was decided to try to induce a state of chronic cadmium intoxication in one animal of each species. In other words, cadmium would be administered until the animal had gross and persistent proteinuria. The second animal of each species would be given just over half the amount of cadmium, in order to have it available in the event of its partner dying.

We reasoned that the most convenient and efficient method of administering cadmium would be to give intravenous injections of cadmium chloride ($CdCl_2\ 2\frac{1}{2}H_2O$). Also the amount to be given would be 2 mg. of cadmium per kilogram body-weight twice a week. This amount was found in previous experiments by Kench et al[14] to

be sufficient, without causing acute paralytic episodes in the animal and subsequent death within 24 hours. The cadmium chloride was thus made up in a solution containing 2 mg. Cd^{++} per ml. NaCl was added to bring the entire solution to a concentration of 0.9% w/v Cl^{-}.

It had been previously shown that direct intravenous injection of this solution caused haemolysis, thrombosis and localised tissue necrosis. A technique had been derived to overcome this. Blood was first taken from the animal - 1 ml. per ml. of cadmium solution, i.e. per 2 mg. Cd^{++} - and the cadmium solutions stirred rapidly but gently into the blood. This mixture was then re-injected. In this way, the cadmium chloride solution is buffered and the cadmium ions bound to the proteins. As a result no localtrauma is caused by the injected solution.

In the rabbits, blood was obtained by nicking the marginal ear vein with a razor blade and letting the required volume drip into a beaker containing 0.5 ml. heparin. The cadmium solution was then added and was thoroughly mixed. This mixture was then taken up in an all-glass syringe and injected into the opposite ear, also into the marginal ear vein, using a $1\frac{1}{2}$" gauge 24 needle.

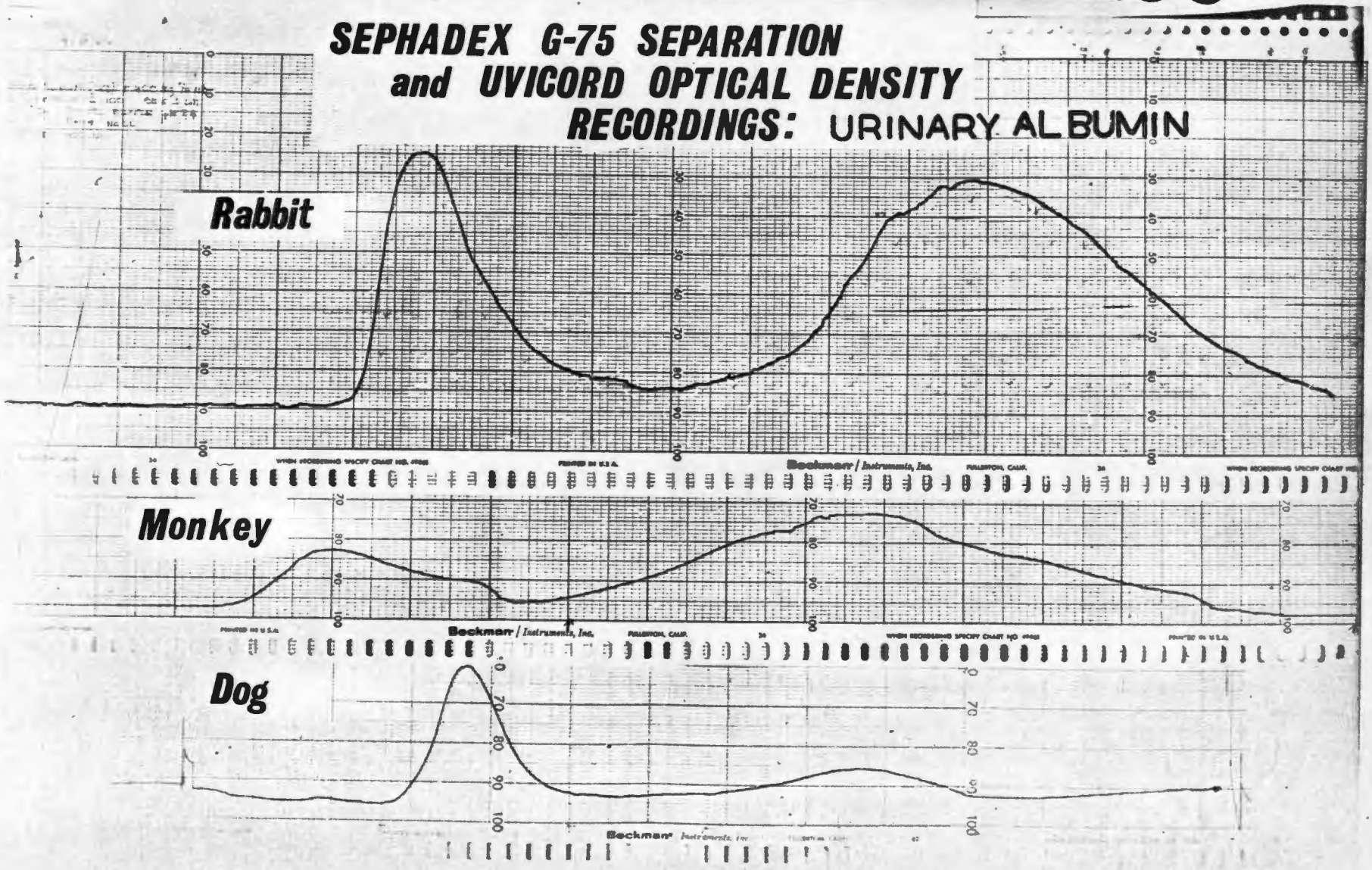
With the monkeys and dogs a different procedure had to be employed as blood could only be taken with a syringe and needle. Also so as to avoid entering a vein too often with the subsequent

scarring, only one needle insertion was made. Using an all-glass 10 ml. luer-lock syringe with an off-centre attachment and 1½" x 21 gauge needles, blood was taken from the appropriate vein. The needle, which had been previously heparinized simply by sucking up a drop of heparin, was left in the vein and the blood-flow halted by placing a luer-lock tap, in the closed position, at the base of the needle. The blood was then displaced from the syringe into a beaker containing 0.5 ml. heparin and well-mixed. The cadmium solution was finally added slowly to the blood-heparin mixture with continuous shaking or stirring to obtain the maximum mixing and buffering action from the blood. The mixture was then sucked up into the same syringe, which was then attached to the tap, the cock opened, and the injection given over a period of about two minutes. The needle was finally removed and stasis was achieved by placing a piece of cottonwool plus "hibertone" ointment on the wound, adhesions being obtained with a length of elastoplast. In this manner each vein was only used once weekly, and complete healing took place during this period. Also cadmium was introduced without local tissue damage, in a form which could be accurately controlled and evenly and rapidly dispersed throughout the body.

The weight of rabbit 1 was 4.0 kilograms and proceeding with twice weekly injections of 6 mg. Cd^{++} per injection (i.e. 12 mg.Cd^{++}/week), the rabbit became proteinuric after having received a dosage of 84 mg. Cd^{++}, i.e. after seven weeks.

FIG 5

SEPHADEX G-75 SEPARATION and UVICORD OPTICAL DENSITY RECORDINGS: URINARY ALBUMIN



The urine was collected daily and, when not analysed directly, it was stored.

In the monkey urinary proteins increased steadily during the course of poisoning, until an average of nearly 200 mg. was being excreted daily. During the intoxication period between 15 and 18 mg. cadmium was injected weekly. When severe poisoning was reached i.e. gross proteinuria, dyspnoea and agitation of the animal, the dose was reduced to 6 - 8 mg. Cadmium weekly.

II 5. QUANTITATION OF PROTEINS IN SERUM AND URINE.

II 5 (i) Standardisation of the Method.

The protein concentrations of both serum and urine specimens were measured daily during the intoxicating period. In order to standardise the biuret method, we used a known pure bovine albumin specimen, and the Kjeldahl method for total nitrogen in the following matter.

A solution of pure bovine albumin was made up in an aqueous dilution to contain approximately 1 mg. N/ml. ($\pm$ 7 mg. protein/ml.). This solution was standardised by measuring the nitrogen content on 1 ml. aliquots by the micro-Kjeldahl technique. On a series of six samples, the nitrogen concentration was found to be 0.746 mg. N/ml. Now, taking the percentage of nitrogen in bovine albumin to be exactly 16% of the total molecular weight, each sample contains

$$0.746 \times 6.25 \text{ mg. protein}$$
$$= 4.66 \text{ mg. albumin/ml.}$$

The much simpler biuret method was applied to these samples as follows: from 1 ml. of the non-estimated protein solution, the proteins were precipitated with 10% w/v T.C.A., centrifuged and the supernatant discarded, the protein dissolved in 1 ml. 40% w/v NaOH, after adding 6 ml. distilled water to the precipitate and, finally, 1 ml. 5% aqueous copper sulphate solution added - Fine (1935)[86]. After the final volume had been brought up to 10 ml., the mixture was centrifuged and the optical density of the supernatant measured at 540 mμ in a Klett-Summerson photometer. The optical density reading, after subtracting the blank, was 67 scale divisions, which, when referred to the actual protein concentration gives 14.4 divisions per mg. protein.

II 5 (ii) Serum.

Serum proteins were determined by the biuret method as followed in the Department of Chemical Pathology, i.e. total proteins were measured by addition of the biuret ($CuSO_4$ - NaOH) reagent, and the colour compared with a Standard and a blank in the Klett-Summerson photometer -Weischelbaum (1946)[86(a)]. To determine the albumin fraction, globulins were first precipitated by adding aqueous solution of sodium sulphate (23% w/v Na_2SO_4) and 2 ml. of diethyl ether added to the solution. On centrifugation, the precipitated globulins form a compact pad at the interface between the albumin solution and the lighter ether phase. The concentration of albumin - containing a small

APPARATUS FOR LYOPHILIZATION

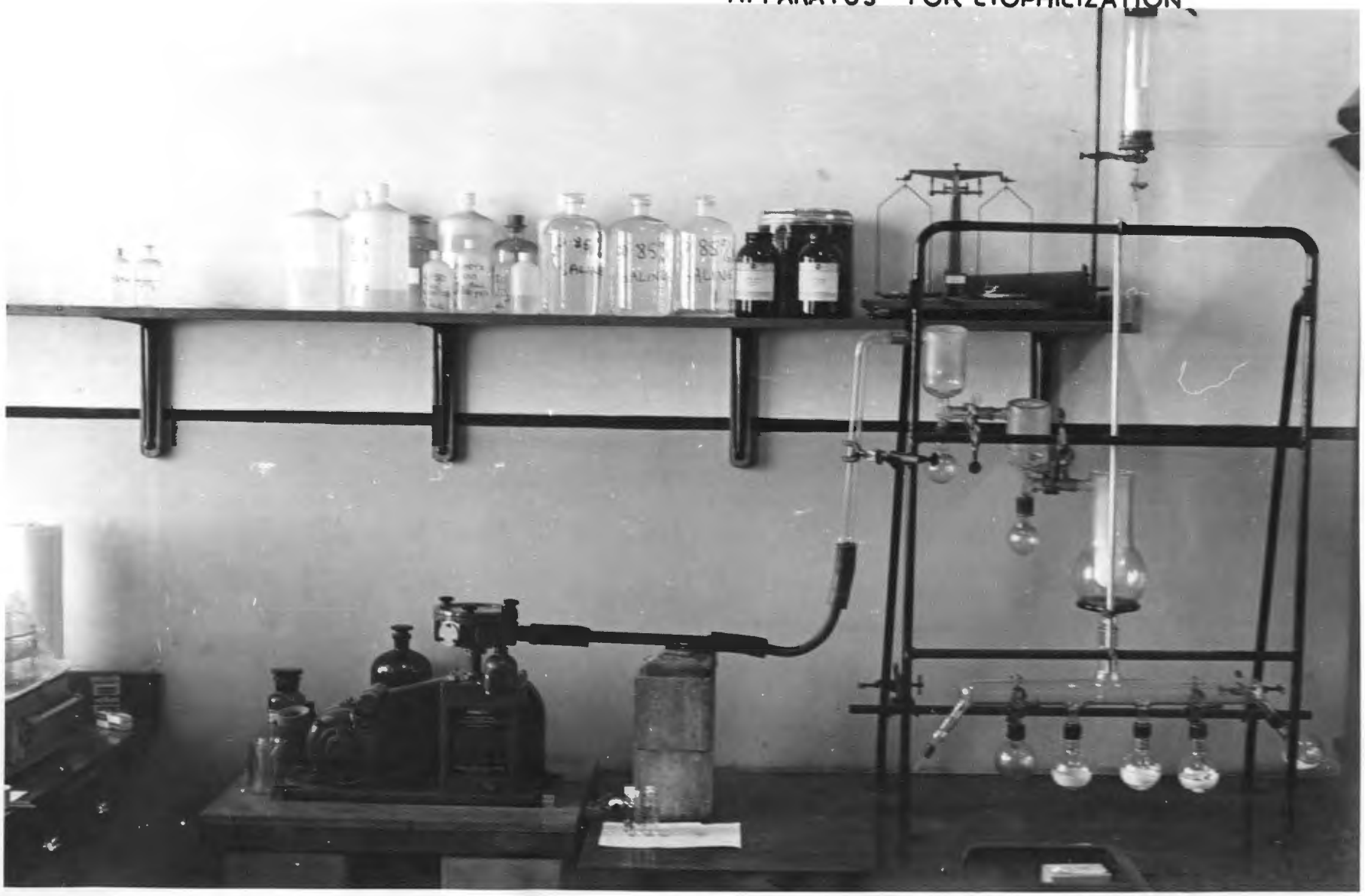
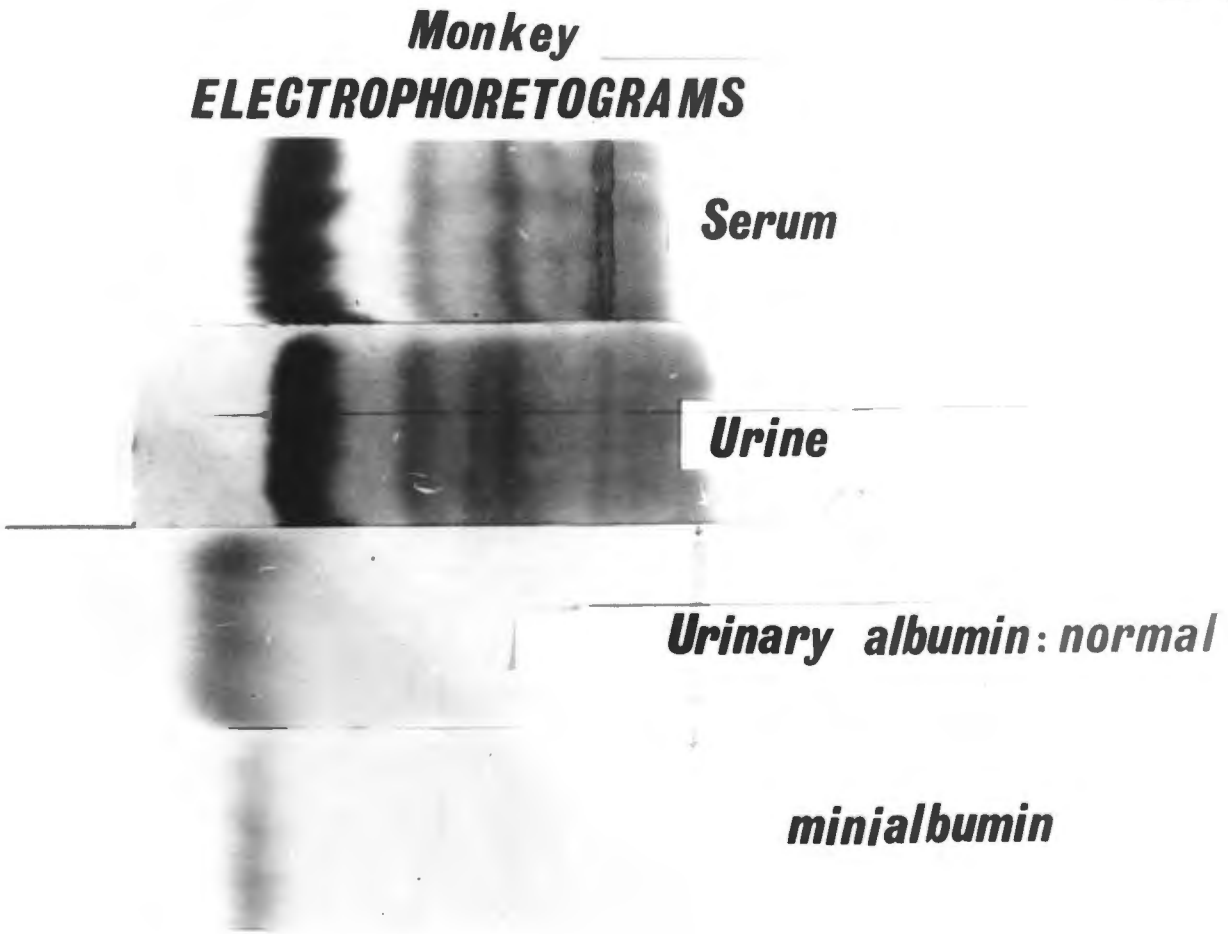


FIG 3


Monkey
ELECTROPHORETOGRAMS
Serum
Urine
Urinary albumin: normal
minialbumin

proportion of globulins - can then be determined by the usual biuret method[56].

The serum protein concentrations for the three species of animals investigated were:

Dogs: Albumin 3.0 - 4.0 g./100 ml.
Globulins 1.8 - 2.5 g./100 ml.

Monkeys: Albumin 3.5 - 4.0 g./100 ml.
Globulins 2.0 - 3.0 g./100 ml.

Rabbits: Albumin 3.4 - 4.4 g./100 ml.
Globulins 1.5 - 2.8 g./100 ml.

These levels remained unaltered during high-dosage administration of cadmium chloride as also during chronic intoxication states.

II 5. (iii) Urine.

Total proteins in the urine were determined and a daily tally kept.

II 6. SPECIAL INVESTIGATIONS OF URINARY PROTEIN.

II 6 (i) Separation of Albumins.

In the proteinuric state of our cadmium-poisoned animals, we had reproduced a state of intoxication which had previously been achieved by a number of investigators; however, Kench and his colleagues[14] had reported the presence of a low-molecular

weight albumin in the urine, and it was this very interesting fact that we intended further to investigate.

The first concern was to prepare the protein in a homogenous form by all criteria, electrophoretic mobility, sedimentation constant, amino acid composition and antigenic behaviour.

At first, specimens were separated using a column of carboxy-methylated cellulose (C.M.C.)[63], a cationic ion-exchange resin, with which, when using a system of buffer with a salt as well as a pH gradient, we could elute a very pure albumin fraction. This method, however, had two disadvantages:

1. during collection of the albumin elution peak in order to get absolute homogeneity, we could examine only the central portion of the peak. This could exclude a portion of the albumin which may be of interest to us.

2. the method involved a lengthy procedure of preparing resins, setting up glass columns and collecting eluted fractions off the column into fraction collections. The recovery was also low, i.e. 50 - 70%.

This albumin specimen was electrophoretically homogenous in that when electrophoresed on paper in a Durrum-type tank, the protein migrated in a single compact band. Staining with amino-swartz showed only a single band. It also retained its full biological activity.

Nevertheless, a simpler method would be preferred; and we

finally found that an extremely pure albumin could be obtained by the technique of Vallance-Owen [61], adapted by Debro et al (1957)[87].

Trichloracetic acid (T.C.A.)Procedure. The urinary proteins were first precipitated en masse with a 10% w/v aqueous solution of T.C.A. which was added in a volume equal to that of the previously concentrated urine. The precipitated proteins were then centrifuged at 2000 r.p.m. for 10 minutes in an MSE major refrigerated centrifuge with a swing-out head at 0°C and the supernatant solution discarded. The precipitate was then washed with 5% w/v T.C.A. To the washed precipitate, 1% T.C.A. in 96% aqueous ethanol was added. Albumin is soluble in acid alcohol mixtures - Delaville et al (1954)[88], Levine (1954)[89] - and thus the albumin was extracted. After extraction, the supernatant extract (S.N.F.) was dialysed in visking tubing * for 24 hours in a refrigerator at 0°C against several changes of distilled water to remove excess T.C.A. along with the alcohol. With this method, the protein at first precipitates during dialysis, but later redissolves again. The precipitation stage was eliminated by washing the total protein previpitate with a 1% T.C.A. solution in 96% aqueous acetone instead of in ethanol.

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* An important procedure in our experiments was to boil all dialysis tubing for at least ½ hour in distilled water. This procedure decreases the pore size of the tubing, preventing the loss of any low-molecular weight proteins (albumins) into the water.

In this manner, a pure aqueous solution of albumin was obtained. Purity at this stage was judged by the single symmetrical peak given on paper electrophoresis in a Durrum tank. Michael (1962)[90] describes the preparation and also comments on the physico-chemical and antigenic properties of the albumin.

The excellent recoveries and the relative simplicity and reproducibility of the trichloracetic acid method were important advantages. This method could be used satisfactorily on both urine and serum specimens.

Any alterations in the albumin molecules during preparation, as to their physico-chemical or antigenic properties could not be detected by previous workers or by us.

II 6 (ii) Zone electrophoretic preparation of albumins.

A very useful technique for checking out albumin preparation was to prepare albumin electrophoretically using the method of zone electrophoresis. We followed the procedure of Campbell and Stone (1957)[60] which is an adaptation of the original method developed by Porath and Flodin (1954)[59]. In this method serum or any other mixture of proteins, is applied to the interior of a column of inert cellulose. A potential difference is then applied to the column. The proteins separate due to their differing electric charges, with the albumin moving furthest down the column towards the anode. The current is then dis-

continued, and the proteins displaced with a buffer. The albumin conveniently comes off first and is collected.

The cellulose to be used in the column must be acetylated, and as the product was virtually unobtainable, we prepared it in the following manner (Flodin, P. and Kupke, D.W., 1956).[91]

150 - 200 g. of the purest absorbant cotton-wool were boiled under reflux for 20 hours in 4 l. of absolute ethanol, to which had been added dry gaseous hydrogen chloride to make the final concentration of HCl 1 M. The alcohol-HCl mixture may alternatively be produced by adding dropwise, with constant stirring, 280 ml. acetyl chloride to 3,700 ml. ethanol. We actually used the latter technique. The mixture must be constantly stirred throughout the procedure. After approximately 16 hours the cotton-wool disintegrates completely and forms a mass of fine particles; these are filtered off after 20 hours and washed with ethanol. Finally, the acetylated cellulose is dried in an oven at 80°C to form a fine pure white powder.

Details of the column and its running procedure were adapted from details described by Porath, J. (1954) [92] and Tiselius, A. and Flodin, P. (1953).[93] We used silver-silver chloride electrodes which were prepared by connecting coils of silver wire onto the anode pole and a platinum electrode to the cathode pole of a power-pack. These were placed in a 2 M solution of potassium

chloride, with 0.1 M H Cl added. A current of 40 - 50 mA was applied for 14 hours.

Buffers. The buffer, which was used for suspending the column as well as in the electrode reservoirs was prepared as follows:

Stock: 63 g. Boric acid (H_3BO_3)
20 g. Sodium hydroxide (NaOH)
Made up to 5 l. with distilled water.

350 ml. of this stock is added to 150 ml. of a 0.1 N HCl solution to make 500 ml. To this solution is added 500 ml. of 1 N NaCl solution and the whole is made up to 5 l. with distilled water.

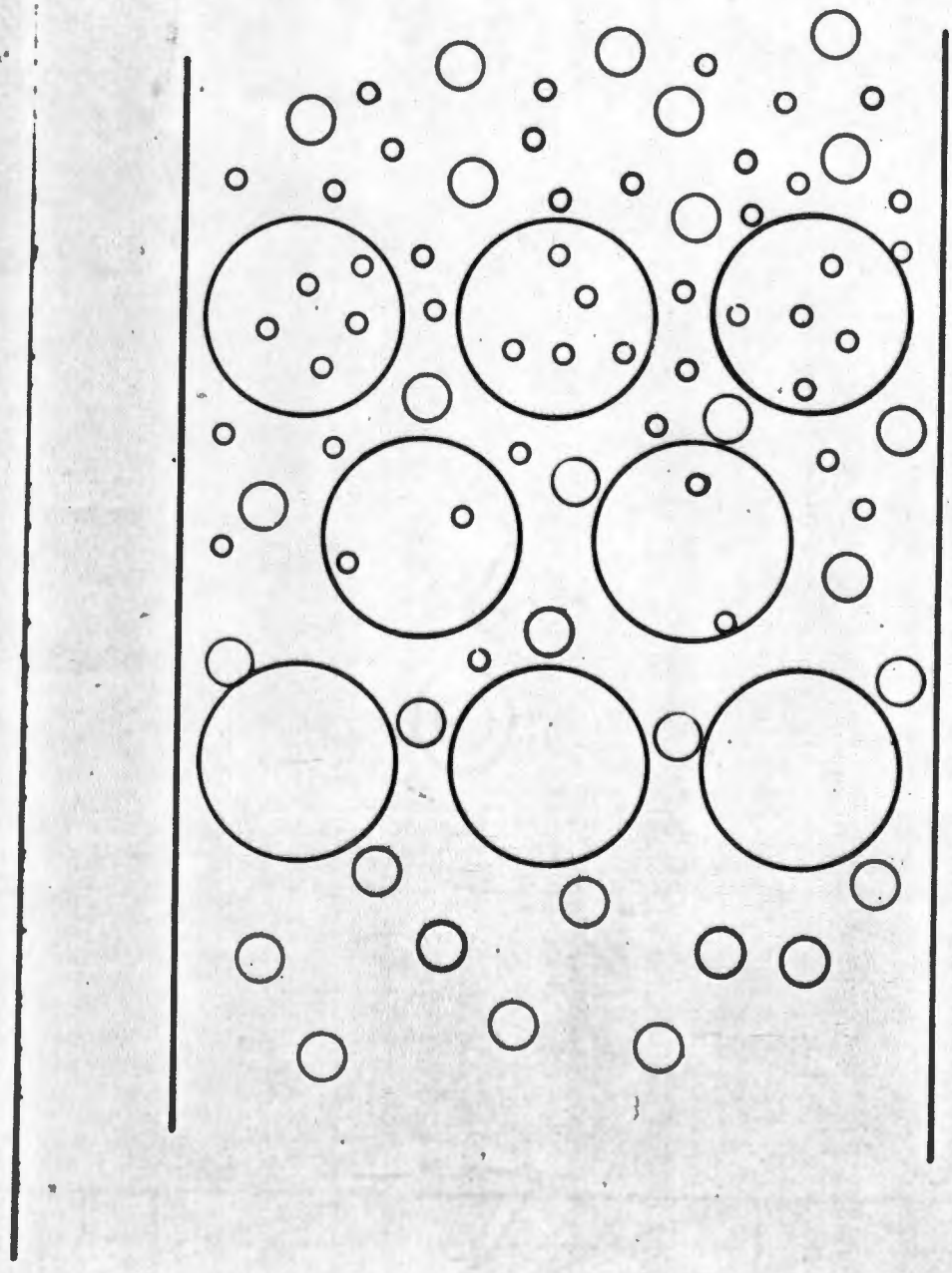
Procedure. The sample is applied, and allowed to sink into the surface of the resin before being washed in with buffer. A current of 160 v is applied for up to 40 hours before the current is disconnected and the proteins are eluted.

The albumin prepared in this way was identical with that prepared by the T.C.A. precipitation method.

II 6 (iii) Separation of albumins of different molecular weight.

The albumin which we had extracted from the urinary proteins of the intoxicated animal, was now investigated, in order to isolate and identify the low-molecular weight fraction described

FIG. 6 MECHANISM OF GEL FILTRATION



in the publications of Smith, J.C., Wells, A.R. and Kench, J.E. (1961) [17] and of Kench, et al (1962).[14] This albumin supposedly differed from normal serum albumin only in its molecular weight.

This molecular sieving technique had become possible since there was now commercially available cross-linked dextran gel, which had the property of separating molecules on a molecular weight basis, and acted as a so-called molecular sieve.

The technique was first discovered in Uppsala, Sweden, and Porath, J. and Flodin, P. (1959) [94] were amongst the first workers to experiment with the substance, which was called Sephadex. These workers, using a solution of serum albumin containing 40% ammonia sulphate, achieved a complete separation between protein and the salt. The gel is also automatically regenerated after each complete operation.

The principle of gel-filtration is that, during the process of cross-linkage, the dextran forms a porous, sponge-like ramification. The gel can now be obtained in fine, medium or coarse granules or beads, each having the aforementioned, porous quality. The Sephadex can be suspended in water, or as normally done, in a weak buffer solution. This slurry of suspended resin is now poured into a column of glass and the resin allowed to settle.

A solution of molecules of varying molecular weights can now

be passed through the column of gel, using a weak buffer as carrying medium. Small molecules are able to enter the actual pores in the beads of the gel and in this manner pass in and out of the pores, retarding their flow. Molecules of a bigger size are not able to enter these pores and pass straight through the column. Separation is thus achieved (Mould, D.L. and Synge, R.L.M., 1954).[95]

If, during manufacture of the gel beads, the cross-linkage of the dextran is varied then the pore size also varies. In this way a series of pore-sizes can be obtained covering a wide range of molecular-weight separatory powers.

Our T.C.A. prepared albumin in aqueous solution was now applied to a column of Sephadex G 75 which would separate particles with molecular weight of under 30,000 from those over 60,000. The solution was applied carefully to the top of the column and allowed to run into the gel. On complete entry, a phosphate-sodium chloride buffer ($\frac{M}{15}$ phosphate buffer at pH 7.2 plus sodium chloride to obtain a concentration of 0.2 M NaCl) was run through the column, acting as an eluting medium. The effluent from the column was monitored at 260 m/u wave-length by running it through a Uvicord, ultraviolet absorbtiometer. In this way any proteins can be detected. The Uvicord was attached to a recording instrument so as to perceive the flow on a graph.

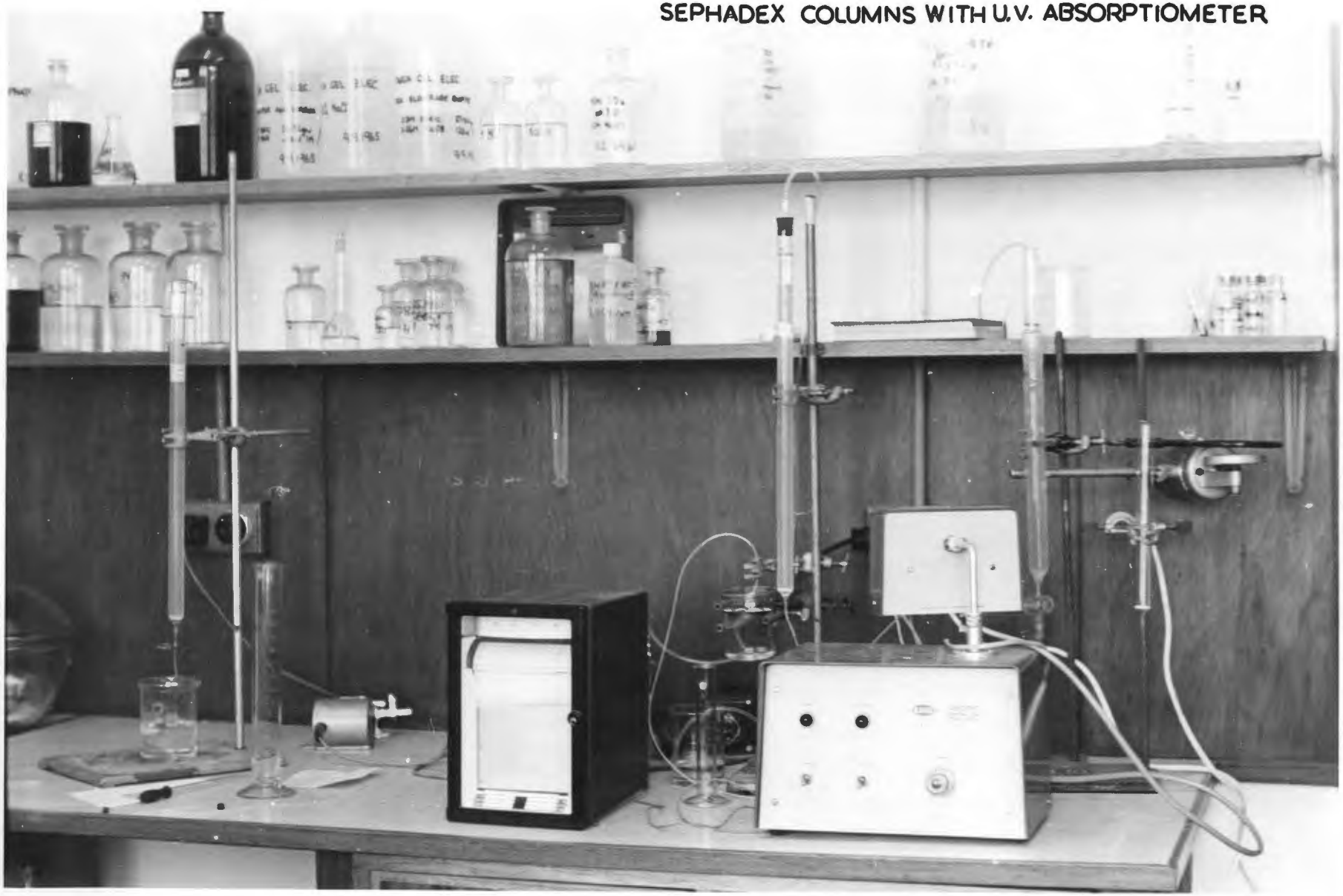
Our solution of urinary albumins produced a graph with two distinct peaks, indicating that there were definitely two proteins of varying molecular weights in our solution. The solution, presumedly of albumins, could be prepared by any of our previously described methods, e.g. C.M. cellulose, zone-electrophoresis or, as normally used by us, T.C.A. method. The same twin peaks were in each case given on Sephadex chromatography.

If, prior to running on Sephadex, the urinary albumin solution was enriched with a solution of normal monkey serum albumin, then the graphic recording of the Uvicord-monitored column effluent had a similar pattern to the urinary albumins alone; but the protein peak first to emerge, i.e. the protein with the greater molecular weight, was higher, with a greater area under the curve. This pointed to an albumin in the urine with a molecular weight similar to that of normal serum albumin i.e. 66,000, as well as an albumin with a lower molecular weight.

II 6 (iv) Molecular weights of albumins by gel filtration.

Many workers have, since the discovery of Sephadex, aimed at predicting molecular weights of proteins for their speed of elution, which is actually the volume of buffer needed to elute a given protein through the Sephadex column. Flodin, P. (1962) [96] and Flodin, P. and Porath, J. (1961) [97] envisaged this application for their dextran-gel, but Whitaker, J.R. (1963) [98] gives us a

SEPHADEX COLUMNS WITH U.V. ABSORPTIOMETER



very good calculation for determining molecular weight on Sephadex. He finds an excellent linear correlation between the logarithm of the molecular weight of a protein and the ratio of its elution volume V_1 to the void volume V_o

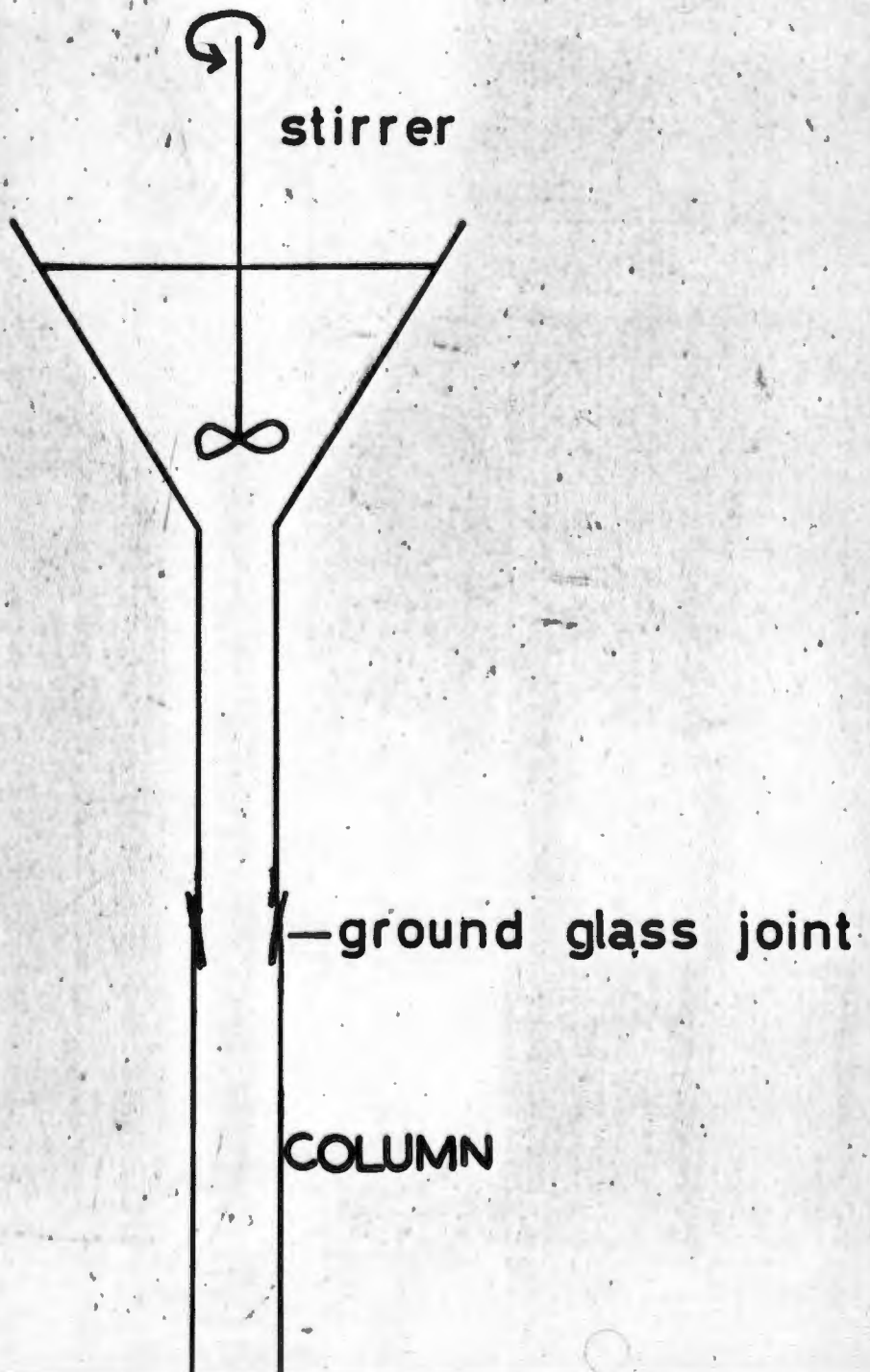
$$\text{i.e. log M.W.} \propto \frac{V_o}{V_1}$$

This correlation seemed to be independent of protein concentration, column size and ion-exchange adsorption. He found anomalies only if the proteins form a complex with the gel, contain an appreciable amount of carbohydrate or dissociates into subunits under the conditions present. Leach and O'Shea (1965) [99] also find a linear relationship between molecular weight of a molecule and elution volume off Sephadex columns.

Procedure. The Sephadex gel was suspended in enough buffer, so that when it was stirred, trapped air bubbles could escape rapidly to the surface. It was then allowed to stand in the buffer for two days. Swelling of the gel particles took place during this period. If this step is neglected, incomplete gel swelling gives a column which has a very slow flow rate. A glass column, with either a sintered glass disc or a wad of glass wool at the bottom, was then filled with a suspension of gel in the buffer. Until approximately 1½ inches of gel had settled on the disc, the stopcock at the bottom of the column is kept closed. The tap is then opened and a quickfit glass tube of similar length and dimensions attached to the top of the column. The whole apparatus

FIG 8

METHOD OF PACKING COLUMNS OF SEPHADEX



is now filled with the resin slurry. The tube (or other holder) acts as a reservoir so the column is poured as a complete unit. After the column is poured, a filter-paper disc is placed on the top surface of the gel to prevent disturbance when the samples are added. The column is then washed overnight with buffer to allow the resin to equilibrate.

The protein was added to the top of the gel-bed in 1 ml. of the buffer. Under flow the protein was run into the gel, and then washed in with 1 - 2 ml. of buffer. Finally, buffer was added above the gel and allowed to run continuously, using a siphon system from a reservoir.

The elution volumes were measured from the initial application of the protein sample to the peak of the eluted protein.

Void volume is determined by running a solute, that is completely excluded from entering the pores of the gel, through the gel. Neither haemoglobin nor serum albumin are absolutely excluded on G 75, as compared with γ-globulins, as both had greater elution volumes, that of haemoglobin being especially high as a result of its dissociation into subunits of approximately 34,000 molecular weight. The elution volume of the γ-globulin peak was taken as the void volume (V_o).

In all experiments the resin used was of the new bead-shape which greatly speeds up flow through the column and allows for finer mesh-sizes to be used.

Using log M.W. $\propto \frac{Vo}{V_1}$, where V_1 is the elution volume of a totally excluded molecule and V_o is the void volume of the column, the molecular weight of the faster moving component was calculated to be ± 67,000 and the molecular weight of the slower moving component to be ± 19,000.

II 6 (v) Acid hydrolysis of urinary albumins.

Our urinary albumins, having been separated, could now be subjected to a variety of analytical and identification techniques. Our first plan was to attack our project directly and try to determine the amino acid composition of the albumins and compare this to the amino acid composition of normal serum albumin.

Our first problem, therefore, was to obtain a reproducible hydrolysate of the proteins. We weighed up the pros and cons of various methods and decided that the use of hydrochloric acid would be the most suitable for our purposes. In the two other modes of hydrolysis, the enzymatic digestion tended to be incomplete, leaving large peptide chains, and the use of alkali tends to destroy or racemise many of the amino acids.

Apparatus. Our first method of hydrolysis was with 6 N three-times glass-distilled constant boiling HCl, in an open reflux in 50 ml. round-bottom long-necked pyrex glass flasks. The flasks were filtered with a water-cooled reflux condenser and all outside air was passed through a tap containing 1 n HCl in order to absorb atmospheric ammonia - Macpherson (1946)[100].

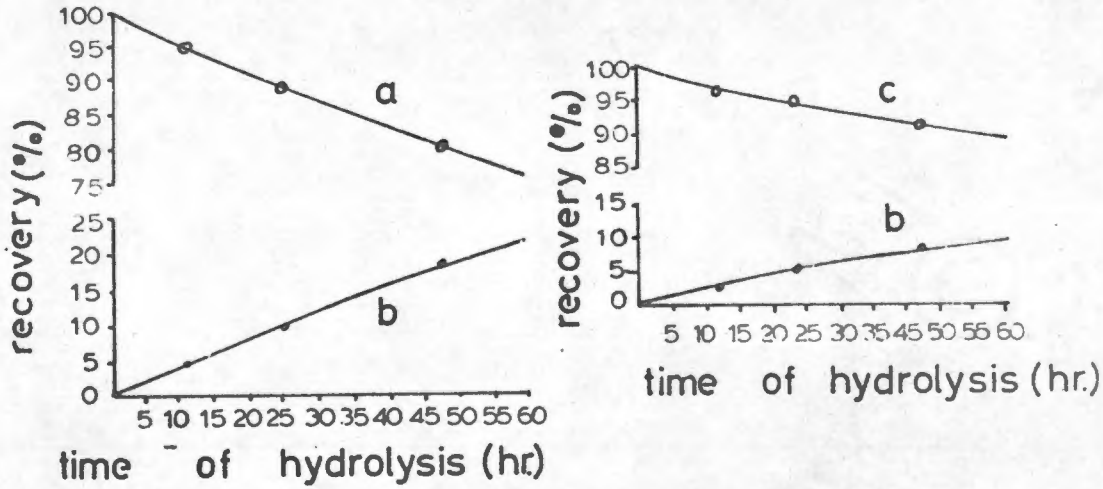
Method. An aliquot of protein solution containing about 5 mg. albumin was evaporated to dryness in a vacuum desicator containing pellets of potassium hydroxide. Five ml. of the 6 N HCl was added plus a few boiling chips. The mixture was then heated for 22 hours under reflux, at a temperature of 110°C. We used a sand-bath for heating, which kept a very constant temperature.

A number of digestions were done using varying time lengths - Rees (1946)[101], Lugg (1946)[102], and 22 hours was found to be most suitable a period. Even at this time, the hydroxy-amino acids, threonine and serine tended to decline in concentration if compared with a 16 hour hydrolysate - (see Fig. 7). But at 16 hours there were also "hard cores" of undigested peptides left in the mixture. After completion of the hydrolysing procedure the mixture was allowed to cool and then placed in a desiccator and evaporated to dryness over potassium hydroxide pellets. The dry residue was then redissolved in 0.5 ml. warm distilled water and brought to a pH of 6.5 by adding sodium phosphate buffer, 0.2 M pH 6.5. The solution was now left to stand open to the air for 4 hours to allow the cysteine to be oxidized to cystine. Finally the pH was adjusted to 2.4 by adding 0.06 ml. 1 N HCl freshly prepared and 2.0 ml. 0.2 M sodium citrate buffer pH 2.2. The mixture was now ready for amino acid analysis.

Sealed hydrolysis. With the above method we tended to obtain slightly high ammonia concentrations on amino acid analysis, on certain occasions. Thus we tried hydrolysing our samples in

FIG. 7

DESTRUCTION OF SERINE AND THREONINE DURING ACID HYDROLYSIS.



a. recoveries of serine at different times of hydrolysis.

c " " threonine " " " " "

b ammonia produced during hydrolysis.

(data of Rees [212])

sealed tubes, following the method of Hirs, Moore and Stein (1954)[103].

Method.

1. A concentrated protein solution containing 2 - 3 mg. per ml. was placed in a pyrex test tube (6 mm x 7 mm) which had been previously drawn out to form a narrow neck.

2. To the solution was added 0.5 ml. constant boiling glass distilled HCl (6 N HCl).

3. The mixture was then frozen in a solid CO_2-acetone mixture and sealed under vacuum. The freezing was to prevent the liquid boiling and spilling when the tube was evacuated.

4. The sealed tube was placed in a sand-bath at 110 $\pm$ 1 °C.

5. Hydrolysis was carried out for 22 hours and for 70 hours.

6. After hydrolysis the tubes were opened and evaporated to dryness in a vacuum desiccator containing potassium hydroxide pellets.

7. The dry residue was dissolved in 0.5 ml. warm distilled water.

8. The pH was raised to 6.5 by adding 0.2 M sodium phosphate buffer, pH 6.5.

9. The solution was now allowed to stand open at room temperature to allow any cysteine to be oxidized to cystine.

10. Finally the pH was brought to 2.4 by addition of 0.06 ml.

1 N HCl which is freshly prepared, plus 2.0 ml. 0.2 N sodium citrate buffer, pH 2.2. The mixture was now ready for amino acid analysis.

Note: Correct values can be obtained by extrapolation of the obtained results to zero time.

Standardisation of the hydrolysis had to be undertaken with each sample. Therefore, a portion of every sample that we hydrolysed for amino acid analysis, was taken for determination of total N-content by the micro-Kjeldahl method - Chibnall et all (1943)[104].

Reagents. Potassium sulphate - B.D.H. (A.R.)

36 N Sulphuric acid - A.R. grade.

Mercuric sulphate solution - 10 g. red HgO dissolved and diluted to 100 ml. with 4 N sulphaturic acid *

Sodium hydroxide-sodium thiosulphate solution 200 g. sodium hydroxide plus 12.5 sodium thiosulphate, were dissolved in distilled water and made up to 500 ml. with distilled water.

Indicator. Solution A 0.2% w/v methyl red in 95% v/v ethanol

Solution B 0.2% methylene blue in 95% ethanol.

Before use, these indicators are mixed in the ratio of

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* The use of mercury as a catalyst: when alkali is added to an ammonia solution containing mercury, as a preliminary to distillation, a considerable fraction of the ammonia is bound by the mercury oxide precipitated. To prevent low results from this cause, sodium sulphide or sodium thiosulphate may be added to precipitate the mercury.

2 : 1 solution A / solution B.

Solution A was stable but the methylene blue solution tended to deteriorate and had to be prepared freshly every month.

Boric acid. 10 g. anhydrous boric acid + 5 ml. mixed indicator. The resultant mixture was then adjusted to 500 ml. with distilled water.

$KH(IO_3)_2$ - A.R. grade 0.01 N aqueous solution.

A standard was also prepared, using ammonium chloride containing 1 mg. per ml. solution.

Method. The sample containing 0.3 - 1.0 mg. of nitrogen was transferred to a 30 ml. micro-Kjeldahl flask - McKenzie & Wallace [105] Fleck and Munro (1965)[106]. 1.5 ml. 36 N sulphuric acid, 1.5 g. potassium sulphate + 0.5 ml. mercuric sulphate reagent were added. The water and the whole digested until clear in a sand-bath at $\pm$ 110°C was boiled away. The sample cleared about five minutes after fuming occurred. Digestion was then continued for a further 15 minutes. The digest was diluted with a few ml. of ammonia-free distilled water and quantitatively transferred to the Kjeldahl distillation flask with several washings of distilled water, taking care that the total volume did not exceed 25 ml. A light application of grease to the rim of the digestion flask helped in quantitative transfer. Now 10 ml. of the $NaOH-Na_2S_2O_3$ solution was added to the distillation chamber and the teflon

stopcork closed.

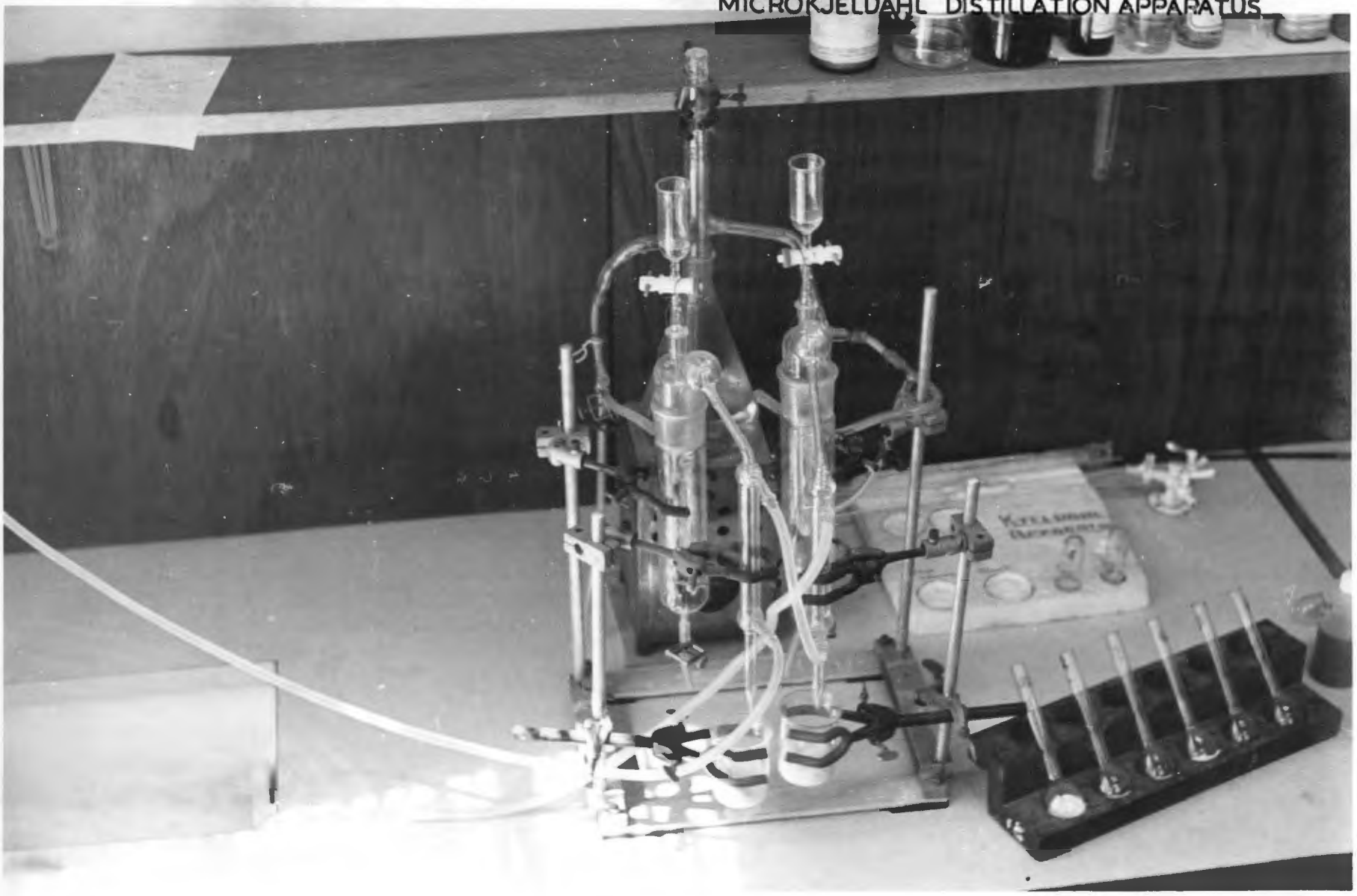
Steam distillation was commenced with the tip of the condensor immersed in 5 ml. boric acid solution, contained in a 5 ml. flask, and graduated to indicate levels of 20 ml. and 25 ml. Distillation was continued until the 20 ml. mark was reached, at which time the flask was lowered so that the tip of condensor is out of the distillate. A further 5 ml. was then distilled over and the tip of the condensor was then rinsed with distilled water, adding the washings to the distillate (see plate 8).

The distillate was finally titrated with 0.01 N $KH(IO_3)_2$ until the blue endpoint of the indicator was reached. The standard and suitable blanks should also be completed in the same manner. Recoveries of the standard NH_4Cl solutions were consistently 100% $\pm$ 0.25.

For the calculation, 1 ml. 0.01 $KH(IO_3)_2$ $\equiv$ 0.1401 mg. N. For albumin, in order to obtain the protein equivalent, we multiplied the total N value by a factor of 6.25 based on a nitrogen content in the molecule of 16%.

At this stage we now had accomplished the isolation of a low molecular weight protein in the urine. This protein had the properties of an albumin in that it could be extracted from a protein precipitate by an acid-alcohol solution and also had a similar electrophoretic mobility on paper as well as on zone electrophoresis. We had as well hydrolysed the protein in a

MICROKJELDAHL DISTILLATION APPARATUS



satisfactory manner, as the same time as obtaining the exact protein content of our hydrolysate via the micro-Kjeldahl total nitrogen estimation. The next step was an analysis of the molecule itself.

II 6 (vi) Determination of amino acid composition.

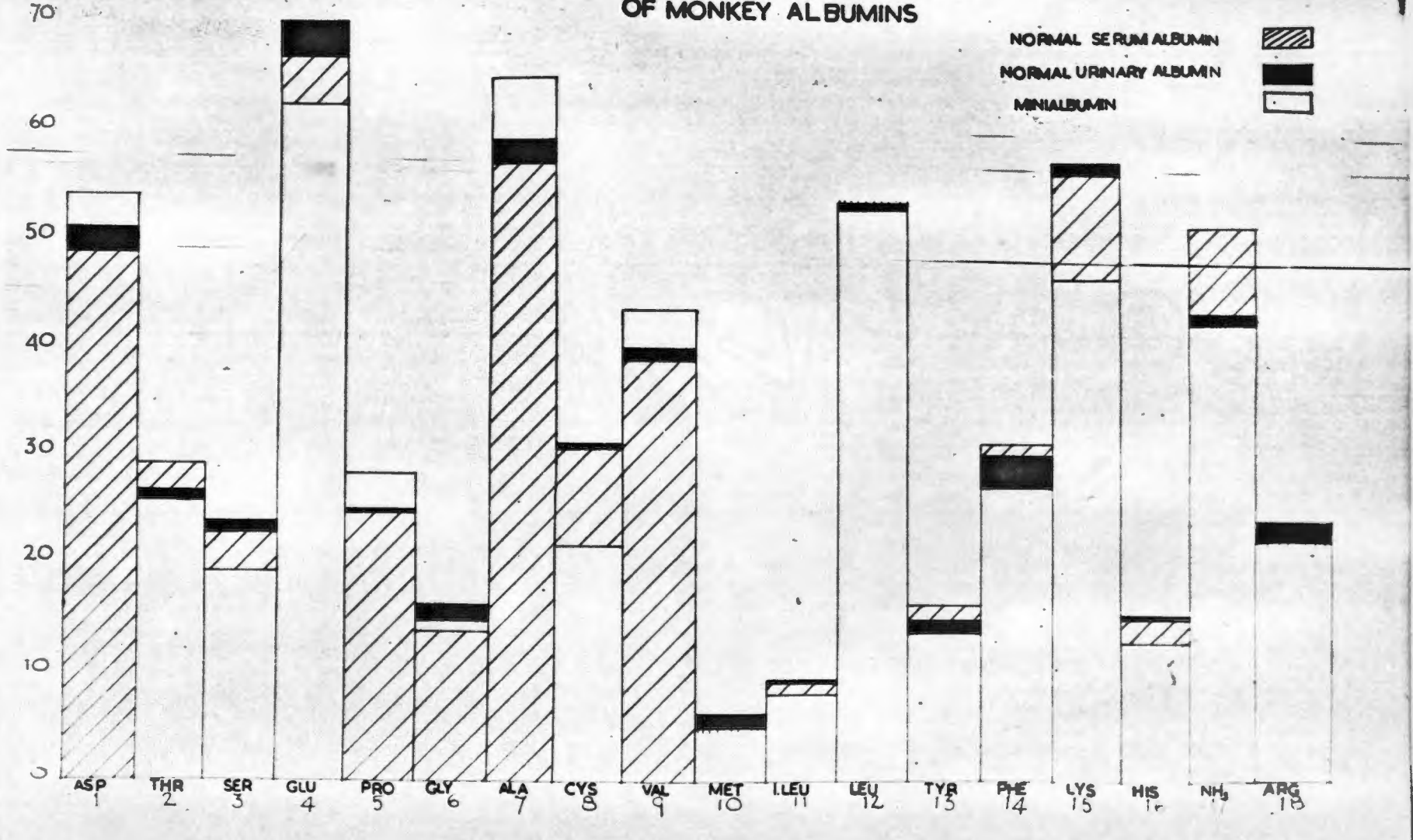
Protein hydrolysates have for many years been analysed to a greater or lesser extent by means of paper chromatography, a technique which can be widely used in the separation and determination of a variety of compounds of biological interest, including fatty acids and carbohydrates as well as amino acids, Richmond & Hartley (1959)[107]. In paper chromatography a strip of filter paper is suspended in a sealed glass jar, with one end of paper dipping in an organic solvent:water mixture. This solvent may be placed in a trough from which the paper strip hangs (descending paper chromatography) or the strip may be suspended from the top of the jar and dip into a trough at the bottom of the jar (ascending paper chromatography). The procedures are carried out in a closed system in order that the atmosphere should remain saturated with both water vapour and the vapour of the organic solvent which is being used.

An extremely small amount, $\pm$ 0.005 of an amino acid solution containing about 0.01 mg. of the amino acid(s) is applied at a marked point, 5 cm. from the end of the paper which is to be dipped into the solvent. The system is allowed to operate for

several hours and the distance, which the solvent has travelled from the marked point at which the amino acid solution was added, is then measured. The paper is then dried and sprayed with a solution of ninhydrin in acetone. This compound produces coloured derivatives of amino acids so that the position of the amino acids on the paper strip can be seen. The amino acids in the mixture will have moved along the paper strip by the organic solvent at varying rates, so that they will be separated from one another. The ratio of the distance travelled by an amino acid to the distance of the solvent front, both measured from the marked point of application, is called the Rf value for that amino acid. Unknown mixtures of amino acids can be compared with known standards and thus identified. Quantitation can be accomplished by cutting out each separate spot, eluting the compound with a suitable solvent and performing a direct colorimetric or chemical analysis.

This method has been modified and advanced[108], until Moore and Stein (1954)[109] utilized an ion-exchange resin and various solvents to accomplish separation on a chromatographic column of all the amino acids in the protein hydrolysate. Modifications in this procedure recently introduced by Moore, Spackman and Stein (1958)[110], permit a complete amino acid analysis of a peptide or of a protein hydrolysate in 24 - 48 hours. This modified system can be used either with a fraction collector or with automatic recording equipment.

FIG 17


AMINO ACID COMPOSITION
OF MONKEY ALBUMINS
NORMAL SERUM ALBUMIN
NORMAL URINARY ALBUMIN
MINIALBUMIN
70
60
50
40
30
20
10
0
ASP 1
THR 2
SER 3
GLU 4
PRO 5
GLY 6
ALA 7
CYS 8
VAL 9
MET 10
I.LEU 11
LEU 12
TYR 13
PHE 14
LYS 15
HIS 16
NH3 17
ARG 18

Whereas on paper, which is neutrally charged, the amino acids are separated only by the difference in their own charges and molecular weights, with ion-exchange resin, the additional separatory force of the resin-amino acid binding power also comes into play with concomitant improved separation.

As we would be studying the amino acid compositions of our albumins very critically, for we would be using this as one of our criteria of identification, it was decided that we had to use the most accurate method of amino acid determination available. Rather conveniently, the method of Moore and Stein mentioned earlier, had just been introduced into normal practical analyses, and with a fair amount of experimentation and adaption to local laboratory conditions we managed to set up an accurate manual procedure for determining the amino acid compositions of our hydrolysed albumins.

(a) Apparatus.

Our apparatus consists of two glass columns, one 0.9 cm x 150 cm and the other 0.9 cm x 30 cm. Each column is fitted with a porosity 2 sintered glass disc at the bottom and the column tapered off sharply to a point under the disc. At the top of the columns were B10 Quick-fit female joints. Each column is fitted with a water-jacket for temperature control. The jackets were fitted with inlets and outlets which were connected to a thermostatically controlled water-bath. The "long column" is

used for the separation of the acidic and neutral amino acids and its setting-up will be described first.

The 0.9 cm x 150 cm column is attached to a frame-work which is in some way anchored firmly to the wall or to a solid work-bench. The attachment of the column is achieved by means of boss-heads and clamps. Care must be taken to have the column absolutely vertical, and a spirit-level or plum-line must be used in this respect.

Other apparatus is:-

(i) three 500 ml. separatory funnels with B24 joints on top. The taps of these funnels must have affixed either spring or elastic clamps to withstand up to 15 lbs/inch^2 pressure.

(ii) a three-way teflon stopcock.

(iii) lengths of 1 cm and 0.5 cm internal diameter teflon tubing.

(iv) a nitrogen cylinder with finally adjustable pressure tap and a pressure gauge.

(v) an external pressure guage which can be a mercury U-tube or a 0 - 30 lb./inch^2 gauge.

(vi) a fraction collector with a siphon or other measuring device capable of collecting $\pm$ 2 ml. samples of constant quantity.

(vii) a 1 ml. pipette. The same pipette to be used always, and only for the application of the hydrolysate to the column.

(viii) a colorimeter. We used a Klett-Summerson colorimeter with a 540 m/u green filter.

ix) various sizes of test-tubes and other miscellaneous glass-ware.

(b) Buffers and Resins.

The buffers used for the running of both columns are citrate buffers of varying pH's.

(i) For the long column on which the acidic and neutral amino acids are separated, the run is commenced using 0.2 M citrate buffer at pH 3.25 and during the experiment, at a time corresponding to the emergence of the amino acid valine, the buffer is switched over to pH 4.25. This procedure ensures a complete separation of the amino acids which emerge from the column after valine.

(ii) The buffer for the separation of the basic amino acids on the short 30 cm. column, is a 0.35 M citrate buffer at pH 5.28. This same buffer is used throughout the run.

Table V.

Buffers.

pH	Na conc.	Citric acid H_2O	NaOH 97%	Conc. HCl	Total vol.
3.25	0.2 M	840 g.	330 g.	427 cc.	40 l.
4.25	0.2 M	840 g.	330 g.	188 cc.	40 l.
5.28	0.35 M	491 g.	288 g.	136 cc.	20 l.
2.20	0.2 M	sodium citrate 105 g.	42 g.	80 cc.	5 l.

The pH 2.2 buffer is used for the pH adjustment of the hydrolysed protein sample prior to application to the ion-exchange column and was described in an earlier chapter.

To each buffer 0.1% pure, distilled phenol is added to prohibit bacterial growth. Also, thiodiglycol is added to the pH 3.25 and pH 5.24 buffers to prevent losses of methionine and cysteine through oxidation. This chemical is purchased from the British Drug Houses in the crude form and has to be redistilled under vacuum to produce a clear, pale yellow liquid which is added to above-mentioned buffers in a concentration of 1 ml./100 ml. buffer.

A detergent is added to the buffers used for the long column to permit faster flow rates, without excessive pressure having to be applied to the column. The detergent used was B.R.I.J., described by Moore and Stein and obtained from Pierce Chemical Company, Rockford Illinois. B.R.I.J. was added to the buffers as 0.75 ml. of a 33% w/v

aqueous solution per 100ml. buffer. It was added after the buffer had been boiled.

Resins and Preparation of the columns.

The ion-exchange resin used in Moore and Stein's original experiments[109] on amino acid chromatography was Dowex 50 x 4 (four times cross-linked) with a particle size of between 200 and 400 mesh. Prior to this inadequate amino acid analyses had been carried out on starch columns. The resolving power of such columns tended to be lacking as well as the final photometric procedure being distorted due to soluble material being washed off the starch.

Markedly improved results, with distinct separation of all amino acids present in a protein hydrolysate, was at last obtained using a strongly acidic ion-exchange resin, namely Amberlite (C.G. 120). This is a finely pulverised sulphonated polystyrene resin with an 8% cross linkage, Hirs (1953)[111].

We used the latter resin which was obtained from the Malenkrodt Chemical Works, New York, U.S.A. The resin is packed in the crude form - the particle sizes varying over a large range. Moore and Stein found that flow rates through the columns were rather slow, especially in the case of the long 150 cm column. One factor which greatly increased the flow rate was having a resin with uniform particle sizes. An ingenious method

used by us and originally described by Hamilton (1958)[112], was found to give resin particle fractions varying in size by as little as 5 /u.

Methodology.

Preparation. One pound of dry resin is suspended in 10 1. H_2O in a large beaker, and allowed to settle for 6 hours. Any fine particles remaining in the supernatant were removed by decantation. This process was repeated three times.

The coarser resin is then transferred to a large Buchner funnel using a hard No. 542 Whatman filter paper. The resin is now washed with 2 1. 4 N HCl and then with 500 ml. water until the filtrate was neutral. The moist resin was then placed in a beaker containing 2 1. of 2 N NaOH and heated on a steam bath for one hour. If the supernatant seemed markedly yellow, this step was repeated. The heated resin was finally washed with water on a Buchner funnel until neutrality and stored in 0.2 N NaOH.

Fractionation. The resin was fractionated in an apparatus based on the design of Hamilton. The principle of the fractionation is as follows:

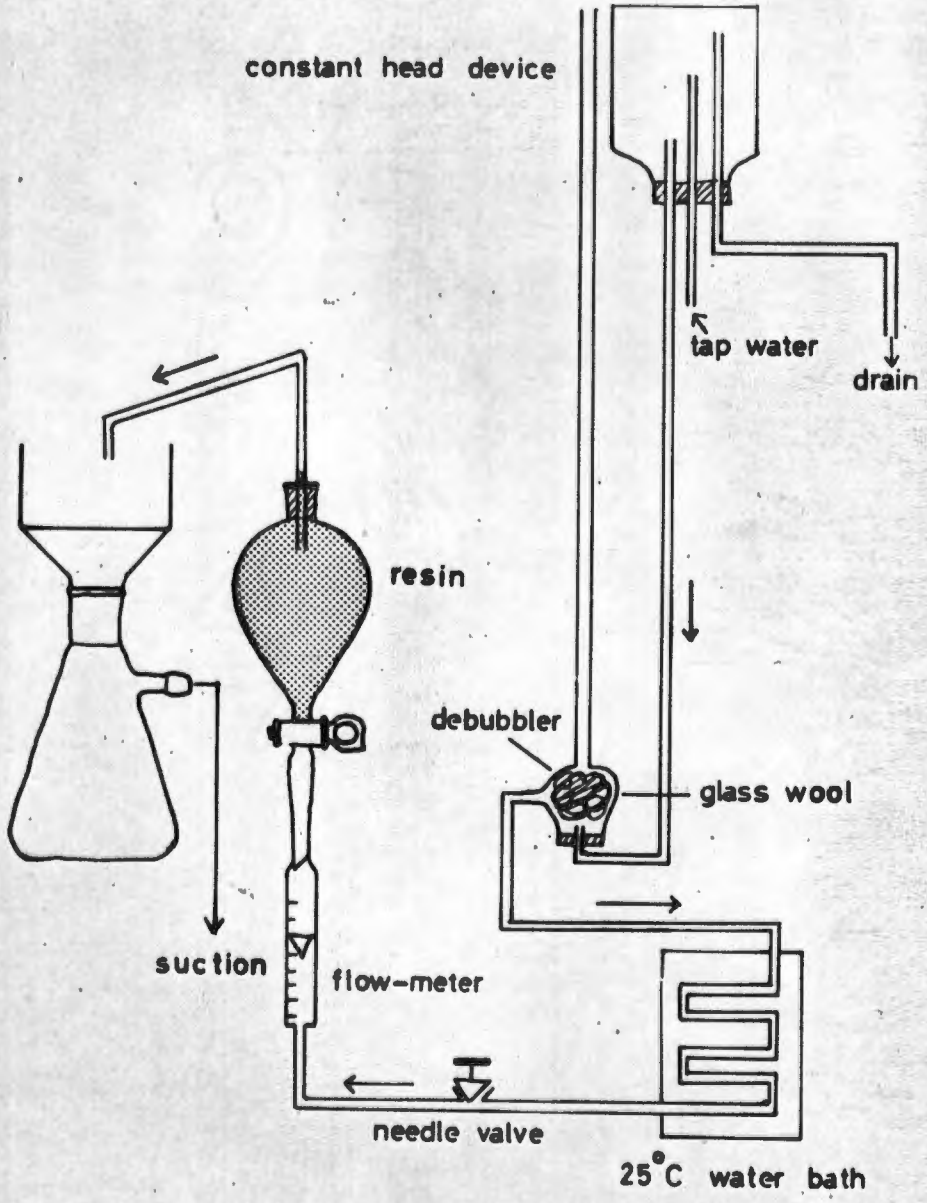
Tap-water is passed through a glass-wool filter into a reservoir placed about 10 feet above the ground. The reservoir has its outlet in such a way that it acts as a constant head device. The water now flows at an even rate through a glass-wool pad in another

reservoir which is open to the atmosphere. This acts as a debubbler, bubbles interfering with the flow of water through the resin. The water is now heated to 25°C by passing it through a series of coils in a warmwater-bath. The temperature of the water-bath must be able to be regulated, as, in order to keep the eventual flow temperature at 25°C during the varying flow rates of the fractionation, the bath has to be slowly heated.

After heating, the water flows through a needle-valve and a flow-meter into the bottom of a 2 l. separatory funnel, which is clamped in a vertical position. The water flows through the funnel and out of a glass-tube through a rubber-bung in the neck of the funnel. The overflow passes onto a filter paper pad on a Buchner funnel. (See Fig. 10).

Procedure and Principle. The thermostatically controlled water-bath is set at 35°C and the constant head allowed to fill. When this temperature has been reached, the resin in aqueous solution is placed in the separatory funnel. The water is now passed through the resin at an initial flow rate of 50 ml. per hour. At this speed only the finest resin particles are carried in the water stream, and pass over onto the Buchner funnel. The water flow was continued at 50 ml./hour until no more resin was transported by the water, and the upper portion of the water in the separatory funnel remained clear. This very fine fraction was discarded as columns packed with such resin have a tremendously slow flow rate. The water flow was now stepped up by means of the

FIG 10 HAMILTON'S HYDRAULIC FRACTIONATOR FOR RESINS



needle-valve to a speed of 110 ml./hour. The flow meter had previously been calibrated by actual measurement of the water flow into a measuring cylinder.

The swifter flow was now able to transport larger resin particles out of the funnel to be collected on the Buchner funnel. This second fraction, or fraction B, i.e. 50 - 110 ml./hour, had a particle size of 25 - 30 μ and although not used by us for the manual method, could be used in to 50 cm. column of the automatic system, where a finer resin can be used owing to the buffer being forced through the columns by a pump. The time taken for the first two fractions to be collected was 3 hours. (See Table VI).

Fraction C, i.e. 110 - 280 ml./hour was collected after the water flow had been increased to 280 ml./hour. This fraction took $2\frac{1}{2}$ hours to collect and was used by us for the short 15 cm column for the separation of basic amino acids. The following fraction, fraction D, was collected after the flow rate had been increased to 580 ml./hour. This fraction had an average particle size of 56 $\mu \pm 9$ μ and came off in $1\frac{1}{2}$ hours, although the major portion thereof was collected during the first $\frac{1}{2}$ hour. This fraction D was used by us for our long 150 cm. column to separate acidic and neutral amino acids. The resin left over was too coarse to use and was discarded.

Table VI.

Fraction.	Speed of H_2O ml./min.	Size.	Use.
A	50	25 μ	-
B	50 - 110	25 - 30 μ	75 cm.
C	110 - 280	40 $\pm$ 7 μ	15 & 50 cm.
D	280 - 580	56 $\pm$ 9 μ	150 cm.

The separate fractions were also sedimented out in a beaker to check their uniformity, which was found to be excellent.

Sedimentation times for the various resin fractions.

Fraction C (110 - 280) - 23 minutes.
Fraction D (280 - 580) - 13 minutes.
Fraction E (580+) - 10 minutes.

Preparation of the columns.

Before pouring the resin into the columns they were thoroughly cleaned by soaking them overnight in a saturated solution of KOH in ethanol 96% v/v. Care must be taken to rinse the columns completely with distilled water to remove all traces of the cleansing agent.

150 cm. column.

After cleansing, the column was clamped absolutely vertically and was now ready for pouring. Fraction D of the

resin (see table) was first equilibrated with 0.2 M pH 4.25 citrate buffer, containing neither thiodiglycol nor detergent and allowed to settle, so that 100 ml. of settled resin was contained in twice this volume of buffer. The resin was now suspended in the buffer by thorough mixing and divided into five or six equal portions in 250 ml. beakers.

After resuspending the first portion of resin, it is poured into the volumn through a funnel. The column has its outlet closed at this stage. The outlet is opened after letting 2 - 3 cm. of resin settle on the sintered glass disc, and a pressure of 30 mm. of mercury is applied to the column from a nitrogen cylinder fitted with a reducing valve and connected to the column by means of polythene tubing passing through a pressure gauge (0 - 20 lb./inch^2) or a mercury manometer. The pressure is maintained until the resin in this section is packed and all but 2 cm. of buffer has passed through the resin. The pressure is now disconnected and the next section poured in the same way. In this way between 5 and 6 sections are poured, or until the final resin height is $\pm$ 155 cm. To facilitate the pouring of the final two sections of resin, an extension tube is fitted to the column. The resin settles down with use and a final height of 150 cm. is obtained.

<u>15 cm. column.</u>

Fraction C of the resin (110 - 280 ml./min) was used for

FIG 9

EXPERIMENTAL ARRANGEMENT FOR MANUAL MOORE AND STEIN CHROMATOGRAPHIC PROCEDURE.

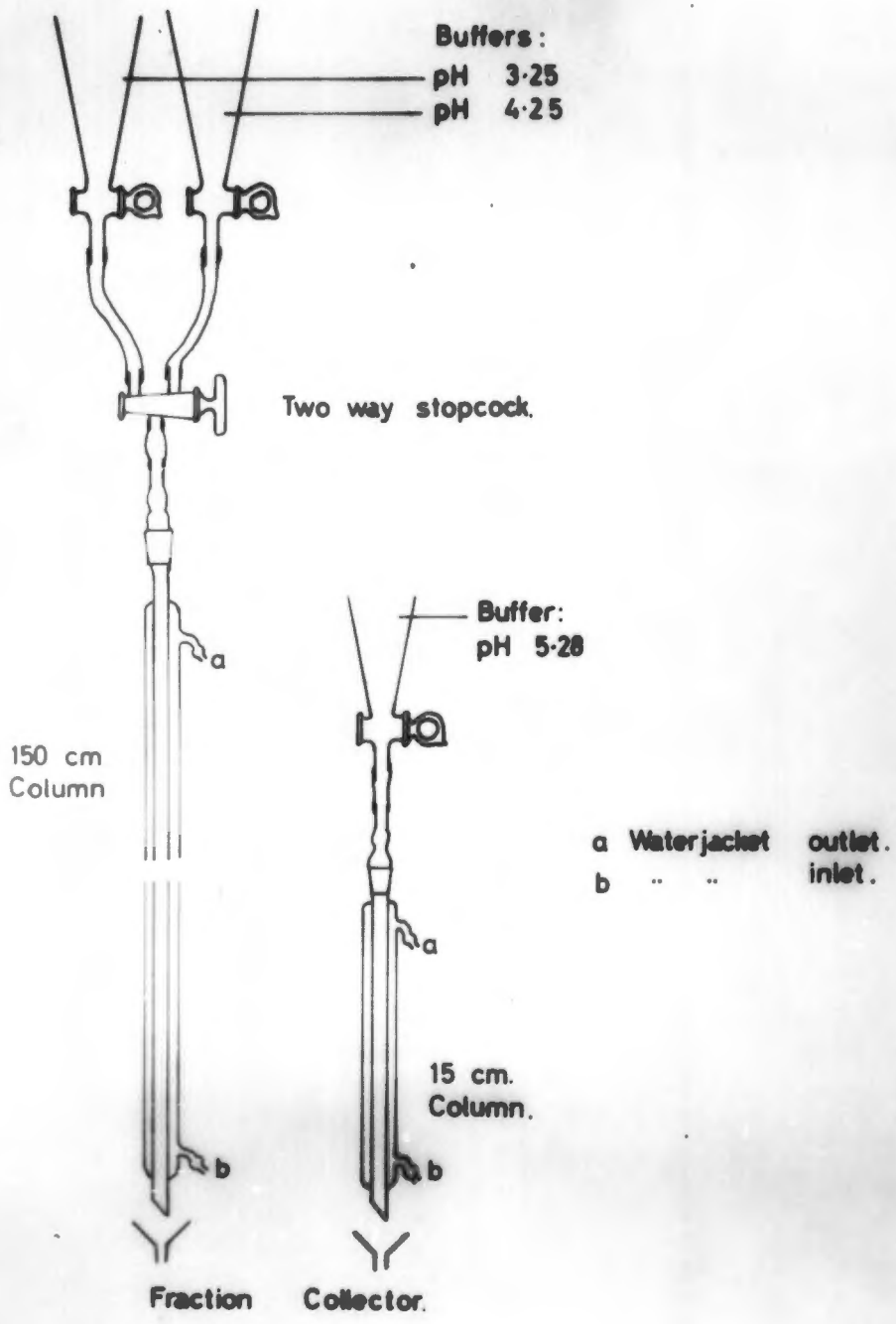
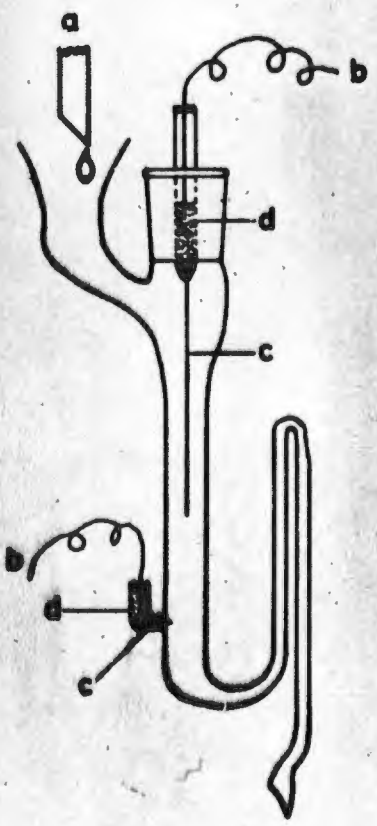


FIG. 9a

FRACTION COLLECTING SYPHON WITH CONDUCTIVITY ELECTRODES.



a Effluent from column.

b Leads to fraction collector circuit.

c Platinum electrodes.

d Mercury bridges.

the short column. The column was poured in a single section using an extension tube. The resin had previously been equilibrated tih pH 5.2 and citrate buffer.

Operation of the Columns.

Assembly of the apparatus.

The apparatus was put up with the column attached to the buffer reservoirs as indicated in the diagram (Fig. 9). The buffers to be used during a run were boiled and placed in 500 ml. separatoryfunnels under a layer of liquid paraffin. During boiling the thiodiglycol was added - 1 ml. per 100 ml. buffer. In the case of the 150 cm. column, where a buffer change is necessary, the two funnels containing the pH 3.25 and pH 4.25 buffers respectively, were connected to the column by means of a three-way teflon stopcock, to allow buffer change-overs. Pressure from a nitrogen cylinder is able to be applied to the top of the buffer reservoirs and therefore all stopcocks and taps must be fitted with pressure-resistant clamps. All joints have also to be fitted with collars or glass "ears" for attachment of clamps.

The whole set-up is mounted above a fraction collector, the fraction cutter being either an electronic drop-counter or a siphon with known delivering capacity. This siphon can work on either a weight and counter-balance system or with electrodes. We found the latter to be the most reliable. (See Fig. 9(a)).

Operation of the 150 cm. column.

Before use during the initial operating run, the resin in the column is regenerated with approximately 200 ml. of 0.2 N NaOH, which runs through the column under gravity. This solution is made up from saturated NaOH, in water which has been deionised or distilled and then subsequently boiled to remove dissolved carbon dioxide gas. The prepared 0.2 N NaOH is stored in a separatory funnel fitted with a soda-lime trap to prevent further uptake of atmospheric carbon dioxide. The resin is then re-equilibrated with citrate buffer, pH 3.25, at room temperature and under slight pressure. After each subsequent amino-acid separation, the resin is regenerated and equilibrated in a similar fashion.

Prior to each run the separatory funnels are filled with the appropriate buffer, which has been boiled. During the boiling, thiodiglycol and B.R.I.J. are added, and the buffer, still near 100°C, is added to the reservoirs by means of long-stemmed funnels, which project under a liquid paraffin layer. The boiling and storage under oil completely deaerates the buffers and prevents air bubbles forming in the actual resin during subsequent heating of the columns. About $\frac{3}{4}$ hour before application of the sample and commencement of the run, the thermostatically controlled waterbath is turned on with the temperature set at 50°C. A centrifugal waterpump pumps the water through the water-jacket

round the column. At this stage the pH 3.25 buffer is running through the column under gravity at a very slow rate i.e. ± 6 ml. per hour. The correct functioning of the siphon and fraction collector is determined at this stage.

The size of the fractions collected is dependent on the resolution or separatory powers of the column; obviously the smaller the size of each individual fraction, the finer the detail that can be observed. However, this leads to a proportionately larger number of tubes to be analysed and, using the manual method, this fact must be taken into consideration. Thus with comprimisation we decided that the collection of 2 ml. fractions would give adequate resolution.

In developing the details of the procedure, a synthetic test mixture or standard of known amino acids was employed. This standard solution was applied to the column in a similar fashion to the protein hydrolysate.

In either case, the sample is applied as a 1 ml. volume, very accurately measured and preferably always using the same pipette. The pH of the sample must be below pH 3.0 if the peaks on the effluent curve of the ensuing chromatogram are to be satisfactorily sharp.

Technique of sample application.

The buffer reservoir is detached from the top of the column

and the supernatant buffer above the resin is carefully removed by means of a pasteur pipette. When the buffer has just disappeared under the surface of the resin, the sample is applied very slowly to the surface, care being taken to add the liquid evenly and without disturbing the surface particles. As the flow through the column is extremely slow, slight pressure is applied to the column from the nitrogen cylinder to aid the penetration of the sample. When the sample finally enters the surface the pressure is released and 1 ml. of buffer is applied to wash the sample into the resin. This washing is repeated three times before the space above the resin is filled up and the buffer reservoir attached, making sure that no air bubbles are trapped in the system. The flow rate of the column is adjusted by means of pressure applied to the buffer reservoir to a speed of 12 - 14 ml. per hour. The effluent from the column is collected in 2 ml. fractions in $5\frac{1}{2} \times \frac{5}{8}$" test-tubes.

The amino acids separated on the column and collected in the tubes, are determined following a colorimetric procedure by employing a reaction which results between the amino acid and ninhydrin to form a dark-blue colour. The concentrations of the individual amino acids are proportional to this colour which develops, and the actual calculations and individual colour values will be described later.

The run being described is continued under the conditions

of 50°C, 4 lbs./inch^2 pressure and pH 3.25 buffer until the emergence in the effluent of the valine peak. At this stage the buffer is changed over to pH 4.25 by means of the three-way stopcock. This buffer change is essential to obtain satisfactory separation of the amino acids emerging after valine. The run is stopped after the emergence of phenylalanine which is the 14th peak. With experience, knowledge to the position and size of each amino acid peak of a certain protein hydrolysate is obtained, and the position in the run easily deduced.

It is a significant fact that any new column gives slightly erroneous results at first and the resolving power is only completely adequate for quantitation after at least four separate runs.

Operation of the 15 cm. column.

The basic amino acids were eluted with buffer of pH 5.28 at 50°C. The sample was applied in the same way as described for the long, 150 cm. column and the pressure through the system regulated to 20 cm. of mercury.

The basic column need not be regenerated with NaOH in between runs as no amino acids are retained with the pH 5.28 buffer.

The Ninhydrin Reaction.

At this stage the photometric determination and especially

the ninhydrin - amino acid colour development will be discussed. Initially, earlier workers had difficulty in rendering this method quantitative, until it was realised that internal oxidation could be avoided by the addition of strong reducing agents like stannous chloride. (Harding and McLean, 1915 [113] and Moore and Stein, 1948 [114]).

Reagents:

Ninhydrin. Several commercial sources provide ninhydrin sufficiently pure to be used without recrystallisation. We used mainly ninhydrin purchased from the British Drug Houses.

Hydrindantin. This product is a reduced form of ninhydrin. In our initial experiments we used $SnCl_4$ to bring about this reduction, but the amount of reduction was dubious and the method unsatisfactory. Thus, later we followed the modified method of Moore and Stein for preparing hydrindantin.[109] The compound is prepared by the reduction of ninhydrin with ascorbic acid.[115][116] To 80 g. of ninhydrin in 2 l. of water at 90°C is added, whilst stirring, a solution of 80 g. of ascorbic acid (Merck) in 400 ml. of water at 40°C. Crystallisation of hydrindantin starts immediately and is allowed to proceed for 30 minutes without further heating. During the next hour the solution is cooled to room temperature under running tap water. The hydrindantin crystals are now filtered off on a Buchner funnel, using Whatman 542 paper, and

the crystals washed well with distilled water. The washed product is dried to constant weight in a vacuum desiccator containing P_2O_5 and protected from the light. The yield is approximately 75 g. and the compound should be stored in dark glass or in a sealed cupboard. The crystallization procedure is carried out rapidly to give small crystals which will dissolve readily in the solvent, methyl cellosolve (2 methoxyethanol).

4 N sodium acetate buffer at pH 5.5. To 2 l. of water add 2720 g. of NaOAc 3 H_2O (reagent grade) and stir on a steam water-bath until solution is complete. Cool to room temperature, add 500 ml. of glacial acetic acid, and make up to a volume of 5 l. We actually made up only 2 l. as only small quantities of the buffer are used. The undiluted solution was found to be at pH 5.5, but if pH adjustments are necessary, 2 g. of NaOH pellets correspond to about 0.04 pH units. The buffer is stored at 4°C in a dark bottle without a preservative.

Methyl Cellosolve ($CH_3 OCH_2 CH_2 OH$). This solvent we obtained from May and Baker, Ltd., Dagenham, England. The various batches tended to be inconsistent, some giving blank colour values of up to 100 colorimeter units due most probably to some ninhydrin-reacting contaminants. By ordering a certain batch we could obtain cellosolve that gave us consistent blank readings of 44 - 48 colorimetric units. For our experiments we used mainly M and B batch 67006.

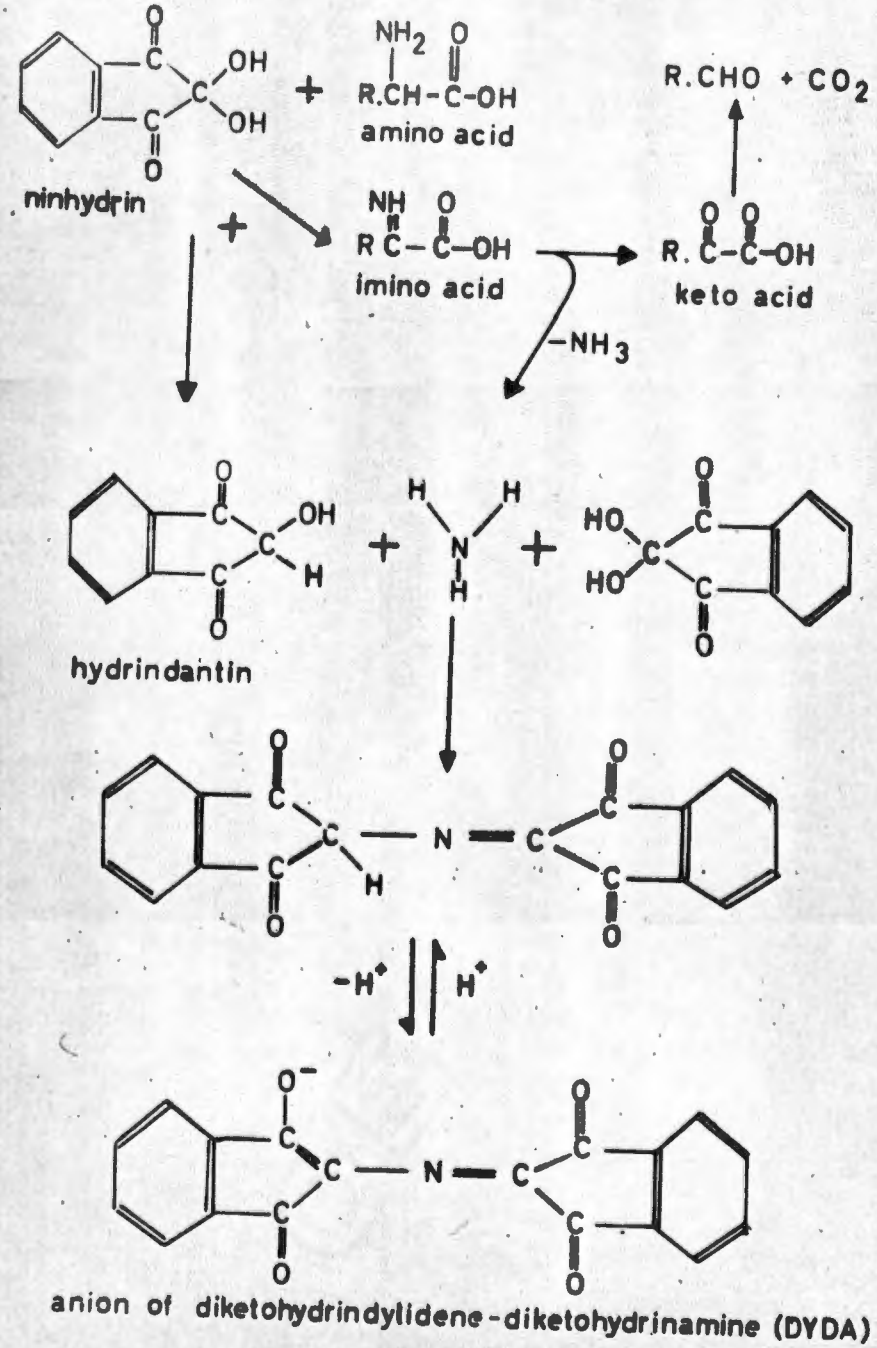
Reagent solution. Just prior to amino acid estimations, the final mixing of the reagent solution commenced.

Dissolve 20 g. of ninhydrin and 3 g. of hydrindantin in 750 ml. of methyl cellosolve. This mixture can be proportionately reduced if samples are analysed on successive days. Add 250 ml. of the pH 5.5 buffer and immediately transfer the resultant deep red solution to a 1 l. dark glass reagent bottle, which is then covered with a nitrogen layer, by blowing nitrogen from a cylinder over the reagent and closing the stopper. A series of reservoirs and nitrogen traps have been described by Moore and Stein [114] to keep this reagent under nitrogen even during use. We, however, used much smaller quantities of reagent at a time to obviate this necessity.

Reaction.

Moore and Stein (1948)[114] studied and isolated the blue-coloured compound which had previously been described by Rùheman (1911).[117] The amino acids in the eluate react with the above reagent mixture to form this compound. The relevant reactions are as follows (see Fig. 11). Ninhydrin or trihetahydrindene-hydrate reacts with an amino acid to form imino acid, liberating water and also forming hydrindantin. The imino acid is unstable in water and breaks down into ammonia and a keto-acid. This ammonia now reacts with one molecule of ninhydrin plus one of

FIG II THE NINHYDRIN REACTION



hydrindantin, with the splitting off of 3 water molecules to form compound A. The addition of a final ammonia molecule gives the final blue-coloured compound. This compound is directly proportional to the concentration of amino acid, the colour-value of each individual amino acid being taken into consideration.[118]

Procedure for analysis of effluent.

We used the completely manual method for our amino acid determinations. The colour-forming reaction was carried out in the actual tubes from the fraction collector to obviate any loss through transfer of the solutions. The samples analysed could be of any volume from 1 - 2.5 ml. as long as they were all identical in volume. In any event, the volume of ninhydrin reagent used would be 1 ml. irrespective of the sample volume.

The tubes were handled 24 at a time. A 25 ml. burette was filled with ninhydrin reagent and a waterbath able to contain 24 test-tubes was set, thermostatically, to boil vigorously.

To each tube containing amino acid effluent exactly 1 ml. of ninhydrin reagent was carefully added, the time being accurately noted. If one tube is handled every $\frac{1}{4}$ or $\frac{1}{2}$ minute, precise timing can be maintained. After shaking for 10 seconds, the tubes are placed in the boiling waterbath, consecutively, at exact time intervals. After boiling for precisely 15 minutes, the tubes are taken from the waterbath and allowed to cool in an

open rack. As they are taken, out exactly 5 ml. of 50% aqueous ethanol is pipetted into each tube to act as a diluent. After cooling for 10 minutes each tube was shaken manually for 30 seconds. At this stage the colour is fully developed and sufficiently diluted for reading. We were very fortunate in that the maximum absorption of the blue-coloured compound was very close to 540 mμ and the Klett-Summerson colorimeter which we used had standard filters at the wave-length. The readings were always carried out in a special colorimeter tube. The machine is set to zero with distilled water and the readings of the fractions commenced. In the run using the long column, no amino acids emerge until ± tube 72, and thus a good average blank value can be obtained. The colour value of the base line tubes should not vary more than 2 or 3 scale units and if standardized reagents are used, the blanks should not read more than 45 units.

After the completion of the run, which usually lasted about 30 hours and 280 tubes (1.4 ml. fractions) we determined the colour density in each tube using the method just described. These values were plotted graphically i.e. optical density against fraction number and a series of peaks, each one representing an individual amino acid, were obtained. The total colour value of each peak was then worked out by summing the total colour value of each point on the peak above the base line of the graph. This summation gives the total colour value of

FIG. 13

SPECTRAL ABSORPTION OF AMINO ACID — NINHYDRIN COLOUR COMPLEX.

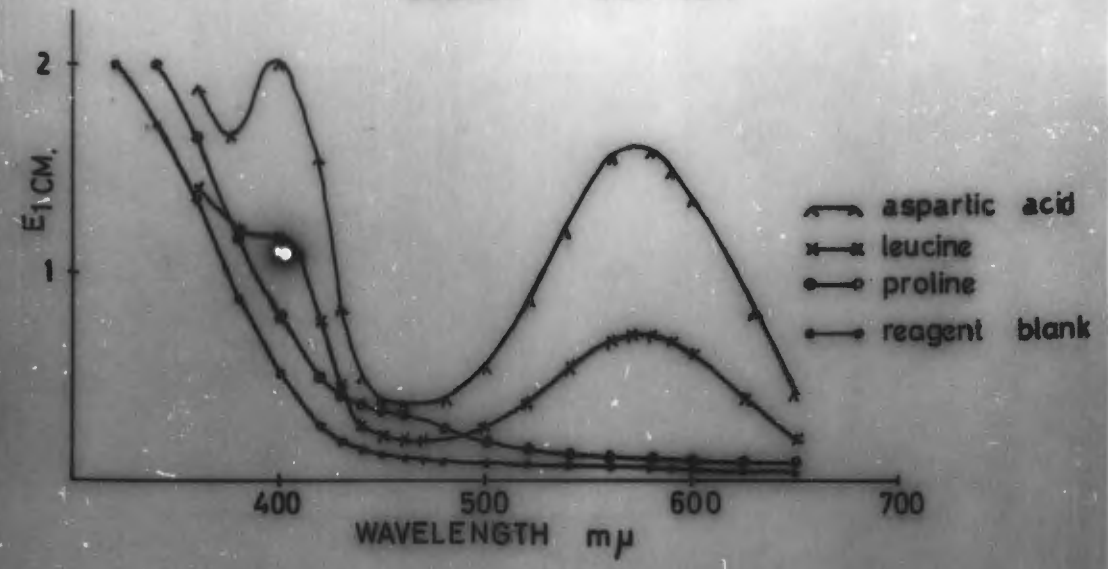
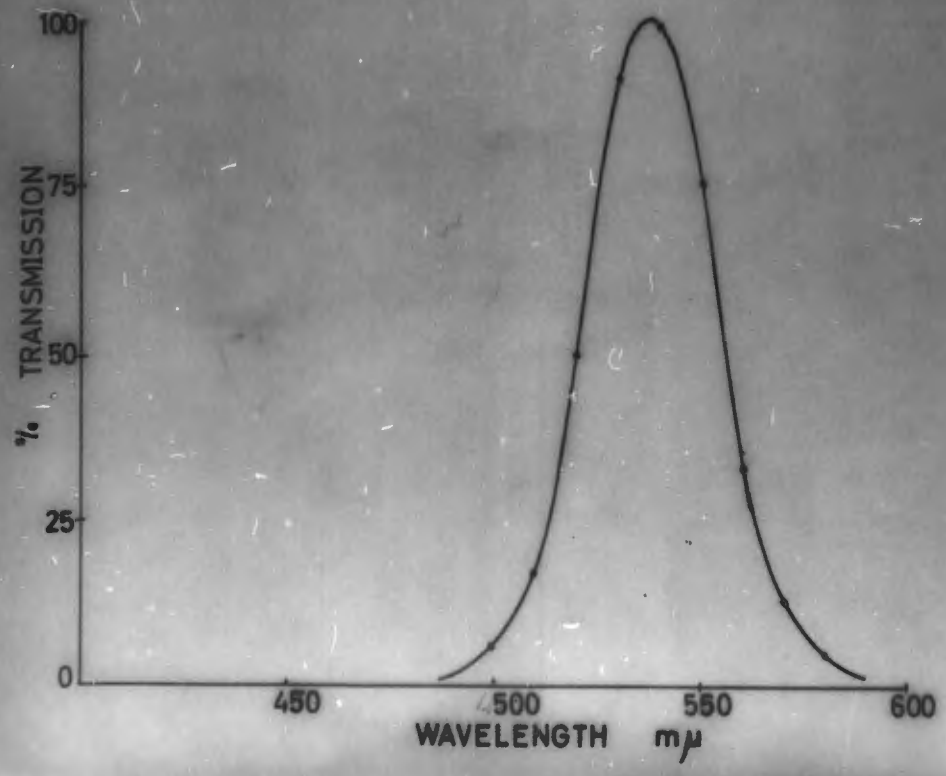


FIG 14.

SPECTRAL TRANSMISSION OF GREEN COLORIMETER FILTER



an individual amino acid. The amount of amino acid can then be calculated by comparing the unknown hydrolysate with a known amino acid standard mixture. When various considerations, which will now be discussed, are taken an accurate degree of quantitization is achieved.

Standardization.

Wave-length. In order to standardize our procedure, many aspects had to be verified. Using the Beckman model D.U. spectrophotometer we first of all plotted the absorption spectra of the ninhydrin-amino acid complexes. The complex tended to absorb quite strongly below 400 m/u, but absorption dropped to a minimum at between 450 m/u and 480 m/u. (see Fig. 13). As stated earlier the colorimetric device used was a Klett-Summerson make, which manufactured filters of various wave-length emittences. The closest to 570 m/u obtainable was one at 540 m/u. This filter gave very good results, however, and when the transmission spectrum of this filter was plotted again in a D.U. model Beckman spectrophotometer, the filter was found to absorb maximally at 535 m/u (see Fig. 14). Also, the peak produced was absolutely symmetrical and completely excluded light below 500 m/u and above 600 m/u. This last fact would cut out any non-specific absorption which could give unpredictable results.

Thus, apart from the slightly lowered sensitivity, there

appeared to be no reasons theoretically against our chosen colorimetric analysis.

Standard curves. In order to calibrate our columns as well as test our recoveries and reproducibility we made up a standard solution of amino acids containing exact quantities of the amino acids normally encountered in a protein hydrolysate. These were checked for purity by paper chromatography and a single ninhydrin positive spot obtained in each case.

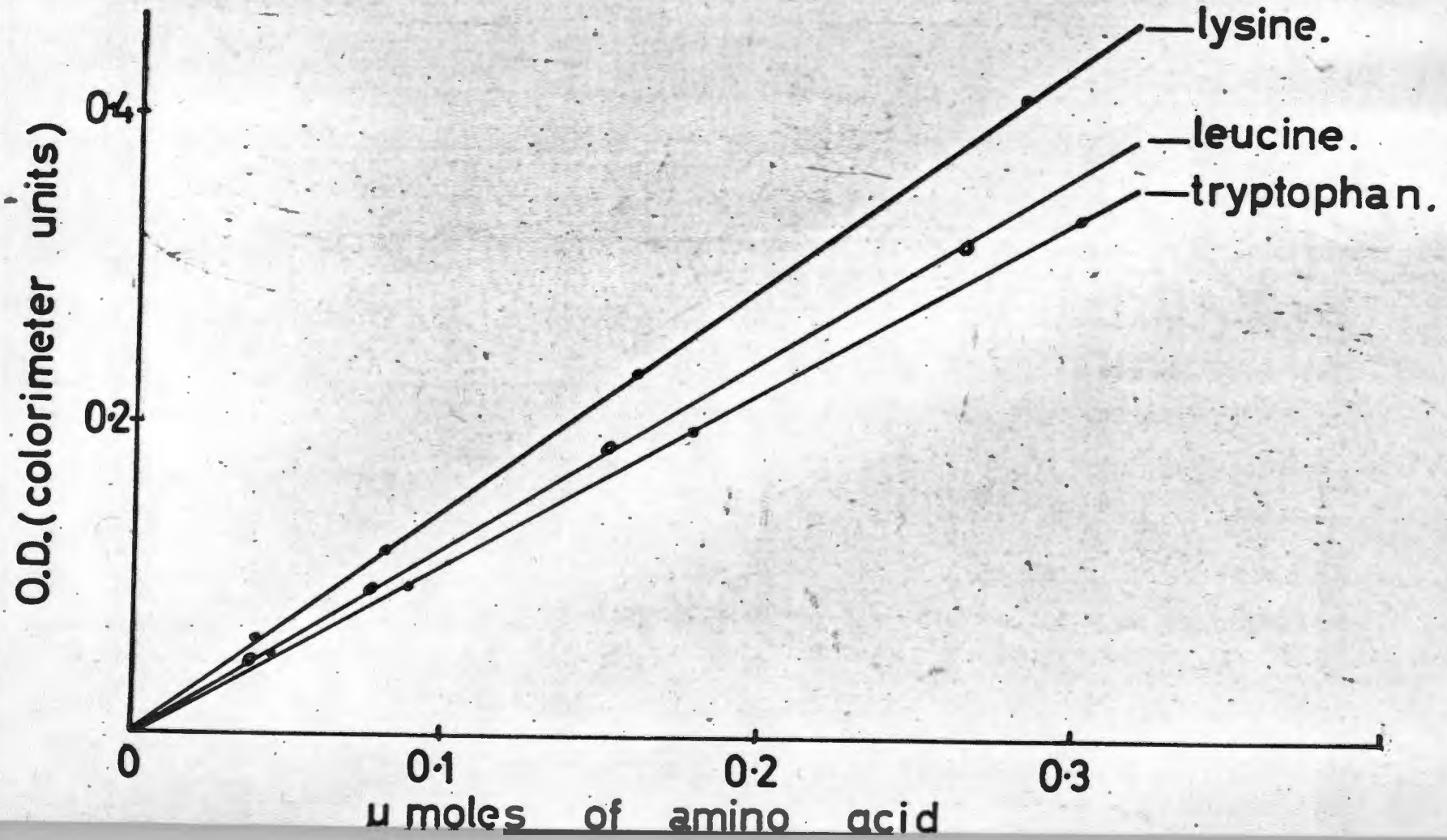
All the amino acids used were Laboratory Grade obtained from the British Drug Houses. The amino acids were dried to constant weight over phosphorus pentoxide (P_2O_5) in a vacuum desiccator before weighing out for use. A stock solution was prepared in 0.8 N HCl (previously 3 times glass-distilled and diluted with ammonia-free distilled water). The concentration of this stock solution was between 5 and 8 /umoles per ml. of each amino acid. Before use dilutions were made so that the concentration of each amino acid was in the range of 0.1 - 0.5 /umoles per ml.

One ml. of this standard solution was analysed in duplicate for each of a total of three different dilutions. The analysis was carried out by the procedure described, and the optical density of the eluate measured in the Klett-Summerson colorimeter at 540 m/u.

We found that for each amino acid, the standard curves were

FIG. 15

STANDARD CURVES FOR SOME AMINO ACIDS DETERMINED BY THE NINHYDRIN REACTION.
(reaction volume 7 ml)



straight lines passing through zero, proving that a linear relationship exists between concentration and colour value. Also, that when dilutions of the colour were carried out, the colour/concentration curve obeyed Beer's Law (see Fig. 15).

A discrepancy was, however, found in the colour yields of the amino acids when compared with each other. Thus, following the procedure of Moore and Stein, we related the colour yield of each individual amino acid to a chosen one i.e. leucine. Thus, taking the colour value of leucine to be 100 (1.00) we could determine the colour yield of all the other amino acids in so-called leucine-equivalent based on a molar basis relative to leucine. A table of the leucine equivalents compared to those obtained by other workers is given (Table VII).

To further standardize our actual protein hydrolysate, we added nor-leucine to the protein before hydrolysis. A known quantity of this synthetic amino acid is added to each sample and the recovery determined as a check to the whole procedure.

Calculations. All the samples from the effluent off the column are analysed colorimetrically and the values obtained are plotted on a graph. We found that our colour values varied slightly from those obtained from other laboratories but were reproducible under our own conditions. We found also that the colour yields were relatively independent of the initial pH of the sample, provided that the substance analysed is dissolved

TABLE VII.

Colour Yields of different amino acids on a molar basis relative to leucine

	Colour Yield	
	Moore and Stein	Present Author
Aspartic Acid	0.94	0.96
Threonine	0.94	0.95
Serine	0.95	0.96
Glutamic acid	0.99	1.06
Proline	0.225	0.35
Glycine	0.95	0.96
Alanine	0.97	1.00
Half cystine	0.55	0.59
Valine	0.97	1.01
Methionine	1.02	1.02
Isoleucine	1.00	0.99
Leucine	1.00	1.00
Tyrosine	1.00	1.00
Phenylalanine	1.00	0.94
Lysine	1.10	1.16
Histidine	1.02	1.01
Ammonia	0.97	0.98
Arginine	1.01	1.02
Tryptophan	0.94	0.93

in 0.2 N buffers at between pH 3 and 7. We used a dissolving buffer at pH 2.2 which slightly decreases the colour yield. This decrease is, however, uniform in all cases for all amino acids and consequently gives no erroneous results.

After the graph has been plotted, an average base-line is calculated for each separate peak. The variation in base-line between peaks up to the valine peak is usually negligible, but after valine, due to the emergence of the pH 4.25 buffer, there is a 2 - 3 unit rise in the base-line.

The total number of points on a peak is now added up giving the total optical density of all tubes comprising a given amino acid. A similar number of base-line values are now subtracted from this total giving the optical density due to amino acid alone.

$$\text{Thus } C = \sum_{1}^{n} O.D. - nb$$

Where C = /umoles of amino acid applied to the column.

n = number of fractions per peak.

O.D. = total optical density value of all the points on a peak, expressed in colorimeter units.

b = average blank value.

The molecular weight divided by the colour yield provides the factor by which the chromatographic results (expressed in leucine equivalents in micromoles) should be multiplied to give micrograms of the substance in question. In other words, after

TABLE VIII.

AMINO ACID COMPOSITIONS IN G. / 100 G. PROTEIN OF MONKEY ALBUMINS

	Serum Albumin	Urinary Normal Albumin	Urinary Minialbumin
Aspartic acid	9.5	10.1	10.7
Threonine	4.8	5.1	4.6
Serine	3.6	3.5	3.0
Glutamic acid	16.9	17.5	16.0
Proline	4.2	4.3	4.8
Glycine	1.6	1.8	1.7
Alanine	7.6	7.9	8.7
½ Cystine	5.6	5.6	3.9
Valine	6.9	7.0	7.5
Methionine	1.2	1.3	1.1
Leucine	10.4	10.6	10.4
Isoleucine	1.7	1.7	1.6
Tyrosine	4.8	4.6	4.3
Phenylalanine	7.5	7.3	6.7
Lysine	12.2	12.4	10.1
Histidine	3.4	3.4	3.0
NH_3	1.3	1.11	1.1
Arginine	5.7	6.2	5.6

TABLE IX.

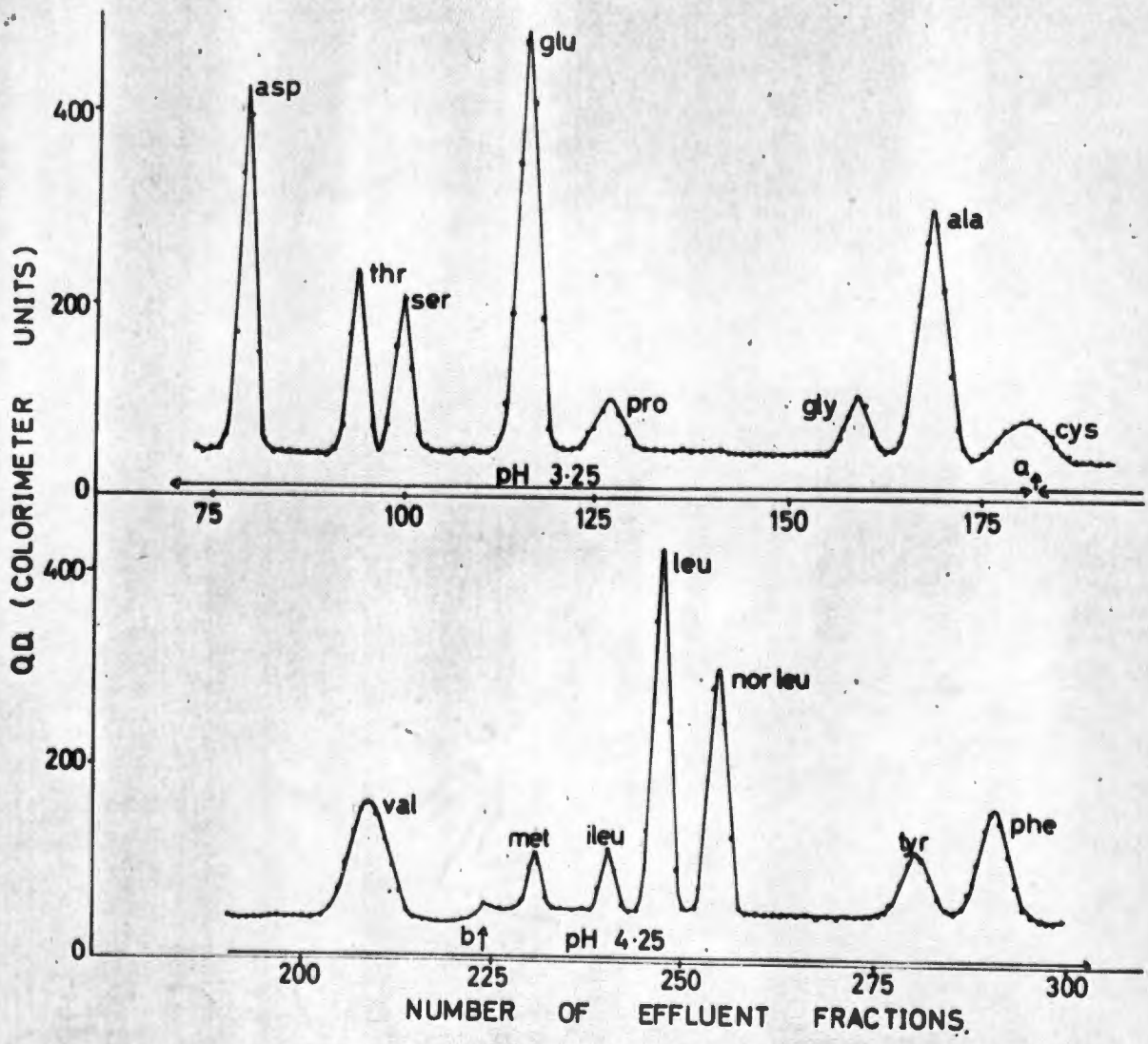
AMINO ACID COMPOSITION OF SERUM ALBUMIN IN DIFFERENT SPECIES IN G./100 G. PROTEIN.

Amino acid	Man	Dog	Monkey	Ox
Glycine	1.6	1.99	1.6	1.8
Alanine	6.25	6.55	7.6	6.25
Valine	7.7	6.11	6.9	5.9
Leucine	11.9	10.99	10.4	14.8
Isoleucine	1.7	0.99	1.7	14.8
Proline	5.1	4.51	4.2	4.7
Phenylalanine	7.8	6.66	7.5	6.6
Tyrosine	4.66	5.26	4.8	5.0
Tryptophan	0.19	0.34	-	1.43
Serine	3.7	3.36	3.6	4.2
Threonine	5.0	3.55	4.8	5.8
Cysteine	0.70			
Cystine/2	5.58	5.12	5.6	6.5
Methionine	1.28	0.78	1.2	0.8
Arginine	6.15	5.35	5.7	5.2
Histidine	3.5	2.50	3.4	16.8
Lysine	12.3	11.30	12.2	16.8
Aspartic Acid	10.4	8.95	9.5	10.9
Glutamic Acid	17.4	16.18	16.9	16.5
Amide NH_3	1.07	0.87	1.3	

FIG 16

SEPARATION OF ACIDIC AND NEUTRAL AMINO ACIDS IN A HYDROLYSATE OF ALBUMIN

(150 CM. COLUMN)



a buffer change 3·25—4·25 (pH)

b. emergence of pH 4·25 buffer

LEGEND TO FIGURE 16 a.

The graphs show an elution curve from a Moore and Stein column of Amberlite resin, of acidic, neutral and basic amino acids from a hydrolysed sample of monkey serum albumin. The ordinate shows the fraction number and the abcissa the optical density values. The peak numbers refer to the following amino acids:

1. Aspartic acid
2. Threonine
3. Serine
4. Glutamic acid
5. Proline
6. Glycine
7. Alanine
8. ½ Cystine
9. Valine
10. Methionine
11. Leucine
12. Isoleucine
13. Tyrosine
14. Phenylalanine
15. Lysine
16. Histidine
17. Ammonia
18. Arginine

FIG 16a

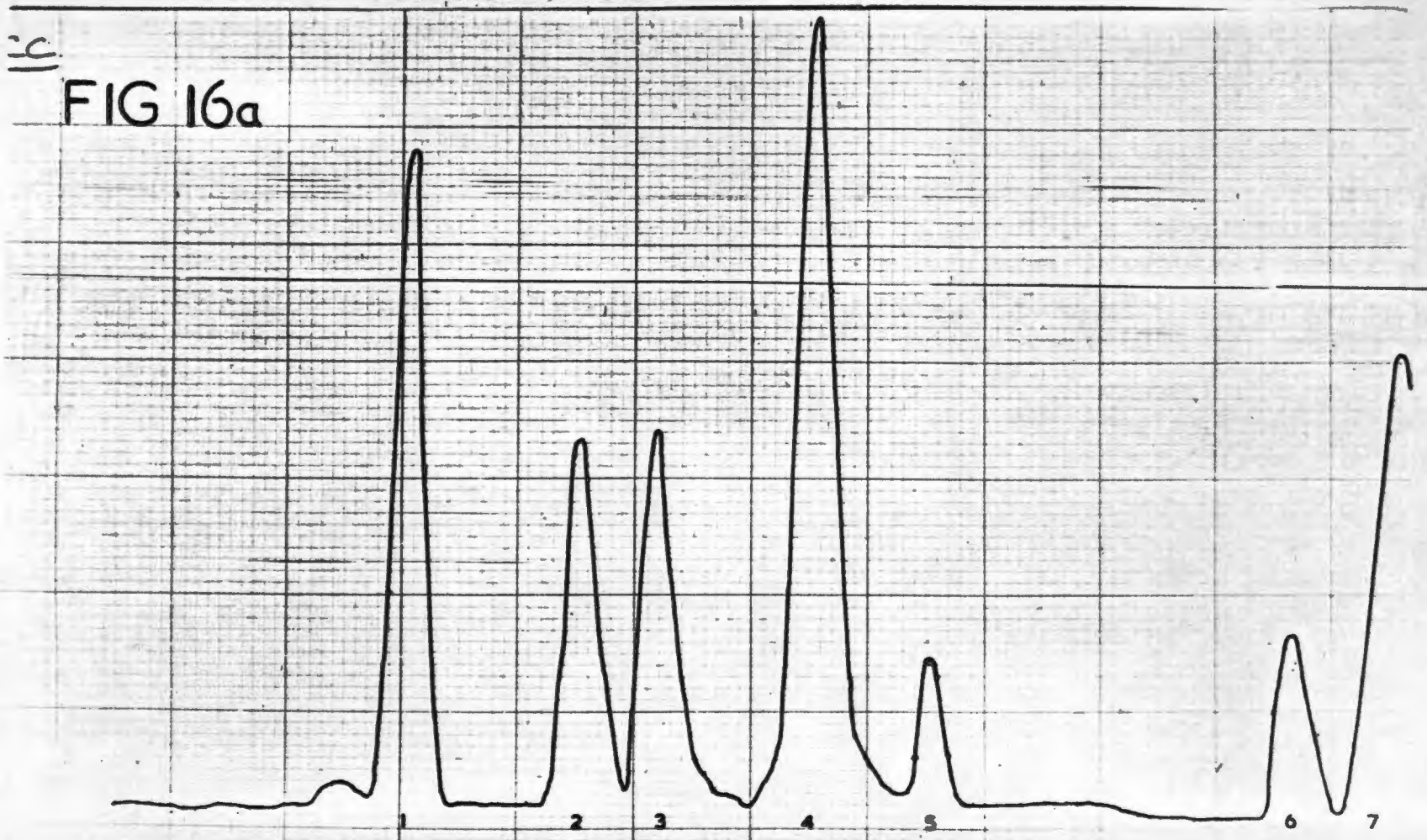


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FIG 16a

Normal Monkey Serum albumin (TC 4)

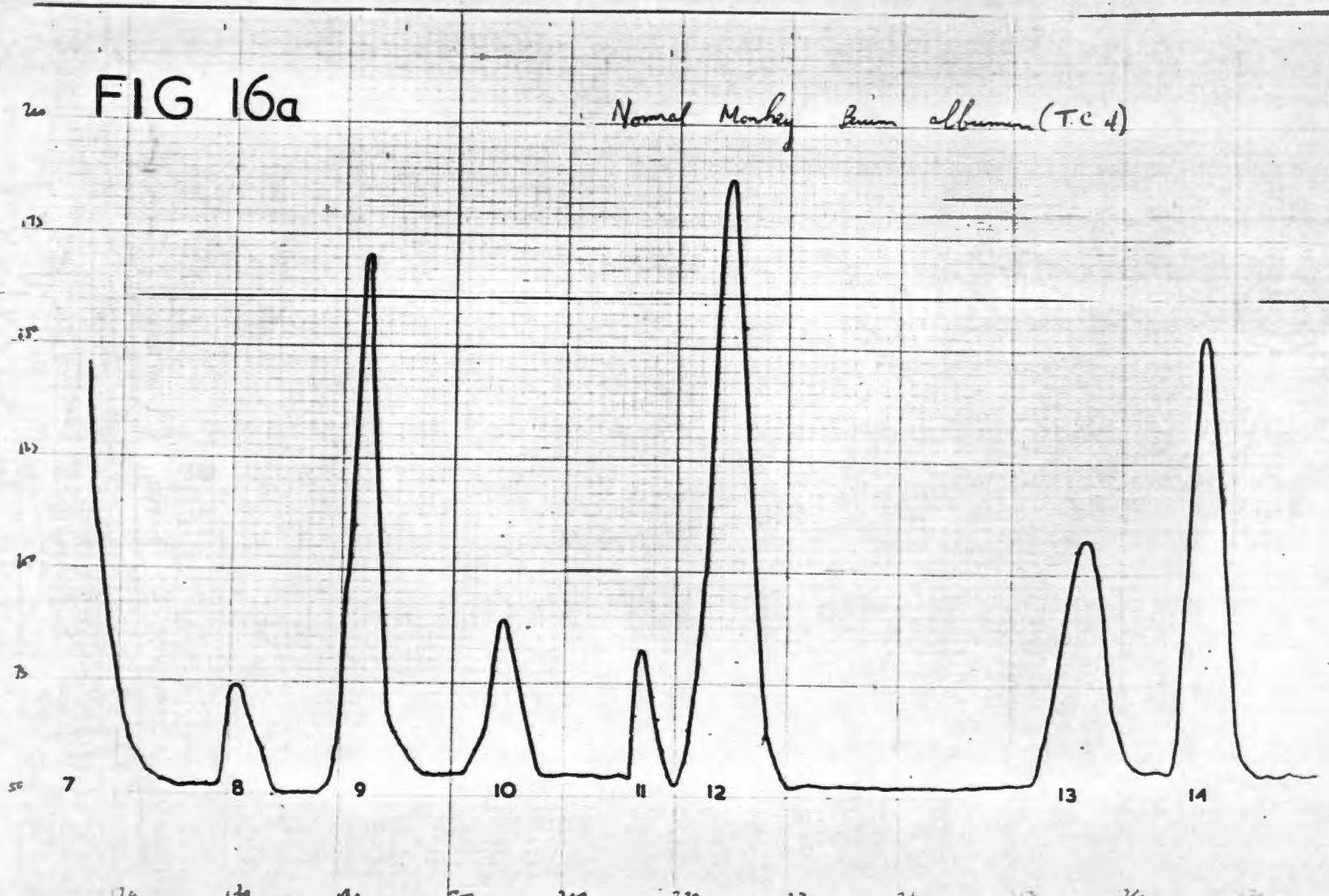

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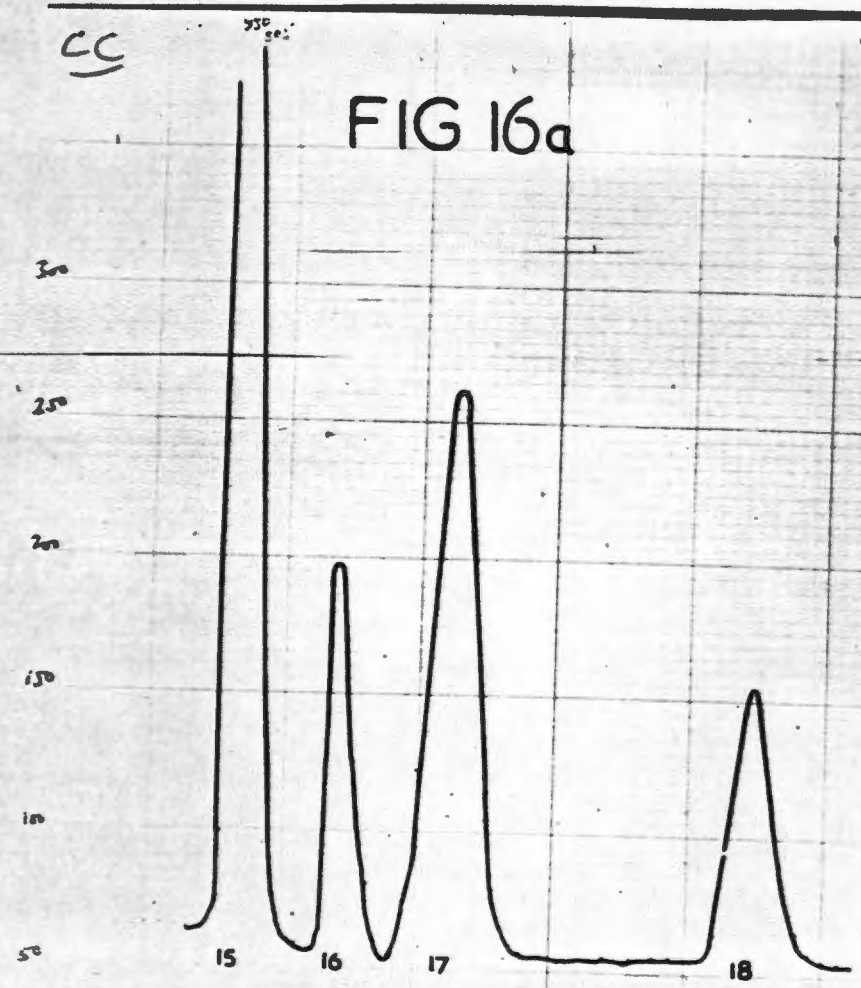
FIG 16a



Normal Monkey Serum albumin
Basic Amino Acids

LEGEND TO FIGURE 16 b.

The graphs show an elution curve from an Amberlite resin column of neutral, acidic and basic amino acids from a hydrolysed specimen of monkey minialbumin isolated from the urine. The ordinate indicate the fraction number and the abcissa the optical density values. The peak numbers refer to the same amino acids as in Fig. 16 a.

FIG 16b

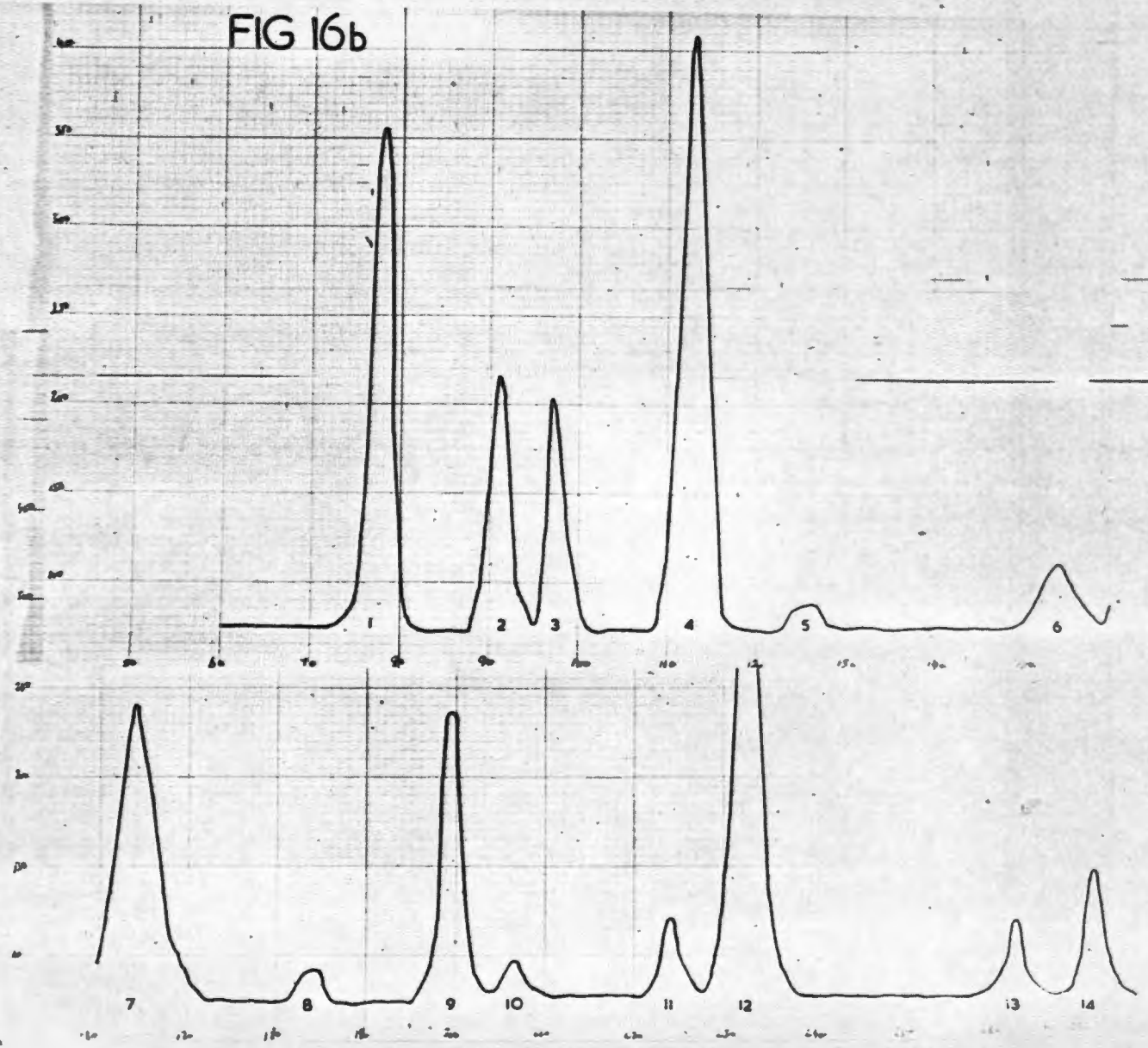
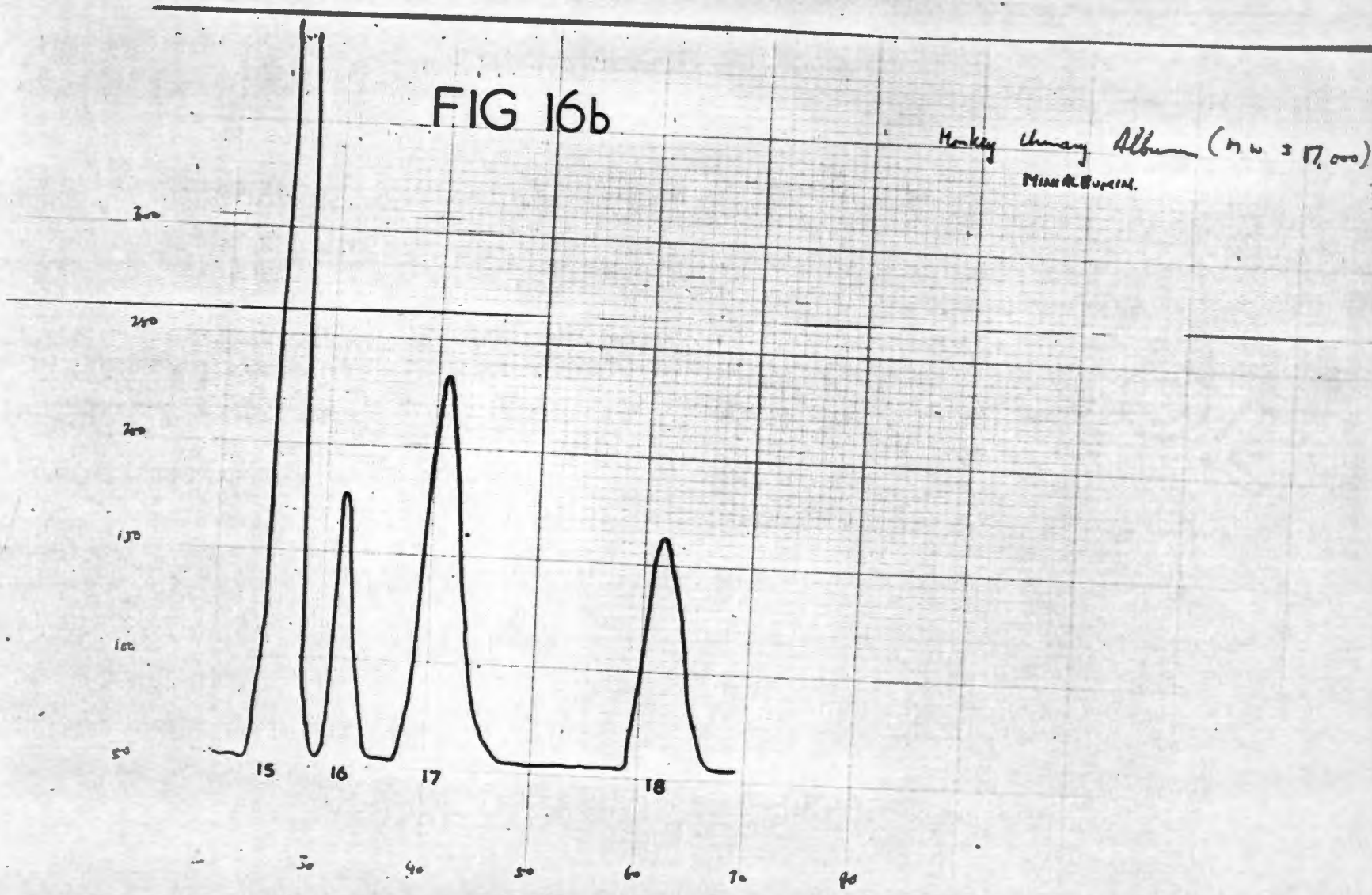


FIG 16b


Monkey Urinary Albumin (MW = 17,000)
MONOMERIC ALBUMIN.
300
250
200
150
100
50
15
16
17
18
30
40
50
60
70
80

comparison of the colour total (C) of each amino acid, with the standard curve, taking recoveries into consideration, an amount in micro-moles is obtained, which is then multiplied by this factor.

Proceeding in this manner we determined the amino acid composition of the normal serum albumin of a control monkey as also the serum albumin and urinary albumins of a cadmium intoxicated monkey. The urinary albumins were prepared and separated on Sephadex as described earlier. The amino acid composition, as a percentage value of the whole protein as well as the calculated residues per protein molecule, are given in the accompanying tables. The amino acid composition of the serum albumin of various other species obtained from the literature is also provided for comparison (see Fig. 16 and Tables VIII and IX).

Conclusions.

With the possible exception of lysine and cysteine, the proportions of amino acids in the urinary albumins and serum albumin are not significantly different from one another.

II 6 (vii) Measurement of the diffusion constant, sedimentation constant and molecular weight of the urinary albumins.

The minialbumin produced in the urine of cadmium poisoned monkeys had now been isolated and separated from the normal albumin in the urine caused by the renal tubular damage. The

minialbumin's amino acid composition agreed remarkably well with that of the serum albumin and at this stage we decided to carry out accurate molecular weight determinations on this protein and at the same time determine some important physical facts concerning its behaviour.

We had at our disposal, thanks to the Virus Research Unit, a Beckman analytical ultracentrifuge, in which we could determine the sedimentation constant of our protein. This data would only give us an indication of the molecular weight. In the calculation of the molecular weight of proteins from sedimentation data it is essential that the diffusion constants be accurately known. In early experiments as also with extremely homogenous protein solutions, the diffusion constants were measured in the ultracentrifuge itself. This was done by calculating the amount of spreading of the protein band at the sedimenting boundary, and the concentration changes were determined from measurements of the light absorption of the solutions - Svedberg (1925)[119]. But in the ultracentrifuge it is impossible to control the temperature to a degree necessary for accurate diffusion measurements. Also, the time of diffusion in the centrifuge cell is far too short for an extended series of measurements. Thus efforts were made to develop an accurate method of measuring the diffusion of substances in solution. Diffusion is a fundamental property connected with molecular size and shape - Lamm (1928)[120,121].

Diffusion constant measurements.

Cohen and Bruins (1923)[122] introduced a method for determination of the diffusion constant still in use today. Their apparatus consists of six circular discs of metal which can revolve around a central axis. The four middle discs have 3 holes of 2 cm. diameter bored into them at angles of 120° to each other and to the centralaxis. The discs are all placed on top of each other so that the holes in successive discs are coinciding. The 3 bottom holes are now filled with solution and the top holes afe turned away and filled with solvent. The whole apparatus is then placed in a water-bath to obtain temperature equilibrium and thereafter the top disc is turned back so that the holes coincide again, and diffusion commences. After completion the plates are all turned so as there is no contact between any of the holes, and the concentrations measured with a interferometer.

The possibility of the relationship between the refraction of light and concentration of solute, in different layers of a diffusion column, was hypothesized by Weiner (1893)[123]. Light of a definite wave-length is passed through a slit, then through a system of lenses and through a diffusion cuvette in which a diffusion process is taking place. Due to the varying refractive indices in the diffusion layer, the light path becomes deviated and the image of the slit becomes distorted. The image of the slit can be photographed and the distortions recorded. From the

displacement of the image from its normal position after known time intervals, the diffusion constant can be calculated. Weiner also derived a relation between the displacement of the light found and the concentration in the different layers.

A modification of this method by Lamm[121] was used for the calculation of the diffusion constant of our minialbumin. He uses a vertical uniform transparent scale which is photographed through the diffusion cell. The refractive index gradient at the diffusion boundary produces a distorted image of the scale, such that the scale-line displacement is proportional to the concentration gradient when the refractive index is a linear function of the concentration.

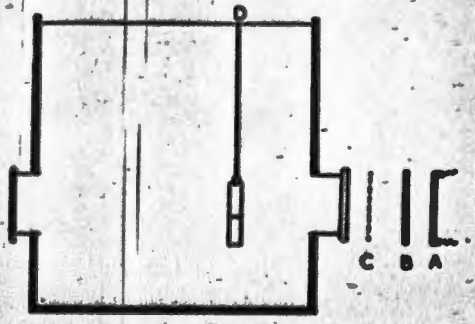
Apparatus. The diffusions were carried out in a perspex holder containing four cuvettes, the sides of which were optically uniform. The bottoms of the cuvettes were connected to a mercury reservoir by means of a system of tubes. The mercury could be let in or out of the cuvettes, varying the volume as also giving a horizontal base. The top half of the apparatus was separate but could be attached to the bottom giving an air-tight fit. Holes were drilled in the top section to coincide withthe cuvettes. This section could also be slided along slightly so that the holes would not coincide with the cuvettes (see Fig. 18).

The perspex housing can be placed in a holder and immersed

FIGS 18 19

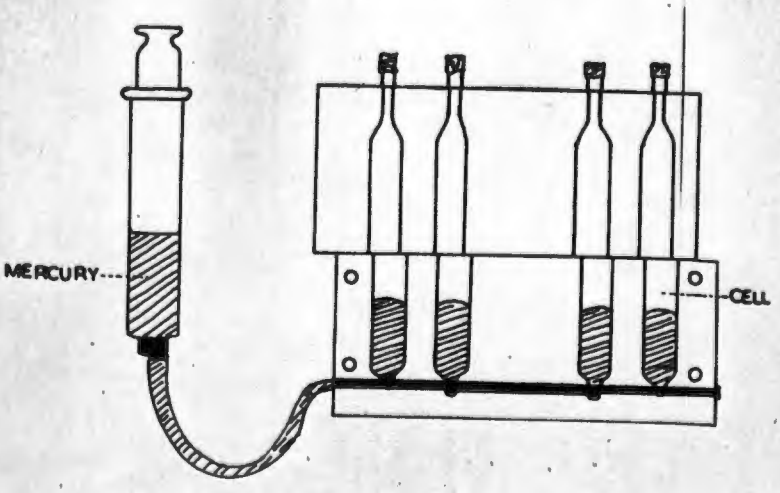
DIFFUSION LAY OUT

F



A: LAMP
B: FILTER
C: SCALE
D: DIFFUSION CELL
E: WATER BATH
F: CAMERA

DIFFUSION APPARATUS



in a water-bath to maintain temperature control. The water-bath is fitted with a thermostat and a circulatory pump, the water passing through a cottonwool filter to maintain clarity. The bath is also fitted with two diametrically opposed optically clear windows. Outside the one window is a mercury lamp placed about 1'4" away. The lamp projects its light through a filter, which transmits light of a wavelength not absorbed by the solution examined, then through the transparent scale, through the water-bath containing the diffusion cuvettes and out the other side. On this side is a camera placed about 5'7" away (see Fig. 19). The camera objective has a focal length of 170 cm. and is placed in such a position that the scale image has a magnification of about one.

Procedure. The protein solutions containing the minialbumin were made up in saline to a concentration of 0.6% (0.2% - 1.0%). A 1% sucrose solution is made up as also an isotonic saline solution. All solutions were de-aerated by applying negative pressure. This step is essential as these solutions are placed in the warm water-bath and gas bubbles liberated would interfere with the diffusion.

The diffusion apparatus and especially the cuvettes are thoroughly cleaned with warm water and teepol and well-dried. The top section is replaced and the mercury allowed to run into the cuvettes until they are just over half-full.

A pure saline solution is now placed, by means of a fine pasteur pipette, into one cuvette, a 1% sucrose solution into the second cuvette and the other two filled with a 0.6% protein solution. The cuvettes are filled until just fractionally past the junction between the top and bottom section. The top section is now slided across so that its holes do not coincide with the cuvettes. The excess solutions are now carefully extracted and the holes filled with saline. The apparatus is now ready for immersion into the waterbath at 37°C. However, the holes are still open to the atmosphere and to avoid the penetration of water, 12" glass-tubes are attached to the holes. The tubes are long enough toproject above the surface of the waterbath and so the interior of the diffusion chamber still remains open to atmospheric pressure. Temperature equilibrium is allowed to take place for $\frac{1}{2}$ hour and then the top section of the apparatus is slided across so that the holes coincide with the cuvettes; this brings the saline in contact with the various solutions in the diffusion cuvettes.

At this stage the interface between the liquids is at the interface of the perspex halves and examination is impossible. The mercury is thus slowly withdrawn from the cuvettes by means of a small electric motor which draws up the plunger of a glass syringe in which the mercury is contained. This causes the solutions in the cuvettes to fall, and consequently the interface drops down slowly into a region about half-way down the

cuvettes where the sides are optically clear and accurate photographs can be taken.

As mentioned, the first cuvette contains saline, which, being similar to the saline above the interface, does not diffuse and acts as a reference. The sucrose solution in cuvette No. 2 diffuses into the saline above it and acts as a standard of known diffusion rate and molecular weight. In the other two cuvettes the two unknown proteins diffuse, and the progress of the diffusion is followed by photographing the scale through the diffusing mixture and thus recording the scale-line deflections caused by the variations in concentrations around the diffusion interface.

Calculations.

The scale displacements obtained by photographing the readings of the distorted scale and a standard reference scale are read off the negative directly by means of a microcomparator. These scale displacements are obtained by subtracting the reference scale from the distorted protein scale. These values are plotted against the comparator readings of the distorted scale, i.e. the rate of change of refractive index with height was plotted against the height (see Fig. 20). The equation for this curve was developed by Weiner (1893)[123] and has the form

$$\frac{dn}{dx} = \frac{n_1 - n_o}{2\sqrt{\pi Dt}} \cdot e^{-x^2/4Dt}$$

where n is the refractive index of the solution,

n_o is the refractive index of the solvent,

D is the diffusion constant,

t is the time since diffusion started,

x is the distance of a point in the cell from the original boundary.

A derivation of the above equation for the calculation of D using the graph obtained as described above and utilizing the optical data in out set-up is

$$D = \frac{\mu^2}{2t} \left(\frac{l - b}{l}\right)^2$$

where μ equals the distance between the two inflexion points on the graph. This position of the inflexion points can be calculated by dividing the maximum height of the curve by $\sqrt{e}$ i.e. $\mu = \frac{H}{\sqrt{e}}$

l = the optical distance from the scale to the camera objective.

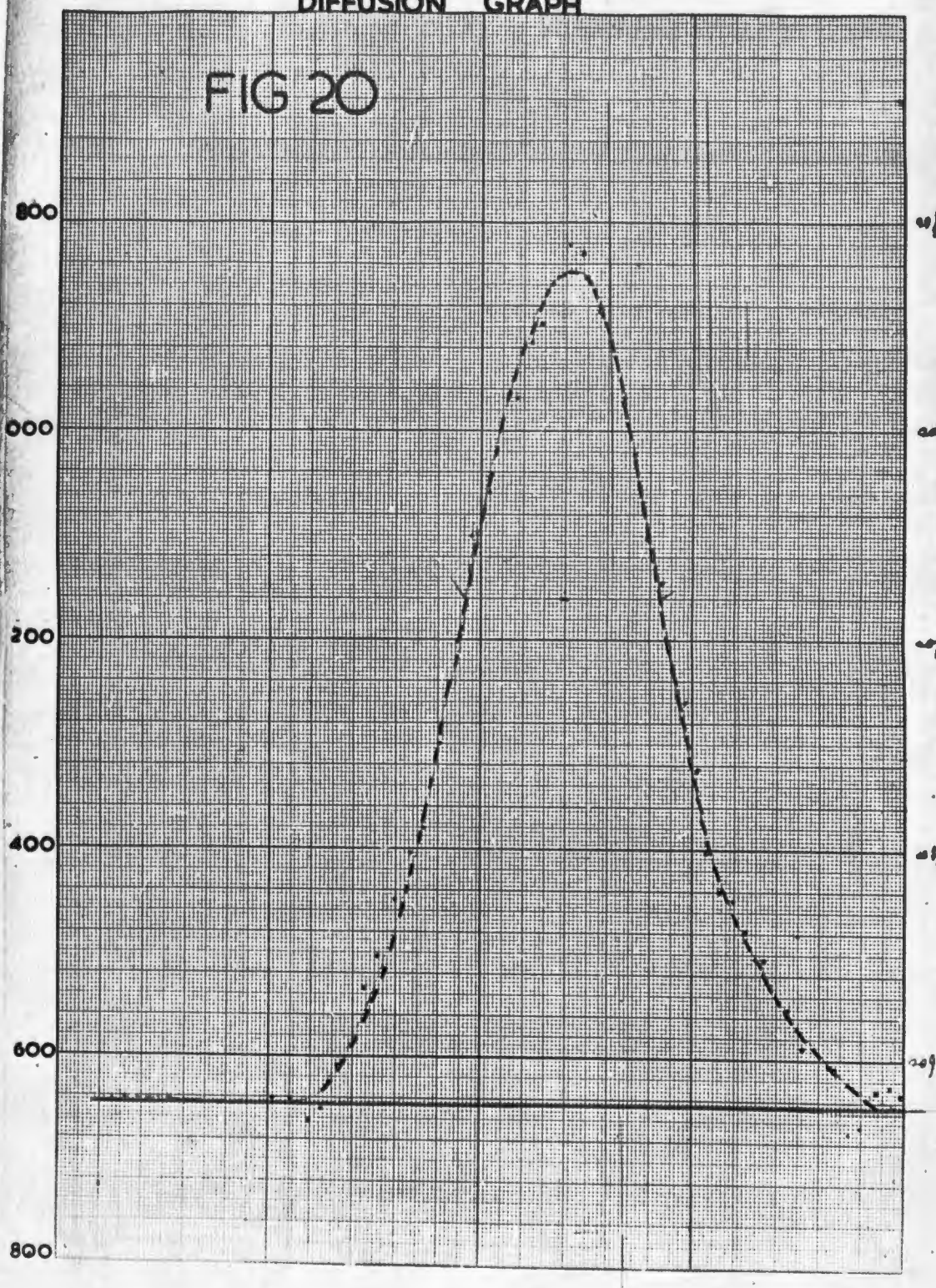
b = the optical distance from the scale to the centre of the diffusion cell and is frequently called the scale distance.

We calculated our constants from an identical formula, bringing in the effect of the photographic enlargement factor.

Thus $D = \frac{\mu^2}{2t} \left(\frac{1}{G} \quad \frac{l - b}{l}\right)^2$

DIFFUSION GRAPH

FIG 20



After drawing our graph and calculating $/u$, using the other constants as measured we calculated the diffusion constant of the minialbumin as follows:

$$D = \frac{(0.32)^2}{2t} \times \left\{ \frac{1}{\frac{640}{1000}} \times \frac{175.9}{240.5} \right\}^2$$

$$D = \frac{0.1024}{12,600} \times \left\{ \frac{1000}{640} \times \frac{175.9}{240.5} \right\}^2$$

$$D = 10.61 \times 10^7$$

The time is calculated in seconds starting at the commencement of diffusion until the photograph was taken. The optical distances are measured in centimetres.

As stated previously, a second physical constant, namely, the sedimentation constant is essential for accurate molecular weight determinations and this we measured next.

Ultracentrifugation and calculation of the sedimentation constant[124,125].

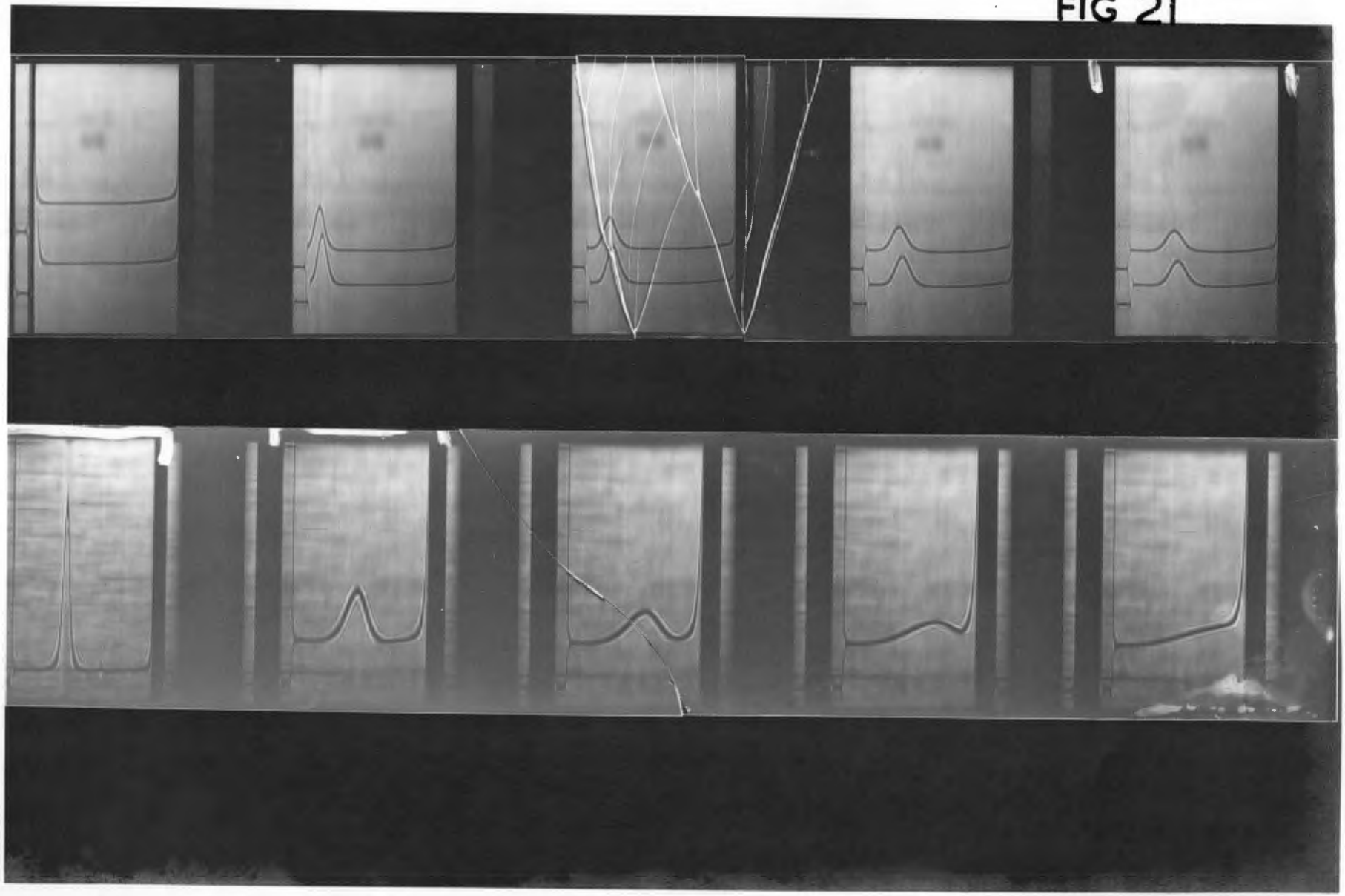
The sedimentation constant of the protein was determined using a Beckman Spinco analytical ultracentrifuge, Model E. The ultracentrifuge employs direct photographing method developed by Philpot (1938)[126] and Anderson (1939)[127]. According to their methods the concentration gradient curve is directly observed by suitable optical arrangements during the run. In this method

LEGEND TO FIGURE 21.

The upper series of ultracentrifuge curves represent the sedimentation of purified normal albumin prepared from Rabbit (top) and a Monkey (bottom).

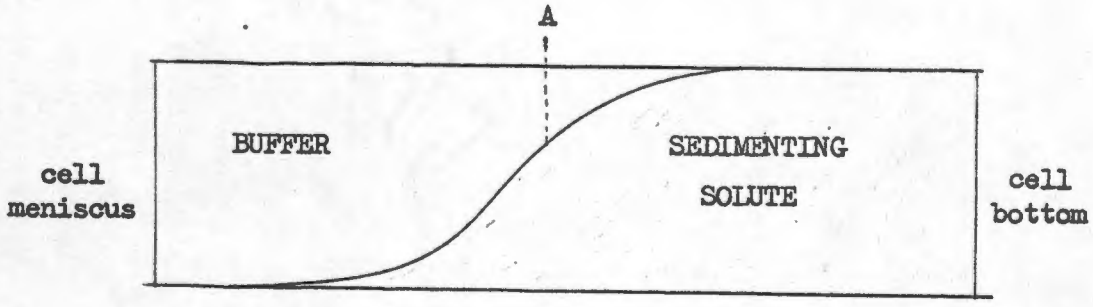
The lower series represent the sedimentation boundary of urinary minialbumin prepared from a cadmium-poisoned monkey. Due to the low-molecular nature of this protein a synthetic boundary cell was used in the ultracentrifuge.

FIG 21



the concentration gradient can be directly observed if light is used of such a wave-length that it is absorbed by the substance sedimenting.

A concentration curve can be drawn depicting the diffusion cell on its side:



direction of g

A unique system of lenses called the "Schliesen" system projects the sedimenting boundary as a peak. The amount of deviation of light passing through any level of the cell is directly proportional to the rate of change of the refractive index and this peak is formed corresponding to point A because the rate of change is greatest at the centre of the sedimenting boundary. Thus by measuring the rate of sedimentation by means of observing the movement of the peak in the cell and plotting this rate against time, we obtain a straight line whose slope indicates sedimentation velocity. (Schachman, 1953 [128] and Kegeles, 1951 [129])

The equation for sedimentation constant was developed by

incorporating the velocity data with data regarding angular spin or rotation (w) and time (t).

$$S = \frac{2.303 \; d \; \log \; x/dt}{t \; x \; w^2}$$

$$S = \frac{(\text{slope}) \; (2.303/60)}{t\left[(2\pi) \; (\frac{\text{r.p.m.}}{60})\right]^2} \qquad 2.$$

We used this formula to calculate our constants. Centrifuge speed was 59,100 r.p.m. and we photographed the peak at intervals of 10 minutes (see Figs. 21 (a) and (b)). Substituting the values into equation (2) we calculated the sedimentation constant as

$$S_{20} = 2.2 \times 10^{-13} \text{ cm./sec./dyne.}$$

Molecular weight determinations.

Using the physical constants calculated as in the previous section we applied the equation of Svedberg (1924),[130] to calculate the molecular weight of the minialbumin.

Svedberg (1929)[131] showed that when dealing with molecular weight (M), a partial specific volume (V) and a molar frictional coefficient (f) then

$$M \; (1 - VP) \; w^2 \; x = f \; \frac{dx}{dt} \qquad 1.$$

but f can be determined from the diffusion constant:

$$f = \frac{RT}{D} \qquad 2.$$

Thus combining equations 1 and 2 and remembering that

$$S = \frac{dx/dt}{w^2 x}$$

$$M = \frac{R\,T\,S}{D(1-V\rho)}$$

where ρ = density of the solvent, V is the partial specific volume of the protein and the other symbols are the same as before.

Calculation. R = gas constant = 8.314×10^7

T = absolute temperature = $293^\circ K$

S = sedimentation constant = 2.2×10^{-13} cm./sec./dyne

D = diffusion constant = 10.6×10^{-7} cm.^{2}/sec.

V = partial specific volume = 0.748 g./ml.

ρ = density of the medium = 1.0000.

Note: The partial specific volume of the minialbumin is taken as the same as that of normal serum albumin due to the fact that their amino acid compositions are so similar.

$$M = \frac{8.314 \times 10^7 \times 293 \times 2.2 \times 10^{-13}}{10.6 \times 10^{-7} (1 - 0.748 \times 1.000)}$$

$$= \frac{8.314 \times 293 \times 2.2 \times 10}{10.6 \times 0.252}$$

$$M = 20{,}060$$

Molecular weight = $\pm$ 20,000.

II 6 (viii) The carbohydrate and sialic acid content of the urinary albumins.

With the molecular weight data in hand we decided that before proceding with radio-active isotope labelling experiments on our urinary albumins, it would be necessary to ascertain whether the proteins had formed, or could form, aggregates with material of a carbohydrate nature. Although our amino-acid determinations suggested otherwise, it could well happen that our minialbumin molecule had attached to it amino sugars of the character of neuraminic acid or its derivative sialic acid.

It is well-known that sialic acids are found widely distributed throughout the animal kingdom, especially as components of mucoproteins present in biological fluids such as blood, milk, mucins etc. and also in certain mucopolysaccharides (Zilliken, 1958).[130, 131]

The sialic acids and neuraminic acid itself, are aminocarboxy-octuloses and so may be regarded both as polyhydroxy- -amino acids and as ketoses. Therefore, these substances are of interest as they are able, theoretically at least, to combine with other sugars through glycosidic linkages and with other amino acids through peptide bonds, the latter case probably being responsible for the formation of mucoproteins. (Kent and Whitehouse, 1955) [132]

Chemically neuraminic acid may be considered as an aldol

condensation product of pyruvic acid with either D-glucosamine or D-mannosamine. The sialic acids are N- and O-acyl derivatives of neuraminic acid and can be obtained from material sources by very mild isolation procedures.

The acids were first obtained and fully characterized as components of the mucoprotein in the submaxillary mucins from sheep, hogs, horses and cattle respectively. They were accordingly named sialic acids (Greek σιαλ os - spittle) and it was from human saliva that we extracted sialic acids for our control experiments. The species source is apparently immaterial as N-glycolylneuraminic acid has been isolated from all above-mentioned species (Blix et al, 1955).[133]

In order to cover the range of products which could perhaps be adsorbed to the urinary albumins, we carried out experiments to test for the presence of neuraminic acid, sialic acid, hexoses and pentoses and for bound carbohydrates. We deduced that a calculated amount of under 4% of bound sugars or derivatives would not appreciably effect the molecular weight determinations.

The methods of estimation overlap somewhat and we decided to combine certain methods in order to determine concentrations of at least two of the substances at once. This cut down extensively on methodology and reagent variety. Also, the fact that we were interested, at this stage, more in the quantity of bound compounds than in their precise chemical structure.

Assay of neuraminic acid and sialic acid.[134, 135]

Neuraminic acid and its N-substituted derivatives, the sialic acids (Blix, Gottschalk and Klenk, 1957),[136] have previously been detected and measured by several methods using reagents such as orcinol, resorcinol and Ehrlich (p-dimethylaminobenzaldehyde). Most of these are modifications of assays which had been previously used for the measurement of other carbohydrates and are still relatively insensitive and in general, have a low specificity. Svennerholm (1958)[137] has succeeded in eliminating interfering material by passing tissue hydrolysates through Dowex-2-acetate columns and thereby partially purifying the sialic acids before measurement. This method, although reliable, is cumbersome and time-consuming.

The use of thiobarbituric acid for the assay of sialic acid and neuraminic acid has given us a method which is not only sensitive but highly specific and the method is very manageable. Although sialic acids are 2-keto-3-deoxy sugar acids, the amino group is always substituted and should not be reactive in the thiobarbituric acid assays. In fact, early workers (Weissbach and Hurwitz, 1959)[138] could only obtain a small amount of colour development with this method. If, however, the periodate oxidation is carried out in strong acid solution and the final coloured solution is extracted into cyclohexanone (Warren, 1959), then very satisfactory recoveries are obtained and the molar extinction coefficient obtained is 12 times higher than the resorcinol method.

Reagents.

1. Sodium periodate (meta) 0.2 M in 9 M phosphoric acid.
2. Sodium arsenite, 10% w/v in a solution of 0.5 M sodium sulphate-0.1 N H_2SO_4.
3. Thiobarbituric acid (B.D.H.) 0.6% in a solution of 0.5 M sodium sulphate.

All these reagent are not readily soluble and must be prepared with warming. They are, however, stable at room temperature for over a month.

Procedure. Since the thiobarbituric acid assay measured only free sialic acid, the protein solutions were heated at 80°C for 1 hour in 0.1 N H_2SO_4. This procedure is known to release bound sialic acid (Warren, 1959)[139] with degradation.

To a sample, containing up to 15 /ug of sialic acid or neuraminic acid, per 0.2 ml., is added 0.1 ml. of the periodate solution. The tubes are shaken and allowed to stand at room temperature for 20 minutes. One ml. of the sodium arsenite solution is added to each tube which are then shaken until the yellow-brown colour disappears. The thiobarbituric acid solution is now added, 3 ml., the tubes shaken, capped with a glass marble and heated in a vigorously boiling waterbath in cold water for 5 minutes. 4.3 ml. of cyclohexanone is added, the colour is extracted by shaking and the tubes centrifuged for 5 minutes.

The clear upper organic phase is red and the colour is more intense than when in water. The optical density is determined in a Beckman, Model D.U. spectrophotometer at 550 mμ. The procedure is also carried out on 0.2 ml. water for the blank determination, and the readings are made against this solution.

In order to have a protein containing sialic acid, we used a mucoprotein solution prepared by precipitating the proteins in 5 ml. salivary mucus with T.C.A. and redissolving the proteins in very dilute hydrochloric acid (Blix, et al, 1955).[133] This dissolved mucin can be precipitated again by dilution with water, it is then washed with water and dried by washing with ethanol, then ether.

One gram of dried mucin can then be suspended in 10 ml. of water and gently warmed with constant shaking for 30 minutes. The mixture is then heated for one hour in a boiling bath with constant shaking to effect hydrolysis. This solution is now heated in a similar way as our protein solutions.

We obtained a sialic acid concentration from the Nutritional Biochemical Corporation and this contained 18.5% sialic acid. By making serial dilutions from 1 mg. - 18 mg.%, we obtained a linear relationship between optical density and concentration up to the latter figure.

Results. Our urinary albumins contained an average of 2.1 mg.% calculated by simple proportion from the linear relationship of

the standard. The two check solutions containing mucin prepared as described above and a mucoprotein prepared in similar fashion from beef liver contained 55 mg.% and 45 mg.% neuraminic acid and derivatives. The latter value compares favourably with figures published by Warren.[139]

Carbohydrate and hexose content.

The determination of these substances bound to protein can be carried out simultaneously due to the fact that the protein-carbohydrate complex can be hydrolysed producing sugars formed from the carbohydrate. The total hexose content of the resultant solution can then be estimated (Schultze et al, 1957).[140]

The principle on which nearly all hexose, and for that matter, pentose estimations hinge, is the fact that when these sugars are brought into solution in an acidic solution, they tend to cyclise to form a ringed compound. This compound is either furfuraldehyde or a close derivative and will react readily with multi-hydroxy benzine compounds to form a colour which can then be colorimetrically measured. Using acids of different dissociation constants and strengths, and gauging the time taken for colour formation, these sugars can be individually identified. But by using a strong acid and heating the reaction mixture the total protein bound hexoses can be determined.

Reagents.

1. 95% ethanol.
2. Orcinol - H_2SO_4 reagent: 7.5 volumes of reagent A mixed fresh daily with 1 volume of reagent B.

   Reagent A: 60 ml. of concentrated H_2SO_4 and 40 ml. of water.

   Reagent B: 1.6 g. of orcinol in 100 ml. of water.
3. 0.2 mg./ml. galactose-mannose standard.

This standard has been used since the best available evidence suggests that on the whole the serum glycoproteins contain only these two hexoses in approximately equal amounts (Shetlar, Foster, and Everett, 1948)[141]; (Friedmann, 1949).[142]

Sialic acid does not give an orcinol reaction and, therefore, our two estimations do not overlap.

Hydrolysis. 0.5 ml. of the protein solution is added to 3 ml. of 3 N H_2SO_4 contained in a vial. The vial is sealed and the mixture is hydrolysed for 8 hours at 100°C in a sand-bath.

Procedure. 1 ml. of the protein hydrolysate, 1 ml. of water and 1 ml. of the standard are heated in similar fashion. Add 8.5 ml. of the orcinol-H_2SO_4 reagent to each tube and mix well by repeated inversion. The tubes are now capped with glass marbles and placed in a waterbath at 80°C for 15 minutes. The tubes are cooled in tap water and read in a colorimeter and either 540 m/u or 420 m/u.

The sensitivity of the orcinol reaction is greater at 420 m/u,

and although the differences between different hexoses are more marked at this wave-length, we were interested in the total value and so used a 420 mμ violet filter for our estimations.

Again a mucoprotein preparation from saliva was used as a yardstick.

Results. The hexose content of the normal molecular weight albumin in the urine was:

Protein concentration: 200 mg.% = 2 mg./ml.

Hexose content: 0.0645 mg./ml.

$$\% \text{ hexose} = \frac{0.0645}{2} \times \frac{100}{1} = 3.2\% \text{ hexose.}$$

The hexose content of the low-molecular albumin component was:

Protein concentration: 71 mg./26 ml. = 2.7 mg./ml.

Hexose content: 0.0618 mg./ml.

$$\% \text{ hexose} = \frac{0.0618}{2.7} \times \frac{100}{1} = 2.3\% \text{ hexose.}$$

Comments: Optical densities are linear with carbohydrate up to a concentration of 500 μg. of galactose-mannose. Protein does not interfere with the determination unless the protein is coloured and has a low carbohydrate content. In such cases, correction can be made by preparing a protein blank without orcinol and subtracting this blank from the protein reading.

The reproducibility of the above procedure is satisfactory, and good duplication is obtained when the same sample is analysed on different days.

Conclusions.

The contribution to the molecular weight of the protein by bound hexose and sialic acid does not appear from these observations to be significantly greater in minialbumin than has been reported for normal serum albumin.

II 7. RADIOACTIVITY EXPERIMENTS

II 7 (i) Application of tracer studies to biological experiments.

The use of tracer techniques for the isolation and study of metabolic pathways was most suitable in the case of the synthesis and excretion of the urinary albumins produced in our cadmium intoxicated animals.

By feeding the stable isotope of nitrogen, N^{15}, in the form of labelled L-amino acids to animals for instance, and then isolating amino acids from different tissue proteins, the fate of unexcreted N^{15} can be followed. Analysis of the proteins reveals that the major portion of unexcreted N^{15} is incorporated in them and isolation of the individual amino acids shows that N^{15} is widely distributed in them with the highest isotope concentration, however, being found in the amino acid being fed. (Shonheimer, Ratner and Rittenberg, 1939[143] and Ratner, Rittenberg, Keston and Shonheimer, 1940[144]).

Later studies involved labelling amino acids with the unstable isotope of carbon, carbon-14 and resultant tracing of the C-moiety metabolism by its radioactivity. For such applications, it is an essential requisite that we know the rate of decomposition of the radioactive element being used in the experiment. The rate of nuclear fission and in the case of carbon-14 the rate of radio-

active decay, as with a great number of other unstable isotopes, have been accurately established.

The rate of decay is a physical constant, which can be expressed in terms of the period of time in which the radioactivity of the isotope would fall to one-half of its value - the half-life of the isotope. C^{14} is a β-emitter with a half-life of 5,700 years. Its long half-life makes it a very valuable tool for metabolic tracing.

II 7 (ii) Concepts of Metabolic Pool and Turnover Rates.

If a radioactively labelled amino acid such as C^{14} lysine or C^{14} glycine is introduced into the body of an animal by a single pulse injection, by repeated pulses, or by continuous infusion or feeding, it mixes throughout the body with chemically identical molecules built up of stable C^{12} atoms. The radioactivity of the compound is thus diluted in the body: the extent of the dilution being determined by the size of the "miscible pool" present already in the animal.

At first, calculations of isotope dilution were made to give a general miscible pool of a compound but latterly, it has been realised that for many or perhaps most metabolites, this is an oversimplified concept. A single metabolite such as glucose, bilirubin, uric acid or amino acid or even proteins such as serum albumin, insulin and other protein hormones, may enter or

leave a number of pools of its own molecules throughout the body of varying extent. In fact, there are a considerable number of metabolic compartments for any individual compound in the cells and tissues of a living organism; and these may vary greatly in size. Not only is the miscible pool subdivided into compartments of different size in a living animal, but the metabolic stability of the substance throughout the body may not be a constant and invariable function (Sprenson, 1949).[145] In some cells, the metabolite may be relatively stable and passive, others it may be in the process of continuous change - incorporation intact into larger molecules, or altered to others of similar size or degraded to smaller fragments, all at rapid and varying rates. In a steady state, for a particular metabolite in a compartment such as blood plasma, the rate at which the compound enters that body plasma is quantitatively equal to the rate at which it passes out. In the case of serum albumin, in a normal person the main factors concerned are the rate of synthesis in the liver and the rate of degradation of the serum albumin to its constituent amino acids, in, at present, unknown sites, but this again may take place mainly in the cells of the liver.

If one infuses homologous C^{14}-labelled serum albumin into an animal, the initial high radioactivity of circulating albumin after complete mixing with the pulse dose falls off exponeratially, due to the summation of two processes, degradation of the high radioactive albumin molecules and dilution of the remaining ones

with newly formed non-labelled molecules. The time which elapses before the radioactivity of the serum albumin has fallen to one-half of the original value, is known as the biological half-life of serum albumin and gives a measure of the turnover rate of the protein. This so-called value is in the range 10 - 14 days, depending on the nutritional status of the animal, being longer on low protein diets (Hansen)[50] and relatively shorter on high protein intakes, and in hypercatabolic states as for example in fevers, in which the turnover rate of body proteins as a whole is increased. (Cohen et al, 1961)[146] The biological half-life of γ globulins is approximately 21 days. (Cohen and Hansen, 1962 [147] and Gitlin et al, 1956 [148, 149]).

If an amino acid labelled with C^{14} is administered to an animal or person, its incorporation into the various body proteins can be followed, as also, catabolism of the proteins. In this manner protein turnover studies and protein half-life determinations can be carried out. Oral administration of the "tagged" compound is obviously the easiest mode of dosage, but then the additional complications of intestinal-absorption equilibria makes interpretation of experimental findings difficult. The advantages of intravenous administration of amino acid are obvious and were made use of in our experiments.

Another consideration is that the compound utilized should be maximally incorporated into a synthetic pathway without losing

its identity or participating in numerous other pathways which makes tracking difficult. For this reason, we chose L-lysine as our labelled amino acid to be injected and its incorporation into serum and urinary albumins traced. This amino acid is essential for optimal body growth and has the advantage that its participation in reversible transamination reactions is minimal. The D-isomer is not utilized by the body, probably because of the non-convertibility of the keto-acid to the amino acid. The fact that transamination reactions are at a minimum, leads to maximum incorporation of actual L-lysine into the synthesizing protein chain. (Norris and Inghram, 1946)[150]

The idea behind the radioactive studies would be to trace the incorporation of the L-lysine into normal serum albumin and into the low molecular weight albumin produced by the cadmium intoxicated monkey. The manner of synthesis and turnover rates of the low-molecular component could be measured as well as the possibility of any precursor relationships with normal serum albumin.

II 7 (iii) Measurement of specific radioactivity of serum and urinary albumins in cadmium poisoned monkey.

The monkey to be the subject of the experiment was brought into a state of chronic cadmium intoxication by twice-weekly intravenous injections of cadmium chloride solution. After

about two months, gross and persistent proteinuria was evident, and the animal was considered as ready for radioactive isotope administration.

Isotope source and description.

For reasons given earlier, we decided to administer L-lysine suitably labelled in an intravenous injection. The radioactive amino acid was purchased from the Radio Chemical Centre at Amersham. The specimen consisted of two ampoules, each containing 0.5 milliCurie of L-lysine monohydrochloride universally labelled with carbon-14. The level of radioactivity was 6.1 mC per millimole and the identification was Batch 61, and catalogue reference CFB. 15.

Administration.

The weight of the monkey just prior to the injection was 5 kilograms, with a calculated blood volume of 350 ml. The normal serum albumin concentration was 3.5 g./100 ml., giving a total of about 12.25 g. of albumin in the circulation. Taking the urinary excretion into consideration as well as the separation of the urinary albumins into two fractions, we calculated that to obtain sufficient labelling of the urinary albumins for optimal detection of the C^{14} we would have to inject the whole milliCurie into the animal. (Marinelli, 1942)[151]

The monkey was not anaesthetized for the injection, but was

maintained motionless by holding it against the side of the cage. Its left leg was brought through a gap in the cage and the calf muscle, which had previously been shaved for the cadmium injections, was sterilized with 70% aqueous ethanol.

The ampoules containing the radioactive L-lysine were then filed open and the contents dissolved, and transferred to a small sterile beaker using a pasteur pipette and sterile isotonic saline. A total of 5 ml. of saline was used to transfer both lots of amino acid into the beaker. With a sterile plastic syringe fitted with a G-21 needle and medium bevel the solution was drawn up and injected over a period of two minutes into the short saphenous vein. The needle was then withdrawn and the wound covered with cottonwool and Savlon and fixed with adhesive plaster.

Collection of specimens.

(i) Urine. The injection had been given at 10 a.m. and the monkey in its cage was placed immediately on a rack over a stainless steel tray. Each individual specimen of urine passed was collected directly; the exact time and volume being noted, and the sample either processed immediately or stored in a deep-freeze. After each urine specimen was passed, the cage was placed over a second stainless steel tray. The urine was then poured out of the first tray into a polythene bottle, capacity 250 ml. A minimum ($\pm$ 5 ml.) of distilled water was then used to wash the section of the tray on which urine had been passed.

(Monkeys in general only secrete a small volume of urine at any one time, seldom totalling over 100 ml. per day). These washings were also collected. The tray was then thoroughly cleaned ready for use again. In this manner contamination between samples was completely eradicated.

Urinary specimens were collected immediately on passing for the first 48 hours and thereafter daily for three weeks. A total of 27 samples were collected during this period.

(ii) Serum. It had previously been decided that due to the very rapid synthesis of albumin, blood specimens would have to be taken as frequently as possible during the initial 24 hours. Times of 2 hrs., 5 hrs., 8 hrs., 14 hrs., 20 hrs., and 28 hrs. were selected. More frequent sampling would necessitate too many intravenous injections and perhaps causing veinous thrombosis. Blood was drawn from alternate short saphenous veins starting low down near the ankle and working upwards.

The blood, after being taken, was ejected from the syringe into a 5 ml. plastic centrifuge tube. The syringe was discarded and the blood in the tube allowed to clot for one hour at 37°C. The blood was then centrifuged at 4,000 r.p.m. for 15 minutes and the clear serum removed with a pasteur pipette, and placed in small glass test-tubes. The samples were either processed straight away or stored in a deep-freeze. A total of 26 samples were collected.

Treatment of the samples.

All the urinary specimens were examined in the same way. A said batch of 8 samples was first concentrated by pervaporation, with intermittent dialysis against distilled water to avoid high concentrations of electrolytes and urea developing. After concentration to approximately $\frac{1}{3}$ of its original volume, the specimens were placed in centrifuge tubes and an equal volume of 10% w/v trichloracetic acid was added. The protein precipitate which formed was then spun down in a refrigerated centrifuge at $0^{\circ}C$.

Albumin extraction was then performed on each sample following the method of Debro et al described earlier. The acetone solution of albumin was dialysed in boiled visking tubing to obtain an aqueous solution, and this solution was finally concentrated by pervaporation to about 5 ml. Albumin concentration was assayed by the biuret method.

Each urinary albumin sample was then passed through a column of Sephadex G 75, thus separating protein molecules below $\pm$ 25,000 molecular weight from those above $\pm$ 50,000 molecular weight. In this way the albumins were separated into two fractions in all our urinary specimens. These proteins would also now be labelled with C^{14}, and we intended to monitor the radioactivity.

The serum specimens were tested in a similar fashion as the

urine, except that no concentration was necessary prior to protein precipitation. The serum albumin was prepared and subsequent preparations were carried out for radioactive monitoring.

As all our specimens were labelled with carbon-14 the monitoring of the specimens necessitated the detection of β-particles which are emitted from C^{14} and equipment for their detection would be utilized.

Detection of β-particles. (Bethe, 1947)[152]

β-emission is a complicated process in which the potential energy is discharged by simultaneous creation of a β-particle and a neutrino. The β-particle as mentioned earlier is a high speed electron which can have energy from zero up to the maximum energy (E max) characteristic of the decay energy of the parent nuclide, which may be several MeV. In the case of C^{14} E max is 0.15 MeV. The neutrino does not cause detectable ionization in matter. (Wollan, 1947)[153]

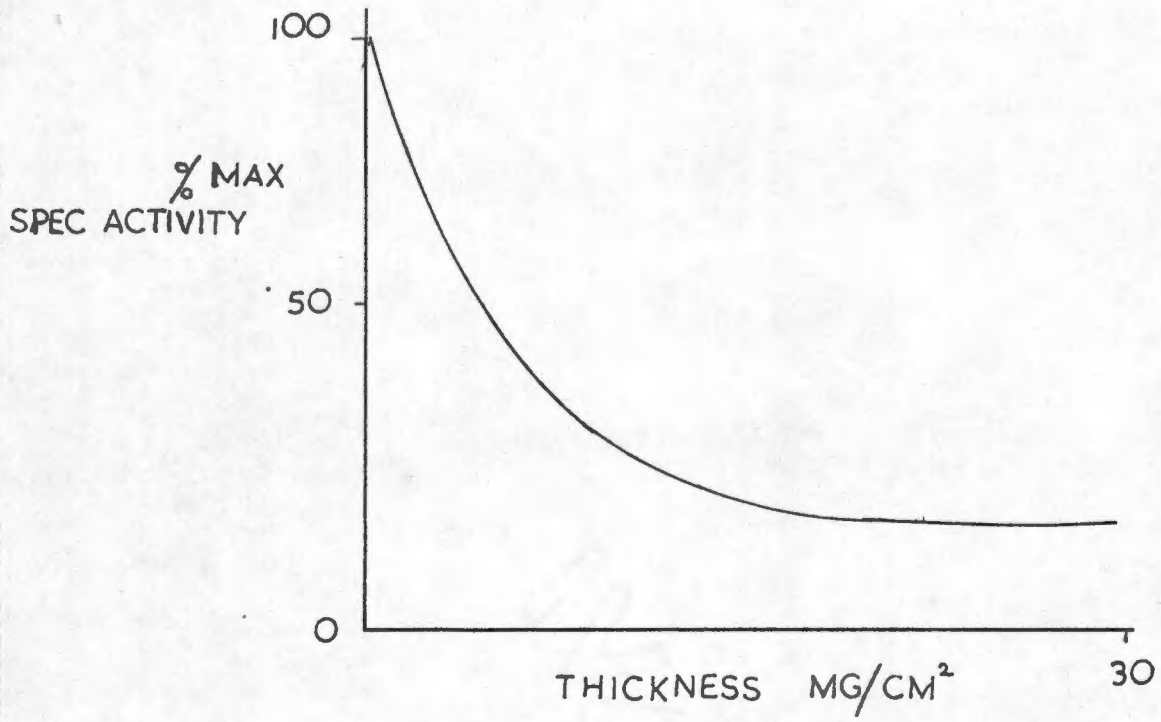
The quantitative determination of radioactivity is based on the ionization or excitation of matter by the radiations emitted by the radioactive compounds. Until 1950, nearly all assay equipment was designed for observation of ionization in gases. The classic example of this type was the Geiger-Müller counter. (Geiger and Müller, 1928)[154, 155] Others were the proportional counter and various kinds of ionization chambers i.e. electroscopes

and electrometers. Since 1950 a new detector, the scintillation counter, which measured the interaction of radiation with either solid or liquid media, has been developed. This detector now challenges the dominant position of apparatus dependent on collection and detection of ions in gases.

Determination of Radioactivity in Serum Albumin.

Our initial experiments on the activity of serum albumin in the cadmium-poisoned monkey were carried out using a G-M counter. Under normal conditions, starting with material of a given specific activity, the source strength measured should increase linearly as the amount of material is increased. Actually, as the thickness of the sample increases, radiations from the lower layers begin to be lost by absorption in the sample material itself. Eventually the sample is so thick that only the top layers contribute to the assay; this sample is then being assayed at "infinite thickness". Thus, if a source radioactivity is plotted against source thickness, the result is not a straight line but a curve that approaches a limiting value. As it is much simpler to prepare planchettes at infinite thickness than at infinite thinness, we prepared our serum albumin samples in the following way. (see Fig. 22)

FIG. 22.



Plating of albumin samples for radioactive assay in a Geiger-Müller counter.

The albumin preparations were precipitated out of their aqueous phase until 10% w/v T.C.A. The precipitate was washed twice in a centrifuge with 5% w/v T.C.A. It is very important to obtain absolutely dry protein for counting purposes as water molecules can absorb much of the -particles emitted.

The precipitate was therefore washed with 95% ethanol and then in a mixture of ethanol, ether and chloroform (2:2:1 v/v). This dehydrated the sample adequately. Finally, the precipitate was suspended in pure diethyl ether and plated onto a small filter paper disc in a modified Buchner funnel.

Prior to the plating, the planchettes, small stainless steel or plastic dishes 1.5 cm. in diameter and containing a filter paper disc (Whatman 541) were weighed. After plating the filter paper discs were removed from the Buchner funnel and replaced in their respective planchettes. These were reweighed to five decimal places and the exact weight of protein determined. The area of the planchette was known and therefore mg. protein/cm^2 area can be calculated (Karmen, 1957).[156] In other words, radioactivity per gram material can easily be calculated as no absorption corrections need be made when using infinitely thick samples, and the value of the ordinate at saturation thickness depends only on the specific activity of the sample.

Actually, after determining the specific activity of the serum albumin samples, we discovered that a series of incongruous results were due to a faulty Geiger-Müller tube, and as we had a liquid scintillation counter (Packard Model 314 EX Tri-carb) at our disposal it was decided to use this instrument. The advantages of scintillation are its efficiency, sensitivity and the ease of sample preparation.

The Scintillation counter.

Introduction. Whereas the G-M type of counter depends on the removal of electrons from atoms or molecules in the gaseous state, the scintillation detector depends on the formation of excited states in which the electrons are retained in the atoms

or molecules. When the excited electrons return to the unexcited or "ground" state, radiation is emitted in the form of light quanta. (James R. Arnold, 1958)[157]

Hence a very important component in a scintillating system is the phosphor or scintillator itself. This substance converts the β-particle energy into light quanta. There are many such substances, the chief requirement being that they must be transparent to their own radiation. Hence, the use of those materials which can be grown as large clear crystals e.g. zinc sulphide or sodium iodide. Usually phosphorescence yields are greatly increased by incorporating a little of a special impurity into such crystals. Thus, sodium iodide crystals may contain a small amount of thallium, and this combindation became very widely used. Subsequential discoveries have, however, brought into use organic scintillators, and, nowadays, there is in use a series of two scintillators, namely primary scintillator and secondary scintillator, the latter acts to shift the wave-length of the light emitted by the primary scintillator to a region where the photomultipliers are more sensitive.

Theory of operation. Having discussed the scintillator itself, the next most important consideration is the solvent in which the radioactive matter plus the scintillator must be dissolved. The primary requisite of the solvent is that it

should permit an efficient transfer of energy from the β-particle to the scintillator and also allow the light emitted from the scintillator to be transmitted with very little absorption. If the solvent possesses these qualities then it does not "quench". There are instances where no suitable solvent can be found. In many of these cases, the material in question can be ground very finely or precipitated and then suspended in the solvent by means of a gel. This technique will be discussed later in the section on the radioactive assay of liver extracts.

Pulse Height analysis. The fundamental assumption underlying the pulse height analysis system of the scintillating counter is that pulses at the amplifier outputs are proportional in height (voltage) to the energy of the β -particles which created them. This is because the amplifiers and preamplifiers are linear devices and the light energy emitted by the scintillator is proportional to the β -particle energy and the photomultipliers produce electrical pulses proportional to the light energy incident on the photocell (Erdtmann, von G. and Herrman, 1960).[158]

Operation. As mentioned earlier we planned to carry out a reanalysis of the isolated serum albumin, monitoring for activity in the scintillation counter.

As these albumins had already been precipitated in planchettes for use in the G-M technique, the following method was followed:

Reagents.

1. Hydroxide of hyamine 10 x (1 Molar in methanol)
2. Analar Toluene
3. PPO (2, 5-diphenyloxazole)
4. POPOP (1,4-bis-2-(5-phenyloxazolyl)-Benzene.

The latter two reagents are both scintillation grade and produced by the Packard Instrument Company, Illinois, U.S.A.

Method. Each protein specimen was weighed out on a micro-balance, the specimen being placed on a preweighed piece of aluminium foil and the exact weight to three decimal places taken. The weights among the 24 specimens varied from between 1 mg. and 8 mg.

The weighed samples were now placed, with the foil, into 25 ml. glass vials which had screw-top plastic lids. The vials are made of special low-potassium glass. All glasses contain K^{40} in varying amounts and this has been found to constitute a significant part of the counting background. Since, for a given concentration of K^{40}, the background will be lower where the vial is smaller, a vial as small as practically possible was used. The sides of the vial should not be extremely thick and should possess uniform optical transmission. (Arnold, 1954[159] and Agranoff, 1957[160]).

Lately, low-background polythelene vials have been purchased,

and if available should be used.

The protein was dissolved in 1 ml. hydroxide of hyamine; (Passman, 1956[161] and Eisenberg, 1958[162]) this took overnight in most cases. 15 ml. of the scintillation mixture was then added, the mixture being made up in the following way: 3 g. PPO plus 0.3 g. POPOP dissolved in 1 l. of toluene. The resultant mixture has a clear irridescent light violet colour.

The 24 vials were then placed in the refrigerated sample holder prior to the operation. At this stage the glass vials must be completely free of any labels or lettering which could interfere with the emission of light.

Internal machine standard and machine setting. The particular machine used by us, i.e. the Packard Tri-carb Scintillating Counter Model 314 EX, could be operated using two channels picking up two different sets of pulse heights. We, using only one isotope, were only interested in one channel, the so-called "Red Scaler" and adjusted the window on this one scaler for optimal efficiency.

In order to check the machine initially and also for daily standardization, a Tri-carb Standard containing C^{14} in toluene with PPO and POPOP added was used. Each vial was individually assayed at maximum efficiency and the assay was furnished. Thus, optimal operating conditions and voltage and window settings could

easily be established. Apart from this standard we also put through daily one of our samples from the previous day in case of fluctuations in the readings.

After setting the machine, the samples in the refrigerator were allowed to pass through for counting. This was a completely automatic procedure and the results were delivered by an automatic printer. Thus, at the conclusion of counting each sample, the printer recorded the sample number, the length of time the sample was counted and the count on each scaler.

Efficiency of the machine. This instrument obviously counted optimally at a certain number of counts per minute, thus when weighing out samples one tried to weigh out more material if it was thought that the counts per mg. would be low. The time of counting was also selected carefully in order to obtain the optimum number of counts per sample. In this way, maximum efficiency was obtained as well as the standard deviation of the counts being as small as possible: due to the fact that

$$S.D. = \frac{1}{\sqrt{counts}}$$

where S.D. = standard deviation.

It is not advisable to have samples giving too low a total count.

In this way the radioactivity of the C^{14} incorporated into serum albumin over a period of three weeks, after a single intra-

venous injection of C^{14}-L-lysine, was determined.

Assay of radioactivity in urinary albumin specimens.

For the determination of the C^{14} incorporation into the urinary albumins, we decided that it would be far more efficient and streamlined to use a counting technique which could assay samples in their liquid form. This would eliminate the protein precipitation procedure involved in the previous radioactive assay on serum albumin.

As described previously, the urinary albumins were prepared by the usual T.C.A. precipitation and redissolution into acid acetone. The albumins were then separated into low and high molecular components on dextran gel and the components concentrated by pervaporation in visking tubing.

Sample preparation.

The protein concentration of each urinary albumin sample - 48 in all, consisting of 24 each of normal and of low molecular weight - was determined both by the biuret method and by micro-Kjeldahl technique.

Aqueous aliquots were then taken from each specimen and the exact protein concentration recorded. Quantities of protein were again taken such that optimal counts were obtained throughout the assay.

Scintillator for aqueous solutions.

The reagent employed for liquids was slightly different from that used previously on dry protein materials and was prepared in the following manner:

In a 500 ml. reagent bottle were placed 400 ml. dioxan (analar) and in this were dissolved 48.9 g. of finely ground naphthalene. To this solution was added 2.8 g. of PPO and 20 mg. of POPOP. Solution was allowed to take place over several hours and the solution then filtered.

Monitoring of samples.

A portion of each protein sample, which had previously been prepared as described earlier was then added to a clean, dried counting vial. The amount taken was dependent on the protein concentration, which would then be proportional to the radio-activity. Volumes of the aqueous solutions, varying between 0.5 ml. and 3 ml. were used containing between 0.5 mg. and 2 mg. of albumin.

To each vial was then added 15 ml. of the scintillating mixture. The total solution remained pale violet in colour as well as optically clear. The problem of pipetting this volatile and nauseating scintillating solution was solved by using a 15 ml. automatic syringe. This device was very accurate and eliminated human pipetting errors satisfactorily.

The vials were then allowed to cool in the refrigerated portion of the counter prior to being monitored, and the machine operated as previously described. Again, internal standards as well as samples that had previously been run, were put through the counter in order to check our results.

Check on quenching of the aqueous protein solutions.

Protein solutions in general tend to absorb the β-decay from the C^{14} incorporated in them. The water itself is also an extremely potent electron absorber or "quencher", this latter fact being one of the main drawbacks in the monitoring of aqueous solutions.

Thus, to determine the amount of quenching in a series of samples, a known amount of radioactive C^{14} was added to selected samples and the percentage recovery was determined. This experimentally checked the amount of radioactivity being lost during the actual counting procedure.

Procedure. To selected samples, say two in a run of twenty samples, presuming that each sample was prepared in a similar fashion, 0.1 ml. of hexadecane-C^{14} was added. The hexadecane solution had a known radioactive count of 2.64×10^4 disintegrations per ml. per minute. Therefore, in adding 0.1 ml. of hexadecane to a sample a total of 2,640 counts could be expected per minute. We counted for a total of 10 minutes per sample in

order to bring the counts into the optimal range, and thus expected 26,400 counts per 10 minutes.

The return obtained from the samples was in the region of 17,500 counts, giving an efficiency of slightly under 66%, which meant that quenching could not rule out the validity of the counting experiments.

II 7 (iv) Possible adsorption of low-molecular albumin to normal albumin.

In order to eliminate the possibility of the minialbumin being adsorbed on to normal albumin to a degree which could give us erroneous radioactivity results we carried out the following experiment.

Method. 2.5 mg. of low-molecular weight urinary albumin prepared from the cadmium-intoxicated monkey following the injection of C^{14}-L-lysine and therefore labelled with C^{14}, were added to a solution of monkey normal serum albumin containing 7.5 mg. of protein, the latter being unlabelled.

An aliquot of these two solutions was counted for radioactivity before mixing, following the usual procedure, they were then mixed and allowed to stand for five hours in a 37°C waterbath. The two albumins were then separated on Sephadex G 75 and the two peaks appearing on the "Uvicord" ultraviolet scanner were collected,

concentrated in boiled dialysis tubing and a similar aliquot monitored for radioactivity.

Results.	Counts before	Counts after separation.
Low M.W.	8452	>8452
Normal M.W.	1723	1637

Background count was 1717 c.p.m.

It can thus be concluded that no evidence of adsorption of the minialbumin onto normal serum albumin was apparent.

Results of radioactive monitoring on serum and urinary albumins after intravenous injection of C^{14}-L-lysine. (See Table XI and Fig. 24).

II 7 (v) Injection of monkey low-molecular weight urinary albumin into a normal monkey.

The low-molecular weight albumin excreted in the urine of cadmium-poisoned monkeys could not be detected in the serum of such an animal. Having, during our previous experiments of labelling the animal with C^{14}-L-lysine, brought about the formation of C^{14}-minialbumin in the urine, we had an excellent tool with which to study turnover rates of the minialbumin.

Method. All available labelled minialbumin fractionated during our previous experiments and not being used during the monitoring procedures was collected and pooled. The total amounted to 79.7 mg.

TABLE XI.

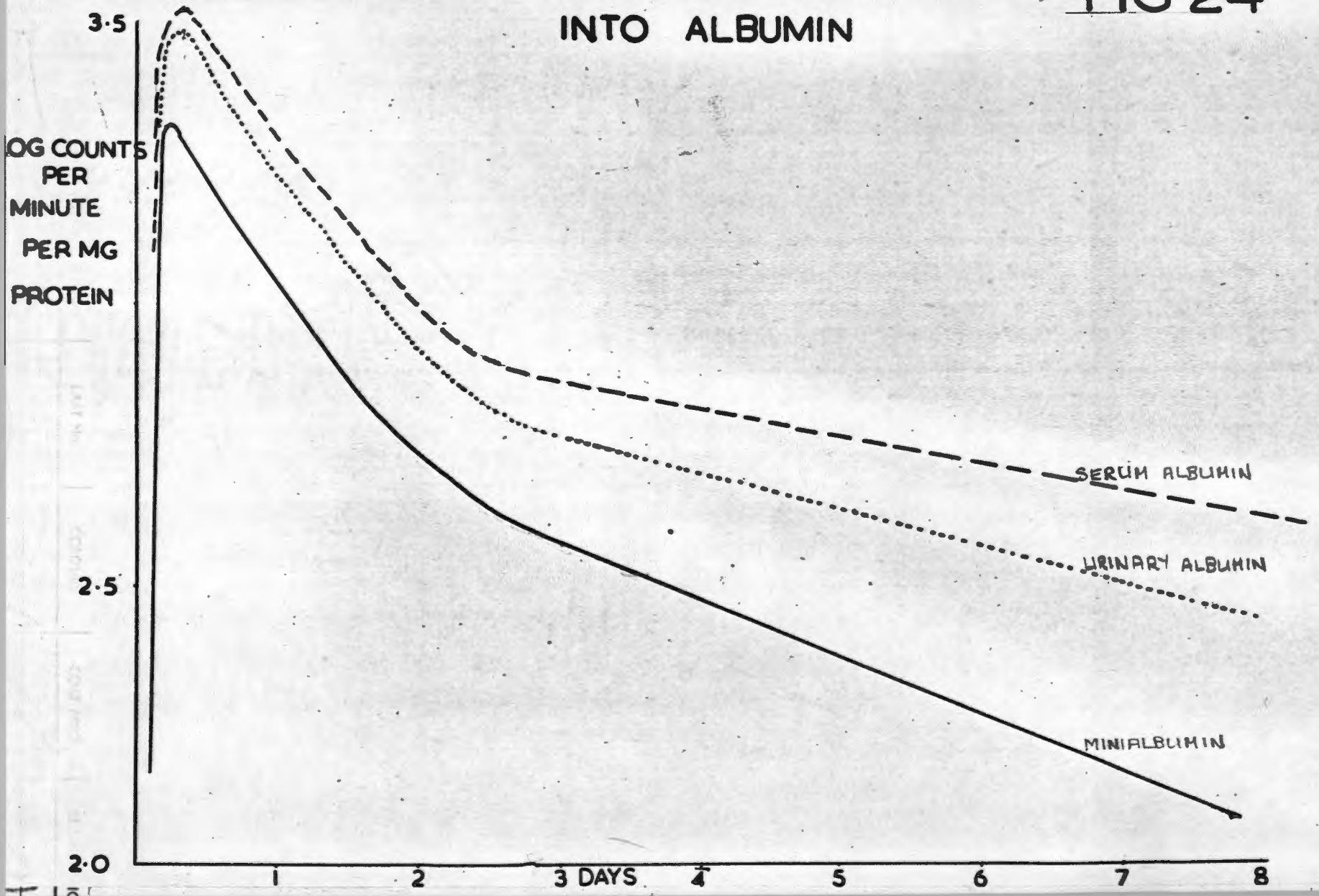
RADIOACTIVITY OF SERUM AND URINARY ALBUMIN SPECIMENS AFTER 1 MILLICURIE INTRAVENOUS C^{14}-l-LYSINE

COUNTS PER MG. PER 10 MINS.

No. of sample	Serum albumin	Urinary normal albumin	Urinary minialbumin
1	1208	1421	1076
2	3022	2071	2113
3	3381	2830	1897
4	2460	2124	1633
5	1774	1847	1259
6	1413	1605	895
7	1239	1333	729
8	1059	1117	567
9	795	711	338
10	708	659	336
11	647	596	252
12	598	532	211
13	596	531	200
14	530	442	182
15	502	384	168
16	465	397	133
17	433	355	135
18	422	349	113
19	356	336	103
20	377	252	100
21	343	220	
22	324	207	
23	311	203	
24	308	183	

INCORPORATION OF L-LYSINE INTO ALBUMIN

FIG 24



with a calculated radioactivity of about 35,000 c.p.m.

The total 79.7 mg. was dissolved in 4 ml. of sterile isotonic saline solution and injected into the saphenous vein of a normal receptor monkey, at 9.15 a.m.

The monkey showed no signs at all of anaphalactic or other shock after the injection. It was hoped to collect blood specimens hourly after the injection, and urine at each individual voiding. Specimens were collected at the following intervals:

TABLE XII.

Time	Time lapse	Blood	Urine
10.15 a.m.	1 hour	5 ml.	-
11.15 a.m.	2 "	5 ml.	-
12.15 p.m.	3 "	nil	25 ml.
1.15 p.m.	4 "	5 ml.	-
2.15 p.m.	5 "	5 ml.	-
5.15 p.m.	8 "	5 ml.	38 ml.
8.30 p.m.	11¼"	nil	-
8.15 a.m.	23 "	5 ml.	60 ml.
3.15 p.m.	30 "	5 ml.	36 ml.
11.15 a.m.	50 "	5 ml.	75 ml.

All urinary serum albumins were prepared with acidified acetone any any variations in the molecular weights of the prepared albumins were looked for during the passing of the albumin solutions through Sephadex G 75 columns.

Urinary Proteins.

Of the 80 mg. of minialbumin injected into the monkey, approximately 40 mg. were excreted immediately and appeared in the urine specimen collected after three hours. This was the only specimen of urine showing the presence of any abnormal amount of protein at all.

Slight radioactivity was detected in the specimen of urine passed at 23 hours following the injection. This was non-protein material presumably products of protein catabolism.

Serum Proteins.

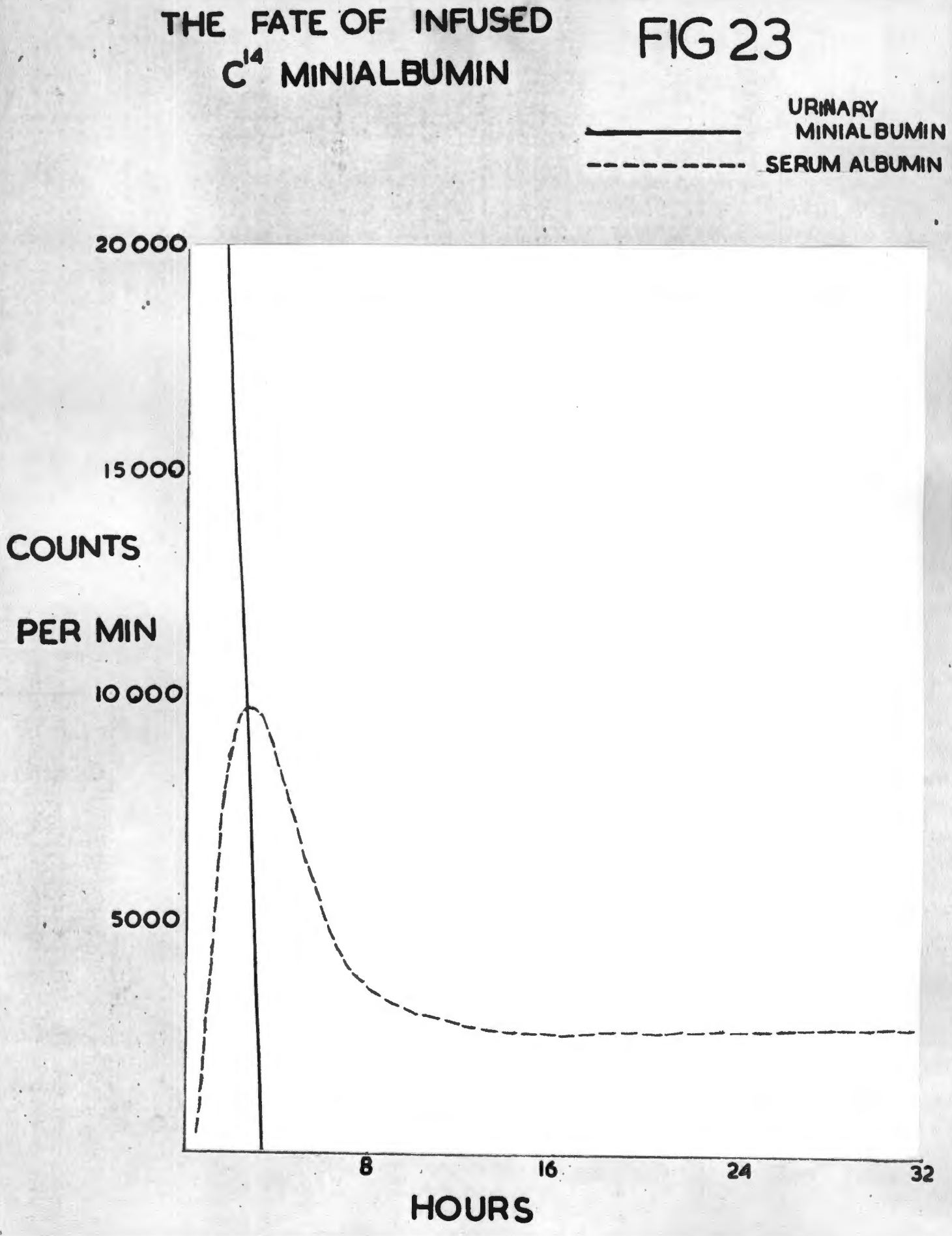
No low-molecular albumin component could be detected in the serum of the animal. The normal serum albumin was, however, isolated and monitored for radioactivity. At two hours after the injection of minialbumin the serum album showed a definite peak in radioactivity, which then diminished steadily over successive hours, and returned to background reading after six days. (See Fig. 23).

Results and conclusions.

The results indicate that the minialbumin was extremely rapidly cleared from the circulatory system of a normal monkey, in fact to such an extent that we were unable to detect the protein in the serum one hour after the injection. The minialbumin was

THE FATE OF INFUSED C^{14} MINIALBUMIN

FIG 23



excreted into the urine almost immediately and could be detected in the first urinary specimen after the injection. No further radioactive protein was detectable in the urine following subsequent voidings of the bladder.

No minialbumin being detectable in the serum, but definite radioactivity associated with the normal serum albumin fraction, seemed to indicate that the minialbumin could either be further metabolised into normal albumin or perhaps adsorbed onto normal albumin. The latter concept was disproved in the case of in vitro experiment, as shown earlier. (II 7 (iv)).

There were no incorporated counts in the serum globulin fraction of the monkey.

II 7 (vi) Fractionation of a Monkey Liver following an Intravenous Injection of Minialbumin.

The problems posed and the conclusions drawn from the results obtained in the previous experiment in which intravenous C^{14} minialbumin was injected into a normal monkey could perhaps be elucidated by a study of the liver of this monkey. Any radioactivity due to residual catabolic products of the minialbumin would most likely be detected in the liver as a whole and if this were the case, the actual fraction of the liver could possibly be isolated along with the radioactive fragments.

Procedure for separation of the liver.

The monkey was killed with a 10 ml. intravenous injection of 5% sodium pentothal. Its throat was then immediately cut and the animal exsanguinated. The blood was not collected, as prior to this operation the counts on the individual serum fractions had returned to the background value.

The thorax of the monkey was then cut open and the liver removed. The liver was placed straightaway into a 0.5 M sucrose solution at 0°C.

Procedure for homogenization of the liver. (Wurst and Novelli, 1964)[163]

The liver, in the ice-cold sucrose solution, was first of all thoroughly washed with 0.5 M sucrose in order to remove all blood clots and extraneous material. (Sachs, 1957)[164] It was then cut up with a sharp cartilage knife into small pieces the size of a pea, and washed further. The pieces were cut very finely and blotted. The weight of liver or the volume displacement in a predetermined volume of sucrose was then measured and, if necessary, as indicated by noticable amounts of blood in the mixture, a further washing was performed. The liver was, finally, suspended in an equal volume of sucrose solution and mixed in a Waring blendor for approximately half an hour, or until the liquid splashing up the sides of the glass container flowed uniformly and easily back without leaving

relatively large pieces of liver behind.

This liquid was then transferred piecemeal to an all-glass hand homogeniser, of the Potter-Elvehjem type, and the aliquots homogenised.

Determination of the C^{14} content of the whole liver homogenate.

Prior to fractionation of the liver homogenate by means of an ultracentrifuge it was decided to monitor the total homogenate.

Method. A portion of the liver homogenate representing about 20 mg. of actual carbon was taken. The carbon content was calculated as close to 5% of the dry weight, the wet weight consisting of 70% water plus 1.5% ash. Of the organic material present, protein was 14% of the dry weight, total lipid 17% and phospholipids 9%. Therefore, 120 mg. of wet liver homogenate was taken, equivalent to 20 mg. of carbon.

This was suspended in twice its volume of distilled water and the proteins precipitated with an equal volume of 20% T.C.A. w/v. The proteins were removed by centrifugation at 6,000 r.p.m., the clear supernatant being removed with a pasteur pipette.

Monitoring of protein precipitate and supernatant fluid.

The radioactivity of the aqueous supernatant fluid was measured in a similar fashion to that of the urinary proteins i.e. an aliquot of 1 ml. was added to 15 ml. of a dioxan scintillation solution in a 25 ml. glass vial. For the monitoring of

the protein precipitate, a more involved procedure was necessary in order to obtain maximum recovery of C^{14}.

As mentioned earlier, an amount of liver homogenate corresponding to 20 mg. of carbon had been used for the protein precipitation. This precipitate was transferred to a thick-walled tube which had a B_{24} female joint. The total protein was now combusted in this tube using a combustion mixture of chromic, iodic, sulphuric and phosphoric acids. (van Slyke and Folch, 1940)[165] This method was unaffected by the presence of nitrogen, sulphur, halogens or alkali metals which tend to interfere with the usual forms of the dry combustion methods. (Meyer, 1938)[166]

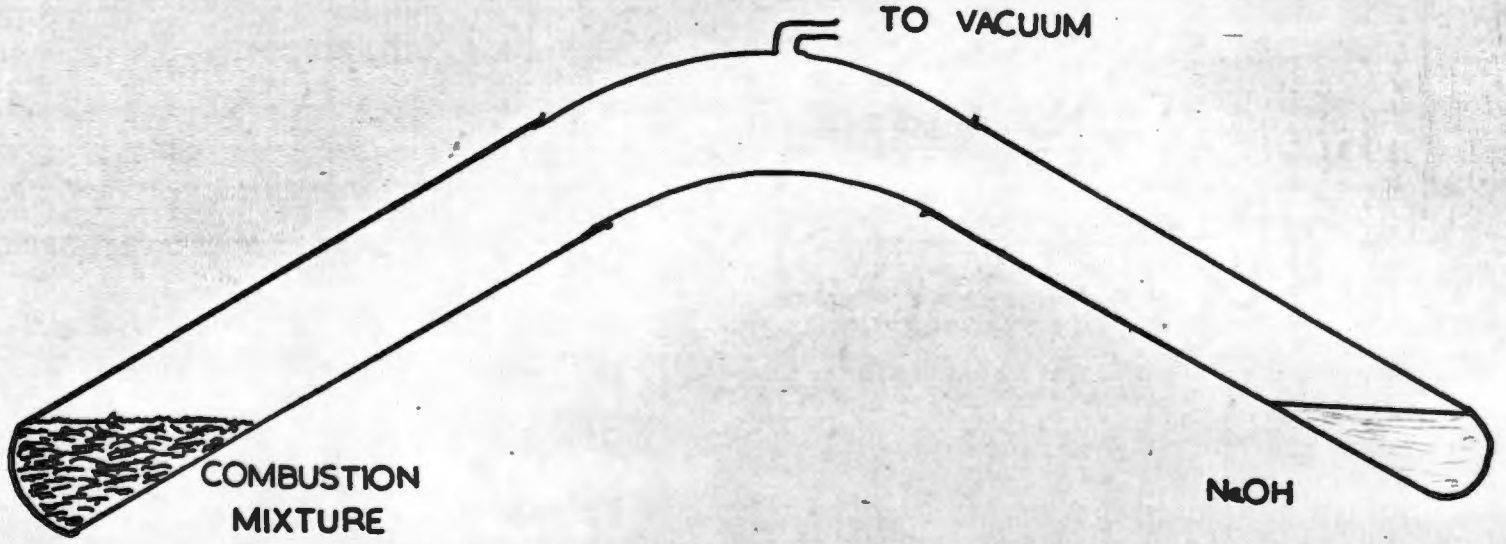
Apparatus and Method. The apparatus employed was extremely simple and comprised two test-tubes joined by means of a quick-fit connection, fitted with an attachment to enable the unit to be evacuated. In the left limb was placed the material to be combusted plus 1 g. of a 2:1 mixture of KIO_3 and $K_2Cr_2O_7$ crystals. To this was added 5 ml. of a combustion liquid of the following composition.

67 ml. fuming H_2SO_4 (= 20% SO_3)
33 ml. H_3PO_4 (S.G. = 1.71)
1 g. KIO_3

This tube was now fitted, by means of the glass bridge to the right limb, which had a B_{19} quick-fit female joint. The

FIG 25

COMBUSTION APPARATUS



B 19 tube contained 5 ml. of 1 M aqueous NaOH, the latter being made from boiled distilled water and a saturated solution of NaOH and was thus CO_2-free.

The two limbs were now joined and the apparatus evacuated and sealed on a flame. The object was now to combust the liver proteins and drive off all formed CO_2 which was then absorbed by the NaOH solution. (van Slyke et al, 1933)[167] (See Fig. 25).

The combustion mixture was warmed very gently at first, and slowly the heat was increased until the mixture was boiling. Initially, fine bubbles of CO_2 begin to rise to the surface as soon as the combustion fluid was warmed. A minute or two was taken to warm the fluid to boiling. Preferably the CO_2 evolution should not be so rapid that it makes, at any time, a foam collar more than 2 cm. high.

Vigorous boiling was necessary to combust the longer chained fatty acids, palmitic and stearic acids, completely but, it was undesirable to boil longer than 1.5 minutes. By the end of this time, the chromic acid had evolved practically all of its labile oxygen and the iodic acid began to liberate iodine and oxygen. If heating were continued the volume of O_2 evolved would appreciably retard the absorption of CO_2. The heating was discontinued when brown fumes started to be evolved. The apparatus plus ingredients were then left to cool and to stand overnight. (Friedman and Kendall, 1929)[168]

This procedure effects complete oxidation of all carbon present, which was totally converted to CO_2 and trapped in the NaOH. (Stanek and Nemes, 1933)[169]

The following day the vacuum was released on the apparatus and to the tube containing the NaOH plus absorbed CO_2 was added 2 ml. of a 2 M ammonium chloride solution and 3 ml. of a 25% barium chloride solution. The precipitated barium carbonate contained the total amount of CO_2 absorbed in the NaOH solution and was thus completely representative of the carbon in the original liver sample.

The precipitate was collected on a millipore filter disc or very fine-grade Whatman filter paper No. 542. We used a millipore filter, Type SC with a 8.0 /u retention limit. The precipitate was collected by means of suction onto the filter pad and then both were placed in a vacuum desiccator for 24 hours to dry out completely. The dry precipitate was then carefully scraped off the pad and placed in a preweighed counting vial. To the vial was add 15 ml. of a special scintillation mixture, which contained a thixotropic gel, "cab-o-sil" to keep the $BaCO_3$ precipitate in suspension and provide the maximum counts to be obtained from the precipitate.

Scintillation reagent for $BaCO_3$-precipitates.

Stock solution:	Toluene	1 litre
	PPO	4 g.
	POPOP	50 mg.

To prepare 180 ml. of scintillation reagent take

45 ml. stock solution

45 ml. Ethanol (Analar)

90 ml. Cab-o-sil.

Any volume can be prepared so long as the volume ratios are 1:1:2.

The resultant solution was very gel-like and after 15 ml. had been added to the vial, the $BaCO_3$ precipitate was evenly suspended by shaking vigorously in a vortex mixer.

Results. Neither the supernatant fluid from the protein precipitation nor the carbonate precipitate from the liver protein digestion itself counted above the background value:

TABLE XIII.

Specimen	Counts per minute	repeat value c.p.m.
CO_3-precipitate	1409	1535
Supernatant	1510	1413
Background (Std. blank)	1584	1647

Conclusions. It is evident from these results that the injected radioactive minialbumin did not leave any residual catabolites in the liver that could be detected.

II 8. IMMUNOLOGICAL STUDIES OF SERUM AND URINARY ALBUMINS.

At this stage it was thought pertinent to carry out various immunological tests on the albumins isolated by us from the urine of cadmium-poisoned monkeys, cadmium-poisoned workmen as well as patients suffering from a variety of renal tubular defects.

We had in our possession Coombs serum, which is anti-human serum prepared by immunizing an animal, usually a goat, with human serum. This antiserum can be obtained from various sources and we used a commercially available product made by Burroughs Wellcome. This product sufficed for the human albumin fractions. No anti-sera to normal monkey serum albumin was commercially available and it was necessary to prepare this specific antibody.

The characterization of human and other animal serum and serum proteins has become an important study with the discovery of serum components with distinct biological, chemical or therapeutic activity. Although a given fraction can be prepared and defined with electrophoretic homogeneity, it can be found to be heterogenous by other physical or chemical analysis. The actual serum proteins present in significant quantity, either in whole serum or fractions of serum, have been difficult to determine by any single method or combination of methods.

Immunochemical analysis, because of the high specificity of antibodies for their homologous antigens, has been employed as a

valid method for testing the degree of homogeneity of human serum protein fractions. Analysis of specific precipitates have been recently performed both in liquid media and gel-media. (Jager et al, 1948[170]; Cohn et al, 1950[171] and Burtin, 1954[172]).

We used two techniques, namely immuno-electrophoresis and Ouchterlony plates. Immunoelectrophoresis enables one to resolve a fraction or fractions of serum or urine electrophoretically on a plate of agar gel. Subsequently, specific antibodies from an animal immunized with the appropriate antigens are allowed to diffuse freely from canals parallel to the axis of electrophoretic migration. When the antibody encounters its specifically combining antigen, the reactants are mutually precipitated forming a white line or precipitin band. The position and intensity of this band is highly specific as to the type of protein as well as its concentration.

The other immuno-technique used by us was that of Ouchterlony plates. (Ouchterlony, 1949, 1953)[173, 174] In this method the gel is also layered on glass plates. Then, holes having been made in the gel, the antibody is placed in the centre hole or "well" and any appropriate antigens in the six surrounding wells. Each component now diffuses freely until meeting its specific antibody where a precipitin reaction occurs.

Materials and Methods.

Preparation of rabbit anti-monkey serum albumin.

Monkey serum albumin was prepared by the method previously described, i.e. using T.C.A. and acetone. It had been decided to prepare rabbit antiserum against monkey serum albumin as we were dealing only with urinary and serum albumins, and it was these that we had to characterize.

To prepare antibodies to the monkey serum albumin, rabbits weighing at least 2 kg. were injected intramuscularly with between 5 mg. - 10 mg. of the albumin in 1 ml. of water, emulsified with an equal volume of an oil adjuvant.

Method. The adjuvant used consisted of two oils, Drakeol and Arlacel A (Mannide mono-oleate) both manufactured by the Atlas Powder Company, U.S.A. A 10% v/v solution of Arlacel was made up in Drakeol and was then ready for use. The sterilization of the adjuvant was achieved by heating in a glass beaker to 100 C and keeping it at this temperature for 30 minutes. The protein solution, containing 10 mg. albumin per ml. was sterilized by passing it through a Millipore filter adaptor fitted to a sterile syringe. The adaptor contained a Millipore filter pad GS with a pore size of 0.22 /u. This effectively sterilized the protein solution.

If the solution was at all cloudy in appearance it was either centrifuged or passed through a glass-fibre prefilter, otherwise the actual millipore filter disc became clogged with the suspended particulate matter.

A millilitre each of both the adjuvant and the protein solution were now mixed and emulsified thoroughly by forcing the mixture in and out of a syringe and 21 G needle. This thorough mixing, until a very thick emulsion was formed, was essential for optimal effect of the adjuvant. The adjuvant was thought to release the protein slowly and over a prolonged period, thereby ensuring a continuous supply of antigen. This saved many repeated injections of the protein alone in order to keep up the continuous supply of antigen.

The injections, of the 2 ml. protein/adjuvant mixture, were performed once a week on male adult rabbits. They were intramuscular and in the inner thigh of the animal.

After five weeks, i.e. six injections, the rabbits were bled from the marginal ear vein and 20 ml. of blood collected. The serum was separated from the corpuscles by centrifugation at 2,000 r.p.m. and the serum used to determine any antibody titre. This was done by making serial dilutions of both the antibody (rabbit serum) and the antigen (monkey albumin) in a so-called block titration. The serum was more dense and was layered at the bottom of narrow, 2 mm. glass tube. The antigen was now carefully layered on top of this, forming a definite interface. The titre of the antibody was gauged by the concentrations at which precipitin bands, at this interface, cease to form. If precipitin bands form at dilutions of 1/32 of each

component then the serum was said to have a high antibody titre.

At this stage of the immunization, the titre was rather low, precipitin bands only forming in the dilutions up to $\frac{1}{2}$ of each component. Therefore, a booster injection was given two weeks after the last injection of the initial series, and again the antibody titre was measured by block titration. After the second booster injection the block titration showed precipitin bands up to 1/16 dilution of both antibody and antigen, showing a satisfactory antibody titre in the rabbit serum. This serum could thus be used for immunological studies.

Preparation of the agar supporting media. (Wunderly, 1959)[175]

Originally the supporting gel on which both immuno-electrophoretic experiments and Ouchterlony plates were run was made from agar, a mucilaginous polysaccharide which, having been dissolved, sets to form a gel. The origin of the gel are various algae, mostly seawater in habitat, which can be extracted to form this product. The agar was predominantly a linear polysaccharide in which the subunits consist of nine residues of D-galactopyranose.

Several commercial forms of agar are available, but lately a new product, agarose, has become available. This compound, a purified form of agar is made locally by Seravac Laboratories (S.A.) and has the advantage over agar in that the process of

endosmosis is is eliminated. A gar consists of two fractions, agarose and agaropectin. Agaropectin has changed particles which during electrophoresis cause endosmosis and badly resolved electrophoretic patterns. When the 45% agarose has been separated and purified it is therefore a far superior supporting medium for immunological work.

To prepare the agarose for applying to the glass supporting plates a 1% gel solution was prepared in 0.05 M Tris-(hydroxy-methyl) amino methane - HCl buffer at pH 8.4. The buffer was made up as follows:

Tris-buffer 0.05 M, 141 ml., to which was added 50 ml. IN HCl. In addition, sodium azide, 500 mg. per litre was added to inhibit bacterial and fungal growth. 200 mg. of agarose was dissolved in 20 ml. of tris-buffer by boiling for approximately 10 minutes. Any liquid loss caused by evaporation was corrected by the addition of distilled water up to a fixed volume. Heating was continued until the solution was visually homogenous, showing no signs of tiny translucent undissolved agarose.

In the meantime, glass slides, measuring 7½ cm. x 5 cm. were cleaned and warmed up in ethanol over a low flame. (Fabey et al, 1963)[176] The plates were then dried and placed on a level surface, where the hot liquid solution was applied by means of a pipette or plastic syringe (10 ml.). With practice the exact volume of solution needed to prepare a gel of 1 - 2 mm. thick

could be determined. This volume was approximately 5 ml. The solution was now left for 20 minutes to gel. (Crowle, 1956)[177]

At this stage, wells were cut in the plates for Ouchterlony tests by means of a punch, giving six wells around a centre well, all 7 mm. in diameter. The plates for immuno-electrophoresis were prepared by cutting two parallel slots lengthwise across the plates about $2\frac{1}{2}$ cms. apart and about $5\frac{1}{2}$ cms. long. Small holes were sucked out of the gel on each side of both slots. These holes are 1 mm. in diameter, between 3 and 4 mm. away from the slots and about $\frac{1}{2}$ cm. off centre. (See Fig. 26). In this way two separate runs can be carried out, each run consisting of duplicates.

Immuno-electrophoresis.

The protein solution to be electrophoresed was applied to the holes with the current on, the application wells being on the cathode side. Phenol red was also added to the wells as a marker, as it ran about 1 - 2 cm. ahead of albumin towards the anode. Electrophoresis was performed in perspex tanks, which could be made airtight and were watercooled. A voltage of 300 V was applied for $\pm$ 20 minutes after which the run was complete. Progress during the run could be gauged by the movement of the phenol red marker. The electrode buffer for electrophoretic tank chambers is 0.1 M Tris-buffer at pH 8.4. (Smith, 1960 and Wunderly, 1961)[178, 179]

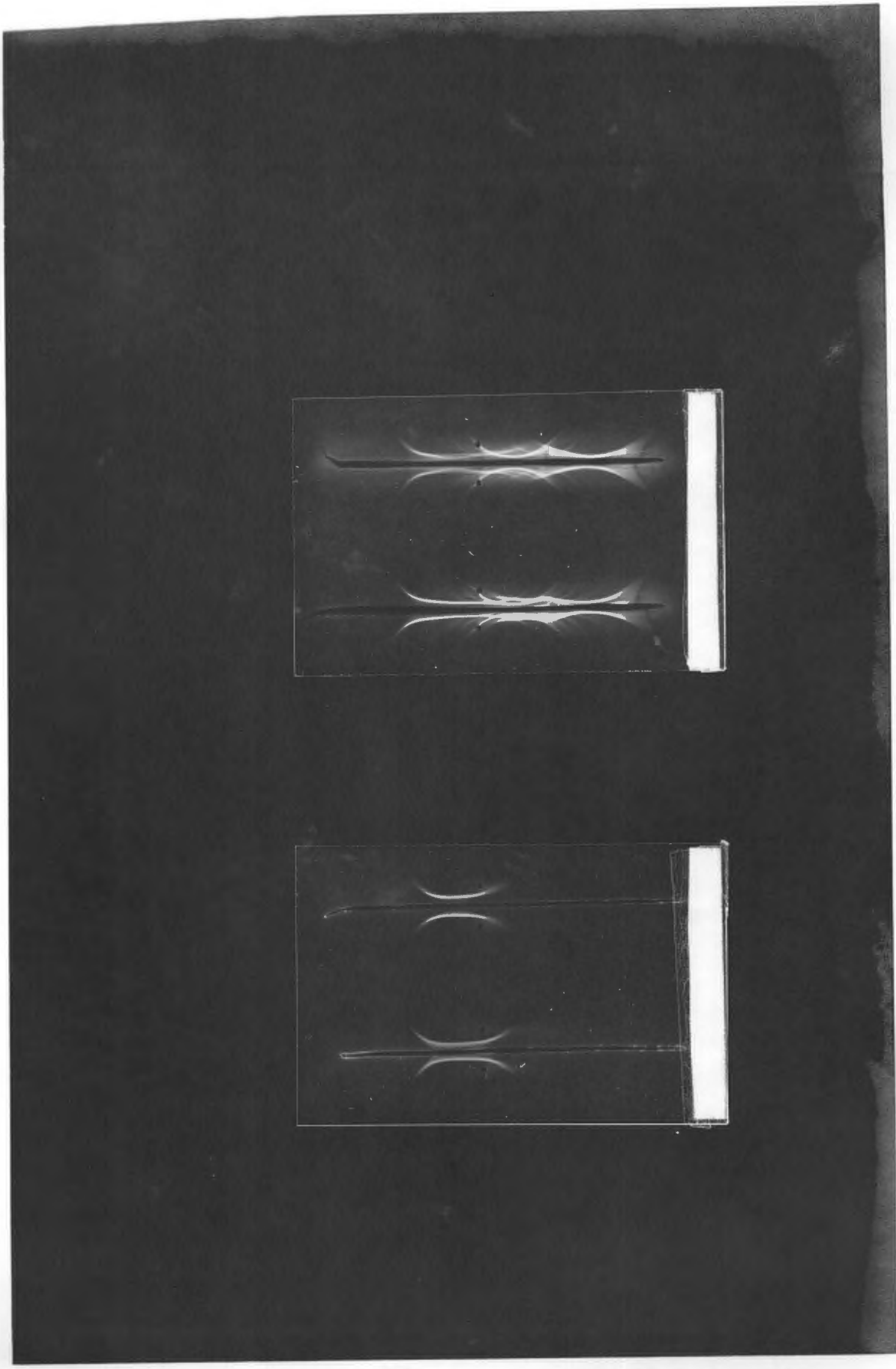
LEGEND TO FIGURE 26.

The top slide represents a duplicate immunoelectrophoretic run of whole human serum against Coombs anti-human serum. The precipitin bands are all clearly visible.

LEGEND TO FIGURE 27.

The lower slide shows a purified γ-globulin fraction run against Coombs anti-human serum.

FIGS
27,26



After removal of the plate from the electrophoretic tank, the antiserum was placed in the slot separating the two runs. Both sets of proteins now freely diffused through the gel until specific antigen-antibody reactions caused precipitin bands to form. The antigen diffused in a radial fashion from a discrete concentration zone, whereas the antibody diffused towards it on a linear front. The lines of specific precipitate were therefore formed in arcs. The precipitin reaction was allowed to continue overnight to completion. Plates were kept in petri-dishes, kept moist with damp blotting paper to prevent drying out.

Ouchterlony plates. (Wilson and Pringle, 1954)[180]

After punching the holes in the gel on the plates, the antibody was placed in the centre well while any antigens or proteins required to be identified were placed in the 6 encircling sexagonal set of wells. Protein migration was again allowed to proceed freely until antibody and specific antigen formed precipitin bands. The reaction was normally complete after 24 hours but with antigens or antibodies of weak titres, 48 hours might be necessary for adequate completion of the precipitin bands. These bands were visible to the naked eye as white lines at varying distances from the central well.

Routine for staining of precipitin bands.

Although in both immuno-electrophoretic plates as well as Ouchterlony plates the white protein precipitin area or bands

were usually visible as faint white lines, it was usually necessary and more satisfactory to stain these areas with a protein dye. Much greater accuracy in perceiving small bands and consequent identification was then obtainable.

The routine for staining was as follows; and was similar in both types of plates.

1. The slides were immersed in normal physiological saline (85% w/v NaCl) for at least 48 hours. This washes out any excess proteins,from the gel, which have not participated in the reactions and would confuse the staining of the precipitin bands.

2. The steps normally followed for dehydrating and drying the slides were omitted by us, as they seemed unnecessary at this stage and did not affect the final product.

3. After removal from the saline solution, the slides were placed in a fixative of acid ethanol (ethanol, water and acetic acid in proportions of 70:25:5 by volume). They remained in this protein fixative for 30 minutes.

4. Thereafter staining was effected by immersion in a suitable stain. We used 0.02% aqueous Nigrosine as it did not give too dark a background colour as Amidoschwartz tended to do. The duration of this procedure was usually judged by direct vision and usually needed some 24 hours under normal room temperature conditions ($\pm$ 20°C).

5. The stained slides were washed in a clearing solution, which removed the stain from the background gel, leaving only the protein bands clearly dark-blue. The clearing solution was 5% acetic acid in water.

Another decolourizing solution, 5% teepol in water, cleared the gel but also tended to destroy the precipitin patterns should the slides be immersed too long.

6. The slides were now removed and dried in the atmosphere under a protective cover to prevent dust particles settling on the damp gel. The slides dried to a very thin film on the glass, which was fairly stable to handling.

7. The slides could now be photographed and the patterns recorded.

Results.

1. Immunoelectrophoresis (See Figs. 27, 28).

2. Ouchterlony Plates (See Figs. 29, 30)

Using both these techniques the urinary proteins from the monkey behaved in similar fashion to normal monkey serum albumin.

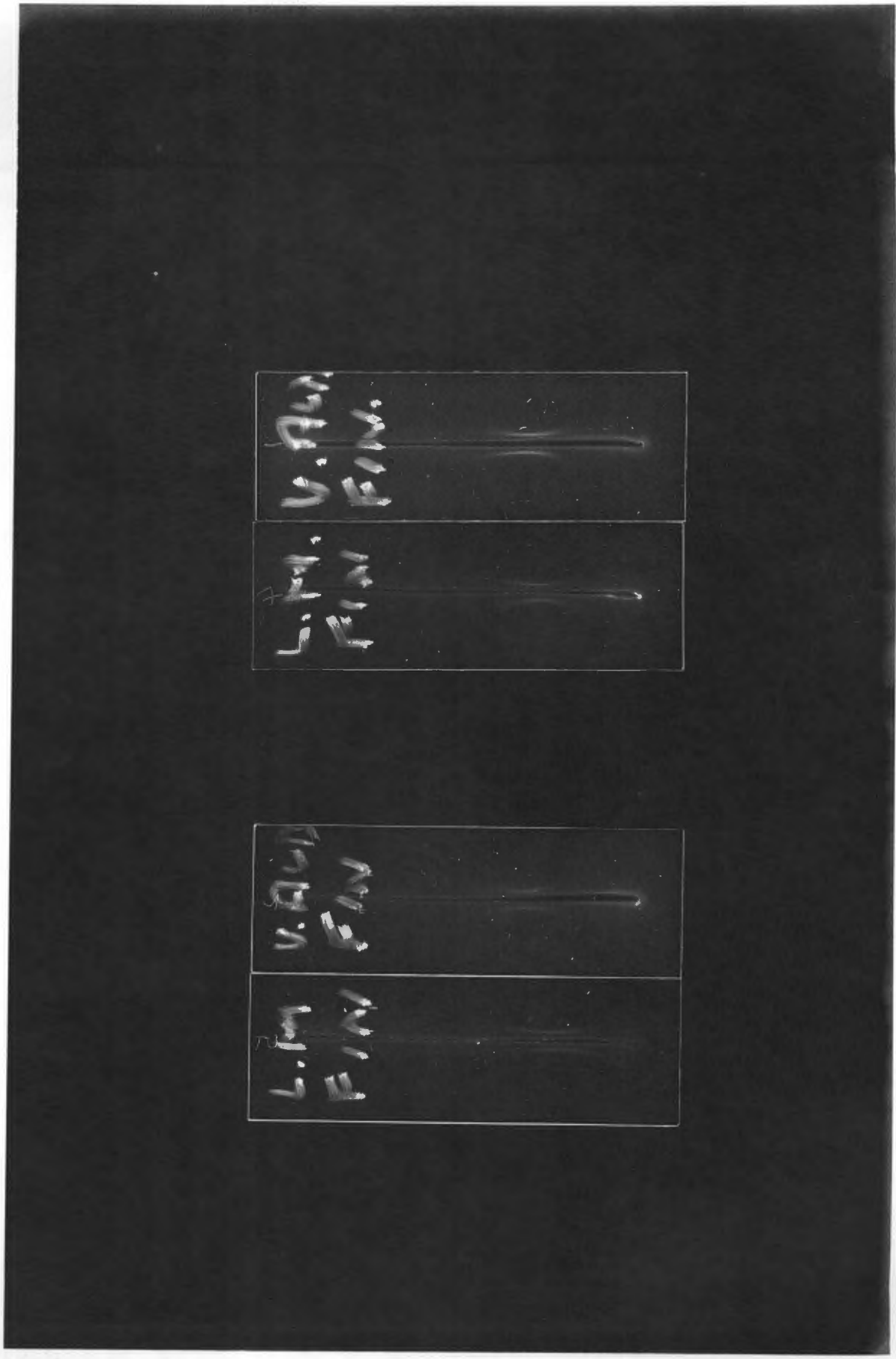
Also, urinary albumins prepared from human patients suffering from various types of renal tubular necrosis was immunologically similar to normal human serum albumin, when run against Coombs antihuman serum.

LEGEND TO FIGURE 28.

The upper slide of each pair is urinary albumin purified by the Trichloracetic acid method described in the text. This albumin was prepared from a cadmium-poisoned worker, Finch.

The lower slide is urinary minialbumin prepared from the same patient and indicating its similar electrophoretic mobility to normal urinary albumin.

FIG
28



LEGEND TO FIGURE 29 (continued)

Plate A.C.

1. Serum albumin: normal monkey
2. Dialysate albumin: nephrectomised, cadmium poisoned monkey
3. Dialysate minialbumin: nephrectomised, cadmium poisoned monkey
4. Dialysate albumin: nephrectomised, cadmium poisoned monkey
5. Dialysate minialbumin: nephrectomised, cadmium poisoned monkey
6. Post-minialbumin peak.

LEGEND TO FIGURE 29.

These Ouchterlony plates are all run against normal anti-monkey albumin, which has been placed in the centre well. The following protein fractions were placed in the respective wells.

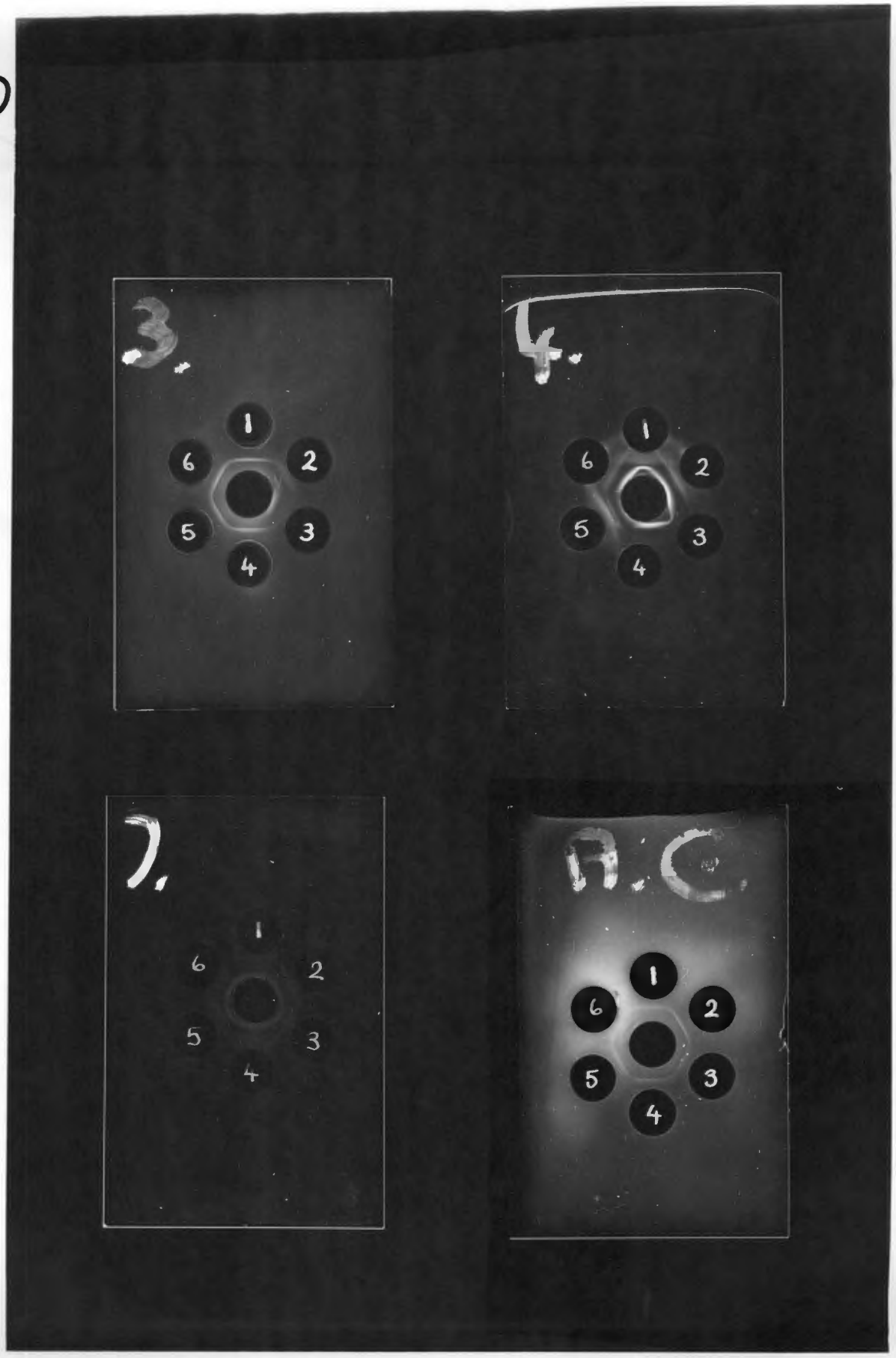
Plate 3.
1. Serum albumin: normal monkey
2. Urinary albumin: cadmium poisoned monkey
3. Urinary minialbumin: cadmium poisoned monkey
4. Urinary albumin (concentrated 10 x): cadmium poisoned monkey
5. Urinary minialbumin (concentrated 10 x): cadmium poisoned monkey
6. Urinary albumin (concentrated 12 x): cadmium poisoned monkey.

Plate 4.
1. Urinary albumin: cadmium poisoned monkey
2. Urinary minialbumin: cadmium poisoned monkey
3. Urinary albumin: cadmium poisoned monkey
4. Urinary minialbumin: cadmium poisoned monkey
5. Urinary globulin peak: cadmium poisoned monkey
6. Urinary globulin peak: cadmium poisoned monkey.

Plate 7. Wells 1 - 6 all contain total urinary proteins

Plate A.C/ ....

FIG
29



LEGEND TO FIGURE 30 (continued).

Plate 6. 5. Urinary minialbumin: cadmium worker (7108)

6. Post-minialbumin peak.

Note: The peaks are those eluted off a column of Sephadex G-75 during molecular sieving of albumin fractions.

LEGEND TO FIGURE 30.

Ouchterlony plates of Coombs anti-human serum in the centre well against the following protein fractions:

Plate 1.
1. Serum albumin: normal monkey
2. Urinary albumin: cadmium worker (Finch)
3. Urinary minialbumin: cadmium worker (Finch)
4. Urinary albumin: Finch.
5. Urinary minialbumin: Finch
6. Serum albumin: normal bovine.

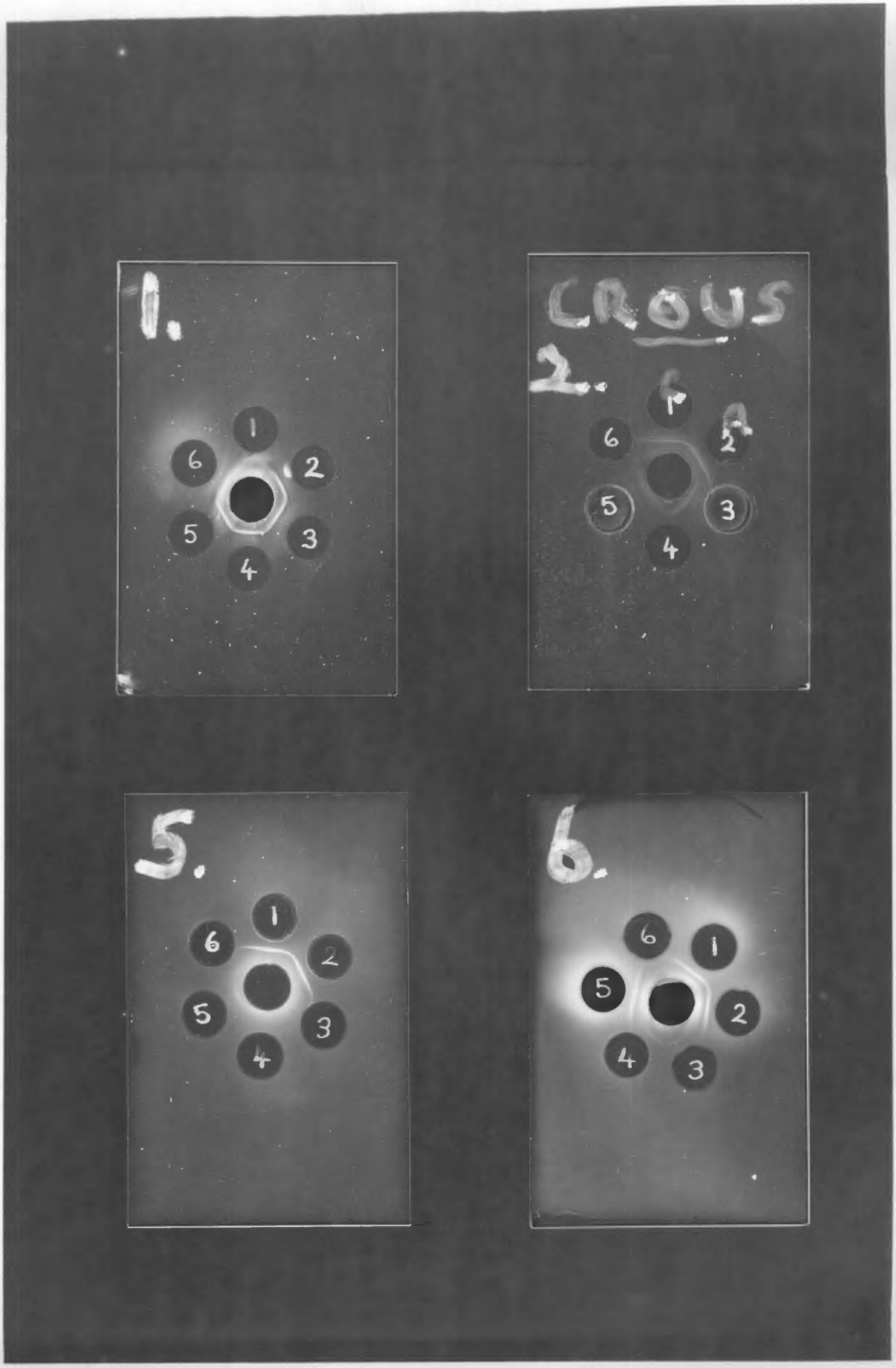
Plate 2.
1. Serum albumin: normal human
2. Urinary albumin: Fanconi patient
3. Pre-albumin peak: Tubular necrosis patient
4. Serum albumin: baby with respiratory distress syndrome
5. Pre-albumin peak: Tubular necrosis patient
6. Serum albumin: baby with respiratory distress syndrome.

Plate 5.
1. Serum albumin: normal human
2. Urinary albumin: nephrotic patient
3.-6. Fractions taken before and after the main albumin peak, i.e. baseline fractions.

Plate 6.
1. Serum albumin: normal human
2. Urinary albumin: cadmium worker (7108)
3. Pre-albumin peak
4. Pre-albumin peak

5./ .....

FIG
30



II 9. EXPERIMENTS TO DISCOVER THE SOURCE OF THE MINIALBUMIN OF CADMIUM-POISONED MAN AND ANIMALS.

II 9 (i) The urinary proteins of cadmium-poisoned workers.

Before proceeding with further experiments to establish the origin of minialbumin, it is as well at this stage to describe the investigations on two lots of freeze-dried urinary proteins from actual workers suffering from cadmium intoxication.

These proteins were given to the investigation by Professor Kench, who had obtained them from patients investigated by him and had preserved them in the lyophylized state in sealed glass containers.

Method.

The proteins were initially dissolved in $\frac{M}{15}$ phosphate buffer at pH 6.8. However, not all of the protein was soluble, some of it obviously being denatured from having been kept for several years at room temperature.

The soluble portion was then removed from the denatured, insoluble fraction, and placed in a separate glass centrifuge tube. The proteins were now precipitated with 10% T.C.A. and the precipitate spun down. The albumin fraction was redissolved in the usual manner using acidified acetone.

After dialysis against water in the cold room, the aqueous

albumin solution was concentrated by pervaporation and screened for molecular weight variations on Sephadex G 75 columns.

The resultant pattern on the ultraviolet absorptiometer showed the presence of two distinct peaks indicating two definite albumin fractions varying in their molecular weights. This was in accordance with results obtained on the urinary proteins of our cadmium-poisoned monkeys and other animals. The presence of a low-molecular albumin in the urine of various cadmium-poisoned species had thereby been correlated.

There had previously been theories propounded that conditions in the body which produced various types of renal tubular involvements and malfunction could also, by virtue of the abnormal metabolism in the actual renal tubular cells, produce abnormal proteins of a low-molecular nature. In other words, our experimental model of a cadmium-poisoned monkey was producing low-molecular weight albumin simply due to the effect of the cadmium on the renal tubular cells themselves and the protein was therefore not an aberration of protein synthesis in the liver.

Renal tubular defects do, in fact, cause many low-molecular weight proteins to be excreted in the urine and there is much evidence and data on this subject. Nevertheless, a low-molecular weight albumin has not been described in these generalized tubular defects and so we decided to investigate the urines of available

patients who had kidney diseases and diseases of a related nature.

Our patients investigated were divided into 4 groups:

1. Nephritic patients.
2. Generalized renal tubular involvements.
3. de Toni-Fanconi syndrome.
4. Hepatolenticular degeneration (Kinnear-Wilson disease).

1. Nephritic patients.

This type of case had previously been investigated by other workers who had found no low-molecular weight albumins in the urines passed by these patients. (Rowe, 1957)[23] We were fortunate to have in our possession a freeze-dried specimen of urinary proteins from a Nephritic patient. This specimen was in the possession of Professor Kench who very kindly let it be analysed as to its albumin content.

As is well known, the outstanding defect in glomerular nephritis and also in the nephrotic syndrome, is the severe urinary loss of protein. Early studies on protein excretion by Bayliss, Kerridge and Russel (1933)[181] showed that for the kidneys of a normal animal a demarcation molecular weight existed, i.e. $\pm$ 66,000, below which value, proteins were excreted into the urine and above which value the proteins were retained in the circulation. Actually as proved later there is a progressive reduction in clearance as the molecular weight is increased. (Brewer, 1951)[18

In nephrotic urines and sera, albumin fractions have been prepared by column electrophoresis (Porath)[59] and all albumins in the urine had molecular weights identical with that of normal serum albumin.[23]

Other urinary proteins in these patients are, however, of a low molecular nature and consist of lower molecular weight members of the groups of proteins that have similar electrophoretic mobilities in the serum.

In our analysis of the freeze-dried urinary proteins, we dissolved the protein in $\frac{M}{15}$ phosphate buffer, pH 6.8, precipitated the urinary proteins with 20% T.C.A., centrifuged the precipitate and then dissolved the albumin fraction in acetone acidified with 1% w/v Trichloracetic acid. The final pH was approximately pH 2.5. The acetone soluble fraction was then dialysed against several changes of distilled water, in a refrigerator or cold room, to remove all traces of acetone and T.C.A. The aqueous albumin solution could now be concentrated by means of pervaporation. A column of G-75 Sephadex was then employed to separate the albumins of varying molecular weights, as had been described in a previous chapter.

No evidence was found of a low-molecular albumin component.

2. <u>Patients with generalized renal tubular involvements.</u>

These patients were selected as having renal tubular defects

of a non-specific type not characterized by any of the other described conditions. For example, the first patient in this category, G.K., presented with renal tubular damage and proteinuria due to septic abortion. In all, four such cases were obtained and their urinary proteins investigated for the presence of abnormal albumins. In each patient, only a urinary albumin corresponding to normal serum albumin, could be isolated.

3. Patients with de Toni-Fanconi Syndrome.

This is a complex syndrome usually encountered in infants and young children. There is a general failure to thrive, and with this is associated a severe form of Vitamin D resistant rickets, acidosis, polyuria, renal glycosuria and a generalized amino-aciduria, indicating renal tubular malfunction. (Dent, 1951)[183]

These cases also present with proteinuria of a mild form at certain times. Of the four cases investigated by us only one, J.C., had detectable proteinuria. The other patients lived very far afield and on their very infrequent visits to us did not present with proteinuria.

The urine of J.C. was again investigated by us and the albumin fraction isolated and screened for molecular abnormalities. The molecular separation on Sephadex G-75 showed only one distinct peak appearing in the region and at the effluent

FIG 31

SEPHADEX G-75 SEPARATION and UVICORD OPTICAL DENSITY RECORDINGS:

Urinary albumin

GENERALISED TUBULAR DEFECT.

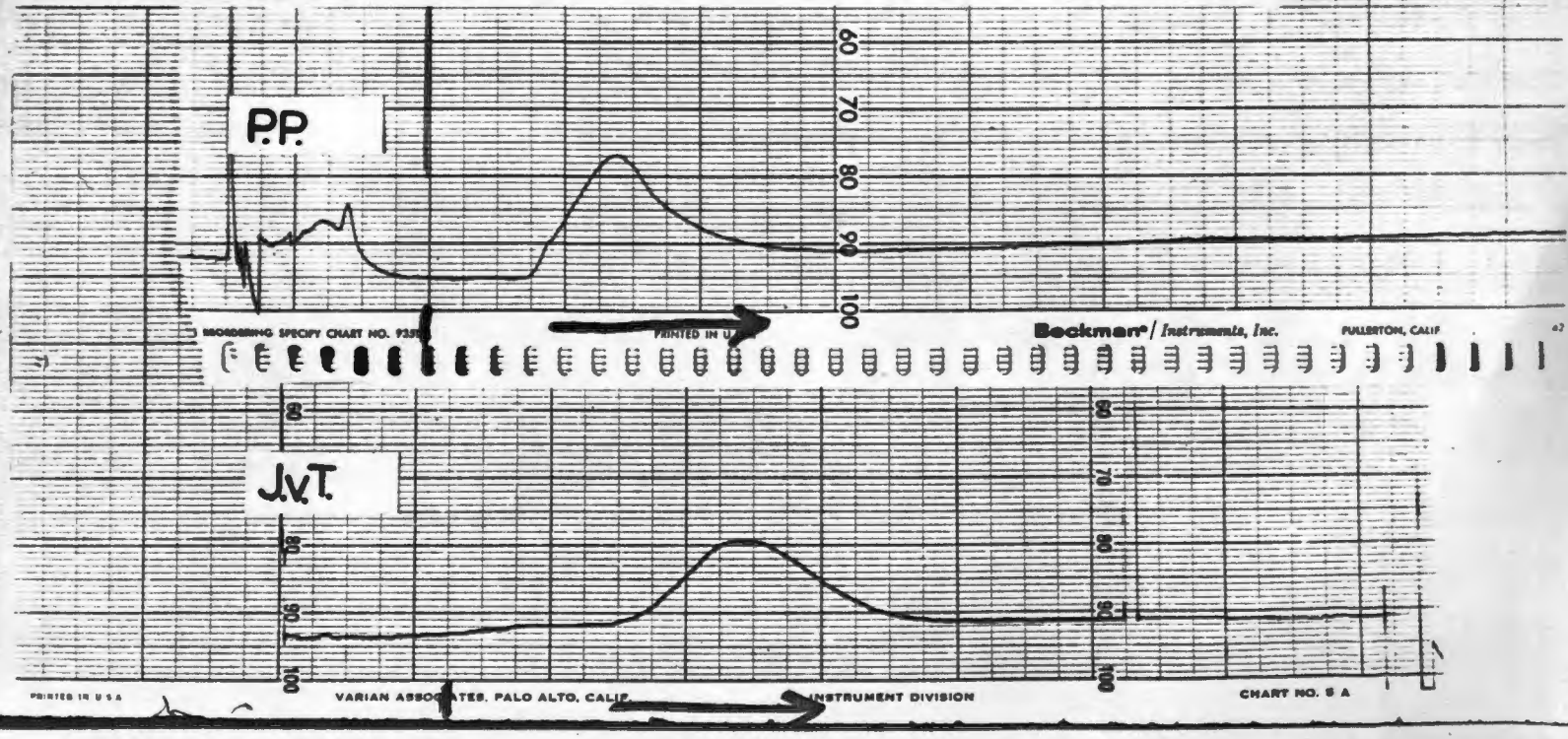
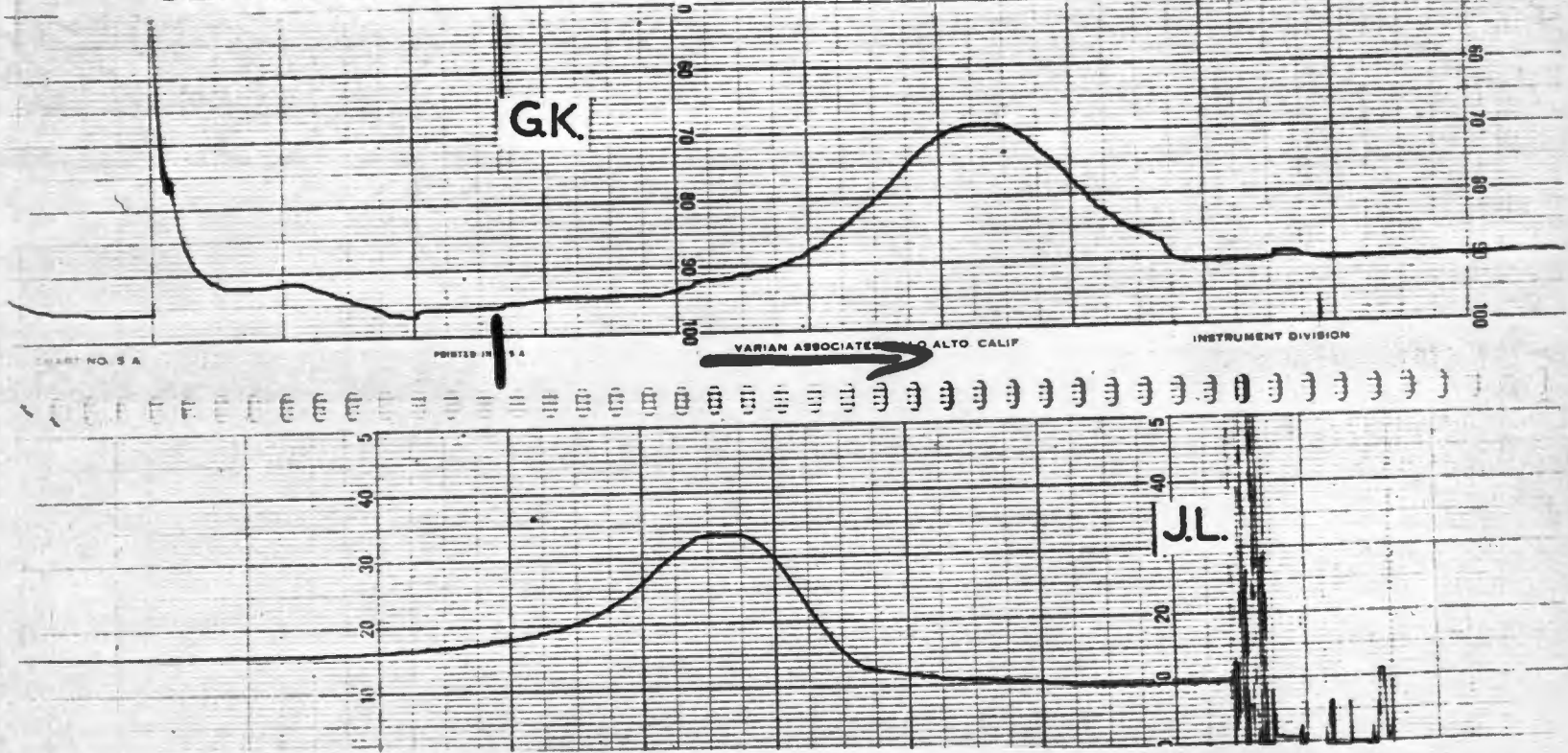


FIG32

SEPHADEX G-75 SEPARATION and UVICORD OPTICAL DENSITY RECORDINGS:

Urinary albumin

GENERALISED TUBULAR DEFECT.



volume associated with albumin of a normal molecular weight. No low-molecular weight albumin was detectable. (See Figs. 31 and 32).

4. Patients with hepatolenticular degeneration.

This is a progressive condition causing renal amino-aciduria and proteinuria due to non-specific tubular damage secondary to a generalized metabolic disease. This condition, also known as Kinnear-Wilson's disease is characterized neurologically by the occurrence of widespread tremor, muscular rigidity and other features indicating dysfunction of the extra-pyramidal motor system. Pathologically, the lesion consists of a bilaterally symmetrical degeneration of the lenticular and caudate nuclei, with some degenerative changes in the nerve cells of the cerebral cortex. Associated with these changes and often preceding them is a progressive multilobular cirrhosis of the liver. A curious feature of the condition is the so-called Kayser-Fleischer ring. This is a zone of pigmentation, brownish in colour, at the periphery of the cornea.

Biochemically, the primary lesion appears to be a gross disorder in copper metabolism causing generalized amino-aciduria plus proteinuria. (Uzman, 1948[184] and Stein et al, 1954[185]) The amino acid content in the serum is always within normal limits; it therefore appears that the aminoaciduria is renal in type and dependent on inefficient reabsorption of amino acids by the renal

FIG 33

SEPHADEX G-75 SEPARATION and UVICORD OPTICAL DENSITY RECORDINGS:

Urinary albumin

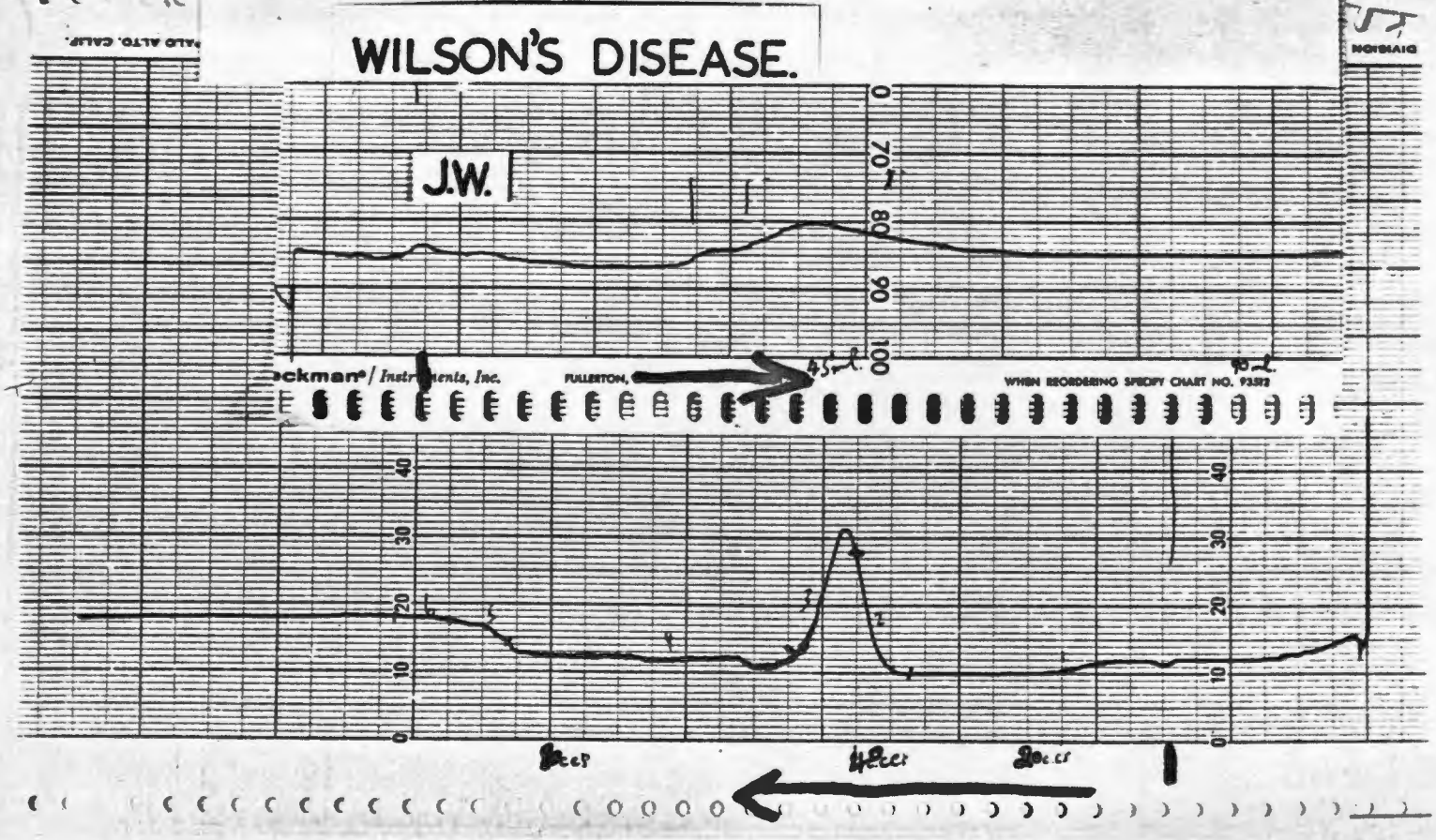
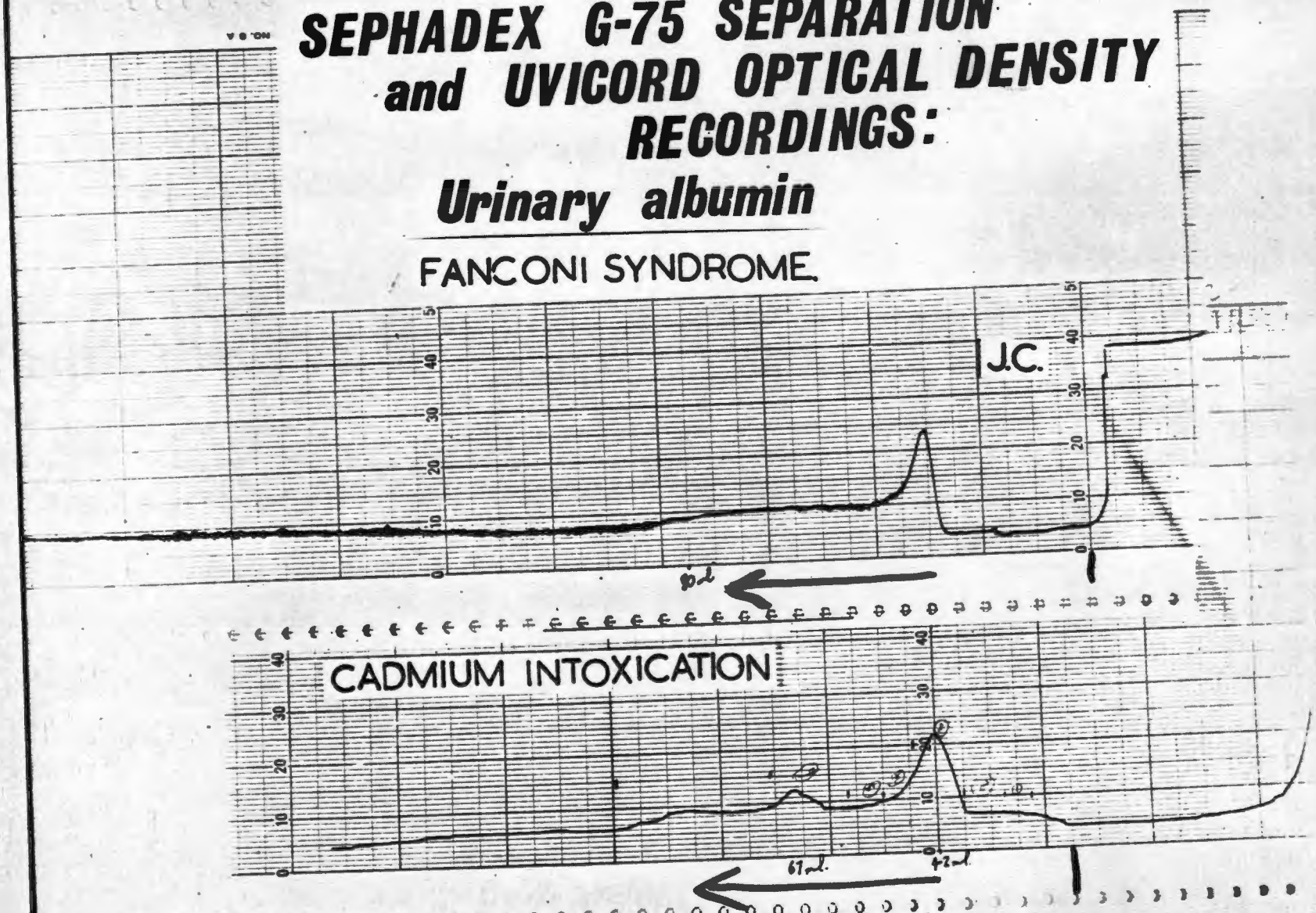


FIG 34

SEPHADEX G-75 SEPARATION and UVICORD OPTICAL DENSITY RECORDINGS:

Urinary albumin



tubules from the glomerular filtrate. (Cooper et al, 1950)[186]

Other renal tubular defects occur in many cases of Wilson's disease. Proximal tubular lesions include a high uric acid clearance and concomitant low plasma uric acid, renal glycosuria, and an increased phosphate clearance and resultant rickets or osteomalacia. (Bishop, et al, 1954)[187]

These cases, therefore, gave us an excellent model of faulty tubular mechanism on which to test the theory that defective renal tubular function itself produced the low-molecular weight albumin found in the urine of cadmium-poisoned individuals.

The patient we investigated had slight proteinuria of 40 ± 5 mg./100 ml. We concentrated one litre of his urine, and then precipitated the proteins with T.C.A. before proceeding with the albumin extraction, in order to have sufficient protein so that small amounts of any component could also be detected.

Carrying out our experiments as before we could again detect no trace of a low-molecular albumin fraction.

Conclusions.

In all cases of renal tubular dysfunction, in patients suffering from various varieties of renal involvement, when proteinuria was present then albumin was found in the urine. This albumin, in all cases, corresponded to normal serum albumin

with regard to molecular weight, electrophoretic mobility and immunology.

No low-molecular weight albumin was detected, leading to the assumption that generalized renal tubular defects do not necessarily cause the formation and excretion of this protein.

The urinary albumins prepared from these patients were identified immunologically on Ouchterlony plates against Coombs anti-human serum. (See Fig. 30).

II 9 (ii) Nephrectomy of cadmium-poisoned monkey.

In part (i) of this section it was described how human patients with renal tubular defects were investigated and the albumin content of any proteinuria analysed. The absence of low-molecular weight albumin was noteworthy, but it was certainly not weighty evidence on which to base conclusions regarding the origin of the protein.

A series of experiments were thus planned during which over a period of time we would remove first one kidney and then the other kidney from our cadmium poisoned monkey and check continuously on the urinary excretion of both normal and low-molecular weight albumins.

Albuminuria prior to first nephrectomy.

Our cadmium poisoned monkey had been proteinuric for many

months at this time and was excreting both normal and low-molecular weight albumins in the urine. The animal had been and was still receiving twice-weekly intravenous injections of $CdCl_2$, containing 6 mg. of cadmium per injection.

We had noticed that although total protein excretion remained fairly stable, the concentration nevertheless fluctuated daily between 140 mg./100 ml. and 220 mg./100 ml. with the urinary volume varying between 80 ml. and 130 ml. It was also clear that the higher values of protein excretion were attained immediately after each cadmium injection, proteinuria forming a bi-weekly peak.

Thus prior to the first nephrectomy we determined, very accurately, a proteinuria baseline with special attention to the actual ratios of normal albumin to minialbumin excreted daily. This value averaging 90:40 mg. (> 2:1) (See Table XIV and Fig. 35) remained fairly constant and between limited values. Even after an injection of cadmium, although the total albumin excretion increased temporarily, this ratio remained within set limits.

Albuminuria after first nephrectomy.

The ratio of normal albumin to minialbumin excreted in the urine following the first nephrectomy was 60:25 mg. on an average, again just over 2:1. The monkey's urine volume remained low for a period of three or four days after the operation, but its remaining kidney very soon took over the extra load.

TABLE XIV.

Urinary albumin concentrations of the cadmium poisoned monkey in mg. per 100 ml.

No.	Urinary albumin	Urinary minialbumin
1	104	49
2	78	36
3	86	37
4	91	36
5	98	50
6	94	40
7	102	40

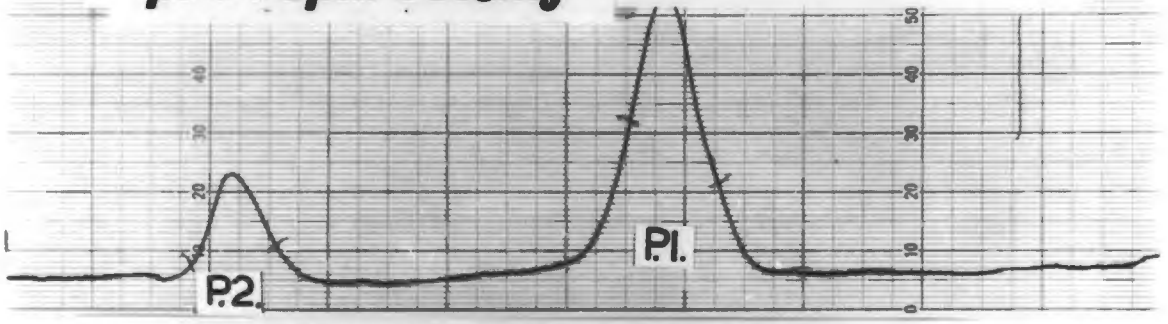
Samples1 and 5 were collected on days immediately after a cadmium injection.

FIG 35

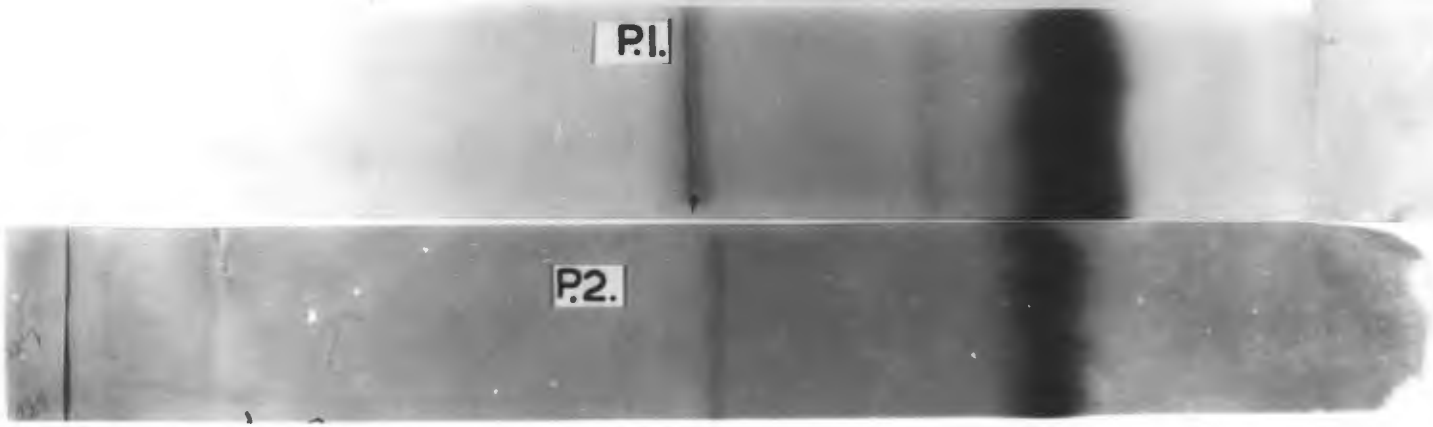
SEPHADEX G-75 SEPARATION and UVICORD OPTICAL DENSITY RECORDINGS:

Urinary albumin Monkey

pre nephrectomy



ELECTROPHORETOGRAMS



The animal lost extremely little blood during or after the operation and its serum electrolyte and urea levels returned to normal within three days.

The kidney, after nephrectomy, was immediately placed in cold phosphate buffer and frozen for subsequent homogenization and fractionation. A small piece of kidney as well as a liver biopsy taken during the nephrectomy were placed in osmic acid to be fixed for electron-microscopy. These tissues had an abnormal macroscopic appearance and texture. The liver was extremely soft and pulpy, while the kidney seemed to be very brittle.

The final fixing of the tissues, however, was unfortunately, not satisfactory for subsequent sectioning on the ultra-microtome. This was most probably due to the abnormal amounts of cadmium in the tissues.

II 9 (iii) Haemoglobin infusion.

Ten days after the first nephrectomy, we injected 5 g. of haemoglobin intravenously into the monkey. The object of this experiment was to ascertain if a load of haemoglobin being presented for absorption by the renal tubular cells could influence the excretion of the minialbumin. (Oliver, MacDowell and Lee, 1954)[188]

The haemoglobin was prepared following the method of Drabkin[189], using 200 ml. of pooled red blood corpuscles collected from several normal monkeys plus the total blood volume from an exsanguinated monkey.

Method. The blood was collected into 3.8% sodium citrate solution, 50 ml. of the latter for the 400 ml. of blood collected. At this stage, the blood from the animals were kept separate in case of any blood group incompatabilities.

The cells were separated by centrifugation at 4,000 r.p.m. for 20 minutes and were then washed thoroughly first with isotonic saline (0.85% NaCl) and then three times with a mixture of 1.2% saline and 0.0025 M aluminium chloride.

Finally, the corpuscles were lysed by the addition of an equal volume of distilled water, thoroughly mixed with 0.4 volumes toluene and left overnight in the refrigerator. The stroma was separated by centrifugation at 35,000 G or 20,000 r.p.m. in a Beckman Model L ultracentrifuge.

The haemoglobin was removed carefully from the stroma by means of siphoning and this solution was placed in glass flasks for concentration and drying by means of lyophilization. The final weight of haemoglobin obtained was 45 g., and in the lyophilized state could be stored without fear of denaturation.

Administration of haemoglobin.

For immediate use the required amount was dissolved in distilled water and passed through a Zeitz filter for sterilization. As mentioned, the first injection contained 6 g. of haemoglobin dissolved in 40 ml. of distilled water (12½% solution). This solution was administered to the monkey by means of an intravenous drip over a period of 10 minutes, whereafter the monkey was again placed on its tray and urine collected.

The following day the urine collected over the previous 24 hours was dialysed in boiled visking tubing and then concentrated to ½ its original volume. A 8 ml. aliquot of this was then passed through a Sephadex G-75 column. The urine was very dark in colour due to the excreted haemoglobin and the descending band could easily be followed visually on the column.

The records of the Uvicord-recorder showed two peaks; the first comprising mostly haemoglobin and normal albumin, while the second peak contained mostly minialbumin. The values being

Normal albumin - 65 mg./100 ml.
Minialbumin - 70 mg./100 ml.

The 1:1 ratio was already a marked change from the 2:1 ratio of these proteins, found in the urine prior to the haemoglobin injection.

On Day three, 5 g. of haemoglobin were again administered intravenously, and the urine collected during the following 24 hours investigated for haemoglobin and albumin content. An interesting feature was that haemoglobin excretion was much less than after the previous injection and that the minialbumin fraction was markedly increased; the excretions being

Normal albumin - 62 mg./100 ml.

Minialbumin - 95 mg./100 ml.

The ratio of normal albumin to minialbumin dropping still lower, i.e. 2:3.

On Day five, a further 6 g. of haemoglobin were injected, the usual investigations being subsequently followed. There was a virtual absence of the first peak emerging from the Sephadex column, showing complete haemoglobin retention as well as a very much reduced normal albumin excretion. A notable feature was the large peak emerging in the 20,000 molecular weight range. This peak contained virtually all low-molecular weight albumin. The respective amounts excreted being

Normal albumin - 30 mg./100 ml.

Minialbumin - 113 mg./100 ml.

with a ratio of nearly 1:4.

Conclusions.

It appears that haemoglobin infusion augmented the excretion

FIG 36

SEPHADEX G-75 SEPARATION and UVICORD OPTICAL DENSITY RECORDINGS:

Urinary albumin: Monkey intravenous Hb. No 1

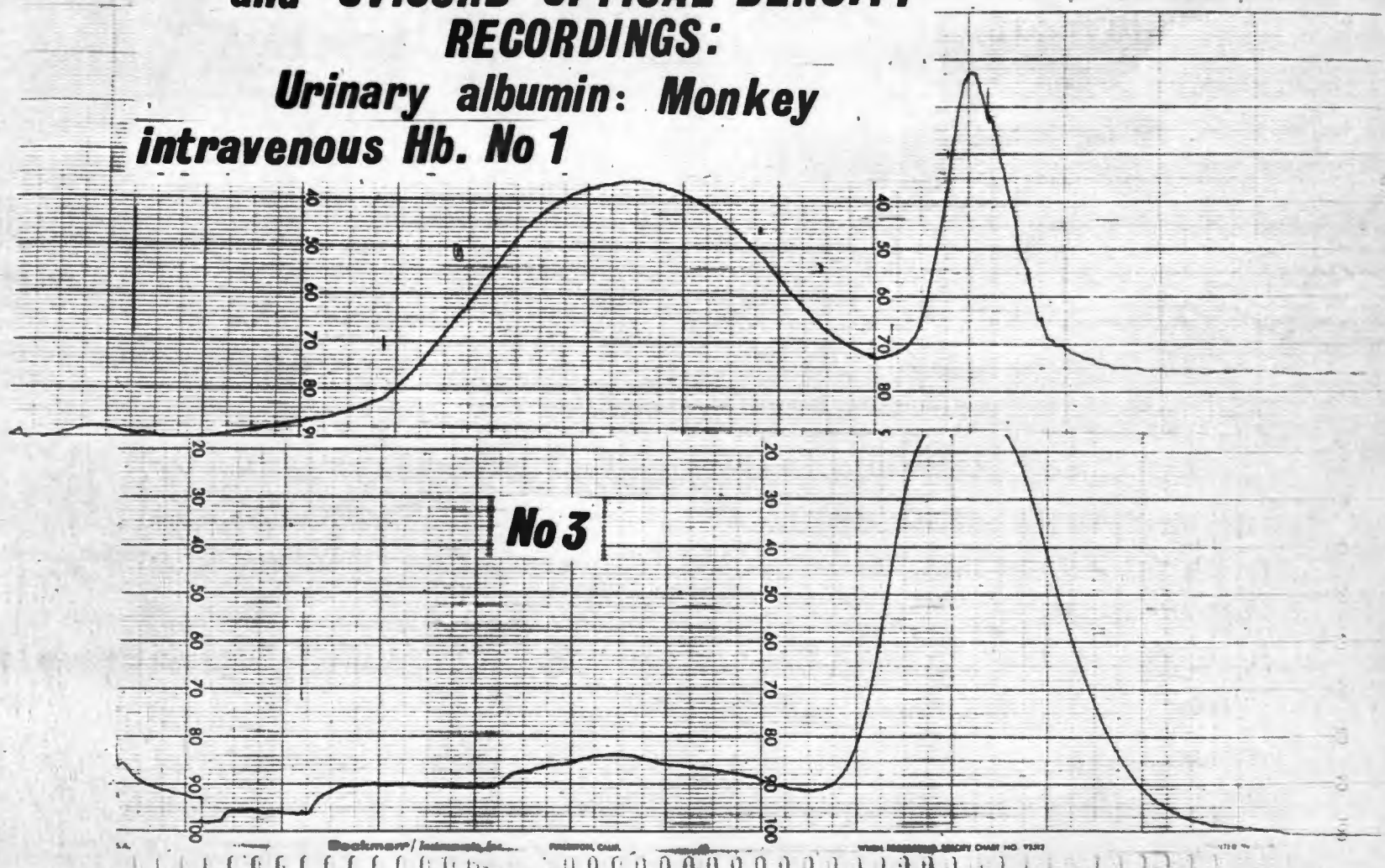
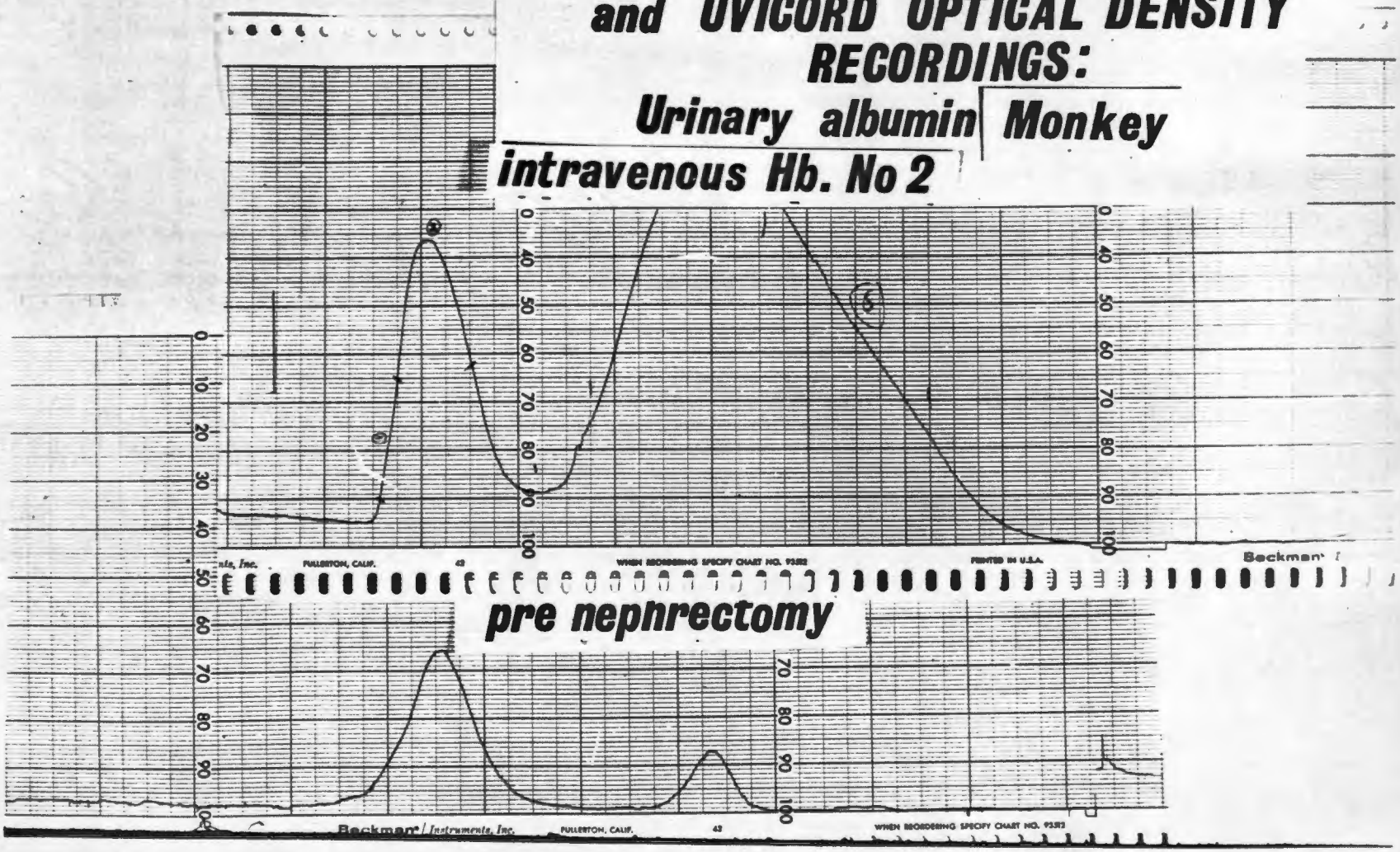


FIG 36a

SEPHADEX G-75 SEPARATION and UVICORD OPTICAL DENSITY RECORDINGS:

Urinary albumin | Monkey intravenous Hb. No 2



of the low-molecular albumin. The quantity of the latter in the urine was much greater following haemoglobin infusion. The reasonable interpretation would be that this effect was on the renal tubular absorption of the albumin, since it is difficult to envisage a direct effect on synthesis in the liver, or wherever the minialbumin is formed. A tubular mechanism appears to be the most likely one involved, since it occurred after heavy infusions of haemoglobin, although at that time the elimination of haemoglobin itself had fallen away. (See Fig. 36).

II 9 (iv) Second nephrectomy on the monkey plus peritoneal dialysis.

For further evidence as to the source of the minialbumin it was decided to remove the left kidney of the monkey, thus completing a bilateral nephrectomy.

The operation was performed three days after the third and final injection of haemoglobin. This period was considered long enough for the monkey to recover from the effects of the injection.

The animal was anaesthetized intravenously with 2 ml. of a sodium pentothal solution ($2\frac{1}{2}\%$ in H_2O) and the kidney removed. At the same time, an indwelling teflon catheter was placed in the peritoneal cavity and the wounds closed. The catheter was placed so that the free end emerged from the wound, in the side

of the animal, and led under the skin, finally emerging through the skin over the spine. The skin was firmly sutured around the catheter, and the free end of the catheter, about 5 inches long, was bound along the ridge of the back. These precautions are very necessary so that the monkey could not cause any complications by tampering with or removal of the catheter. The operation was performed on a Monday morning and a full 24 hours lapsed before the first peritoneal dialysis was undertaken.

Post-operatively, the monkey fared very well, and by the next day was sitting up in his cage. It was envisaged that 4 l. of dialysis fluid could be exchanged peritoneally during each dialysis session. Each litre would be administered individually by means of the catheter, allowed to remain in the peritoneum for 1 hour and then run out into a collecting flask. We calculated that this procedure repeated four times would give sufficient exchange of unwanted electrolytes and other catabolites, and, at the same time, provide a fluid in which circulating minialbumin might be detected.

Composition of the dialysis fluid.

The solution that was used for dialysis was commercially available and came in one litre flasks. The flasks were fitted with rubber stoppers so that a drip-set could be easily attached and the fluid run in under gravity.

The "Dianeal" peritoneal dialysis solution contained 1.5% dextrose - 150 g. of dextrose per litre. Electrolytes were present in the following quantities:-

Sodium : 142 m.eq./l.
Magnesium: 1.5 m.eq./l.
Lactate : 45 m.eq./l.
Calcium : 3.5 m.eq./l.
Chloride : 101 m.eq./l.
Bisulphite: 1 m.eq./l.

The solution contained no potassium ions as retention of these constituted a major hazard to the experimental subject, and its removal at maximum efficiency was imperative. Blood urea levels were also extremely critical in cases of bilateral nephrectomy, and it was for the prevention of high blood urea concentrations that 4 l. of the fluid were required for exchange during each dialysis session.

Administration of the peritoneal dialysis fluid.

The dialysis fluid was administered from a "vaculitre" bottle by means of a drip-set. This consisted simply of a teflon tube running from the fluid into the catheter and the dialysis solution flowing in under gravity.

Once the fluid has been administered and allowed to remain in the peritoneum for an hour, the catheter was unplugged and the

fluid ran out into a bottle below the animal in order to obtain a good siphoning action. We had some difficulty with the removal of the dialysate fluid as the catheter frequently became blocked due to pieces of clotted lymph. With care, we managed to administer and recover 3 l. of dialysis fluid on three occasions.

Course of the dialysing procedure.

As has been already mentioned, the first dialysis session took place on the day following the nephrectomy. It was hoped to dialyse the animal on alternate days, but, although this procedure was followed, the monkey died 6 days later unexpectedly. We thus dialysed the animal on three occasions, i.e. 2nd, 4th and 6th days post nephrectomy. Each time the procedure described was followed and the dialysis fluid collected and investigated for its protein content.

As the dialysis was performed on alternate days, each portion of fluid collected was analysed immediately and no storage was necessary.

Analysis of the peritoneal dialysis fluid.

The main objective now was to investigate the peritoneal dialysate for abnormal albumins. We reasoned that if the mini-albumin was not a product of abnormal renal tubular function, then

it should still be manufactured in the bilaterally nephrectomised monkey and be present in the dialysis fluid. Following the analytical procedure described earlier, all specimens of dialysis fluid were examined.

Minialbumin was found unequivocally to be presented in all peritoneal dialysates, and the amount present did not appear to fall during the 6 days subsequent to complete nephrectomy. Moreover, when a single dose of 6 mg. cadmium was injected intravenously at 10 p.m. on 5th day post nephrectomy, there was a considerable rise in output of minialbumin in the peritoneal dialysate collected 10 hours later: the rise in concentration was from 110 mg./100 ml. to 180 mg./100 ml. dialysis fluid. (See Figs. 37, 38 and 39).

FIGS 37, 38

SEPHADEX G-75 SEPARATION and UVICORD OPTICAL DENSITY RECORDINGS:

Monkey albumin: POSTMORTEM SPECIMENS

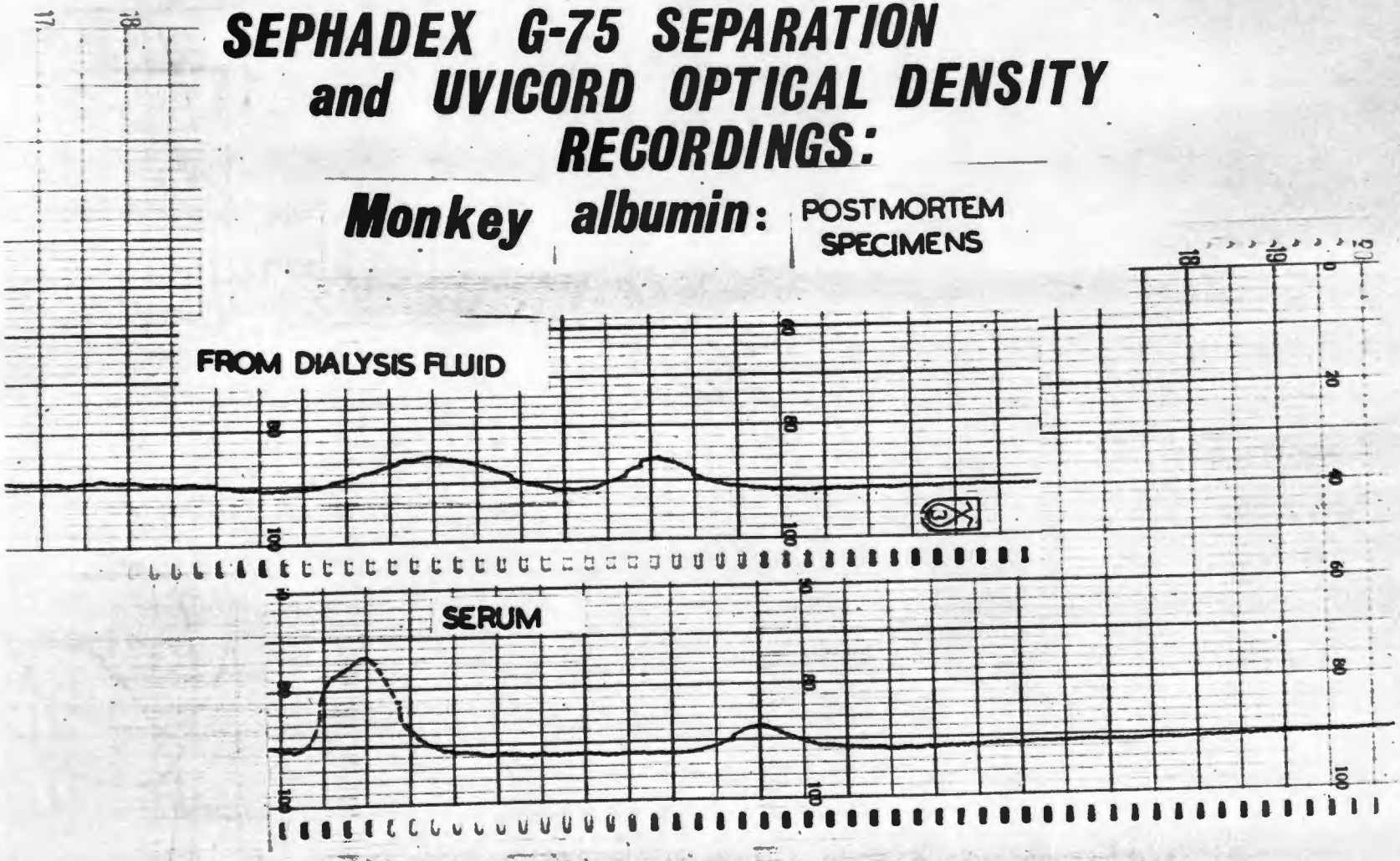
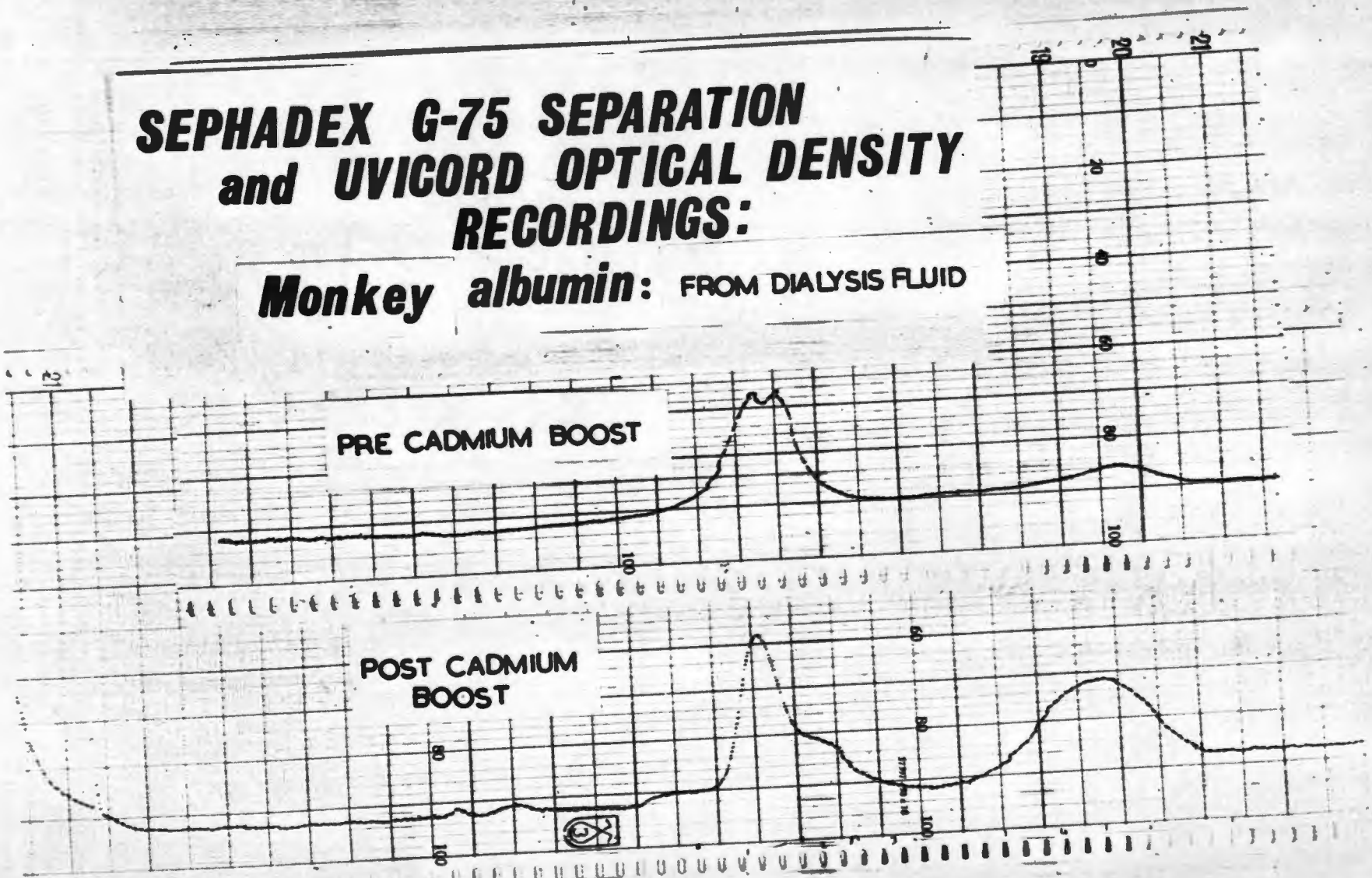


FIG 39


SEPHADEX G-75 SEPARATION
and UVICORD OPTICAL DENSITY
RECORDINGS:
Monkey albumin: FROM DIALYSIS FLUID
PRE CADMIUM BOOST
POST CADMIUM
BOOST

PART III.

DISCUSSION.

In the previous section, experiments are described which were undertaken to shed more light on the nature and origin of the urinary protein excreted by man and animals poisoned by cadmium. These questions are still not resolved definitively although the present work has clarified a number of aspects of the problem, which may now perhaps, be placed with the context of the whole picture of cadmium poisoning in man.

The signs of cadmium poisoning are very much influenced by the route of entry of the metal into the body. Cadmium compounds taken by mouth cause severe gastroenteritis, whilst chronic inhalation of cadmium oxide dust or fume gives rise to severe emphysema leading often to cor pulmonale. In all types of cadmium poisoning, proteinuria may be found. No strict correlation was observed between the degree of emphysema and that of proteinuria, which appeared to exclude lung tissue as a source of the urinary protein. Typically, too, extensive necrotic lesions of tissues lead to urinary excretion of mucoproteins, such as the Tamm Horsfall protein of much higher molecular weight than is encountered in cadmium poisoning. More recent studies have shown that a similar type of proteinuria occurs when no lung lesions are observed. This again points to a dissociation between the emphysema of cadmium workers and the urinary protein excreted.

As regards the nature of the protein itself, some investigators have stressed the non-specificity of the pattern as a whole

(Creeth et al), whilst others (Kench et al) have found particular interest in the protein which migrates electrophoretically as albumin does in serum. The molecular weight of the protein excreted by the cadmium poisoned monkey as now measured is approximately 20,000, rather less than the 25 - 30,000 value reported for the urinary protein of cadmium workers, and even less than that reported (36,000) for the traces of albumin detectable, by special procedures, in normal human urine. On dextran gels, such as Sephadex G 75, the urinary albumin fraction of poisoned monkeys can be separated cleanly into low and normal molecular albumins, and in this case, as in persons suffering from nephritis, nephrosis or mercury poisoning, the larger albumin has a molecular weight similar to that of normal serum albumin. Electrophoretically, the two albumins behave similarly, and they are alike in amino acid composition, with small differences in contents of lysine and cysteine. Such a finding is difficult to reconcile with the observations of Press and Porter who have, following enzymic degradation of serum albumin, isolated fragments as large as 7,000 molecular weight much more basic than the albumin molecule as a whole.

Their work would appear to exclude the possibility that a minialbumin could be a subunit of the larger normal serum albumin, which could arise during synthesis or degradation of the normal prototype. This aspect of the problems is of obvious importance, and many more investigations are needed on the amino acid composition

of individual low molecular albumin preparations.

Separation of the albumins by the trichloracetic acid acetone procedure has consistently produced an albumin fraction free of globulins. Where traces of α_1 globulins have been detected, this has been due to faulty technique - in particular, carrying away a small protein of the globulin precipitate along with the supernatant albumin solution. The antigenic behaviours of the low molecular albumin, towards Coombs antihuman serum or to specific rabbit antimonkey serum albumin, has been indistinguishable from that of its larger counterpart, both in immunoelectrophoresis and on Ouchterlony plates. The antigenic sites of normal serum albumin appear, therefore, to be present in minialbumin. We have not, however, so far checked whether traces of globulin contaminants can be detected by challenging the preparations with rabbit anti whole monkey serum proteins. This is necessary and about to be done: Coombs antihuman serum does not give a precipitation reaction with monkey serum globulins although no contamination was detected in urinary albumins prepared likewise from cadmium poisoned workmen. The evidence so far available, and much of it is described in the present thesis, consistently favours the view that we are dealing with a true albumin. Whether it is closely related to the metabolism of normal serum albumin remains yet to be established. The rate of urinary excretion of minialbumin was boosted following massive

infusion of haemoglobin, which suggested that reabsorption in the renal tubular cells could play an important role in its urinary excretion. This is circumstantial evidence only, and the normal molecular albuminuria was not aggravated by this manoevre. When both kidneys had been extirpated, minialbumin was found in the peritoneal dialysis fluid 6 days later, the amount did not fall off rapidly with time as would be expected if it had originated from the renal tissues, and its concentration in the peritoneal fluid was much elevated shortly following a cadmium injection. All this suggests that the minialbumin arises from tissues other than the renal tubular cells, and it seems logical to suppose that the liver is its source.

Usually minialbumin does not appear in the urine until the monkey has been chronically poisoned for 2 - 3 months with approximately 120 mg. cadmium. This could be understood if cadmium had a primary deleterious action on the liver leading to raised circulatory levels of minialbumin, some impairment of renal tubular reabsorption occurring at a later stage of poisoning to allow the escape of the protein into the urine. The observed experimental fact, that C^{14}-labelled minialbumin injected intravenously into a normal monkey, or as heretofore (Kench et al) into rabbits, was rapidly cleared through the kidney, is not in conflict with the above proposals. The peritoneal dialysate of a normal monkey contains no detectable minialbumin, and presumably,

therefore, very little is formed, circulating or reabsorbed in the renal tubular cells. The quantity of minialbumin excreted by the poisoned animal has been of the order of 35 mg./day. The injected dose administered as a single pulse into a normal animal was 80 mg. which exceeds the amount of minialbumin presented to the renal tubular cells over a period of 2 days rather than the short time (2 minutes) required for the injection. Many aspects of the biochemical mechanisms leading to the production of minialbumin remain to be explored.

One would like, especially, much more information on the following:

1. The amino acid composition and primary structure of minialbumin and the terminal amino acids of the protein, as determined on specimens produced at low and high intensities of cadmium poisoning: this would indicate whether cadmium produces a specific biochemical lesion.

2. The precursor relationships, if any, between minialbumin and normal serum albumin in the biosynthetic or catabolic pathways.

3. If the minialbumin arises due to aberrant synthesis, what is the genetic mechanism involved - incomplete production of albumin molecules on a normal ribosomal RNA-template, or abnormalities arising in nuclear DNA, messenger RNA or at some other point in protein biosynthesis, or even, perhaps, facilitation

of operation of a genetic code normally in abeyance?

4. The specific part played by cadmium ions at intoxicating levels in cells and maybe revelation of a physiological role of the ion in regulation of protein metabolism: mutual interrelations with other cations within cellular structures, such as mitochondria, microsomes, lyzosomes and in the free cytoplasmic fluid compartment.

These larger issues will come under review in turn (Kench and Sutherland - personal communication), but indulgence in further speculation at this time would not seem to be profitable. What we hope is that cadmium may provide an important experimental tool for the probing of the metabolism of serum albumin in man and other animals.

PART IV

CONCLUSIONS.

The conclusions that can be drawn from the results of the investigations described in this thesis may be listed under a number of headings as follows:

1. From its behaviour variously in T.C.A.-acetone, on immunoelectrophoresis, in Ouchterlony plates and from its amino acid composition (established in earlier work), the low-molecular protein component in the urine of cadmium workers, which migrates electrophoretically on paper as does serum albumin, is in all these respects, apart from molecular weight, similar to human serum albumin. Therefore, it has been named minialbumin.

2. No minialbumin could be detected in the urine of patients suffering from non-specific renal tubular damage, as for example, an advanced case of hepatolenticular degeneration, Fanconi syndrome, acute tubular necrosis or nephrotic syndrome. This was a very small series, and unfortunately, no further cases could be traced. The question whether minialbumin may be found in such patients in the future by the methods here described must be left open at present.

3. Rabbits, dogs and monkey can be chronically poisoned by intravenous injection of cadmium chloride and, after some months, these animals too excrete minialbumins closely similar in all the respects already mentioned with their own normal serum albumins and the normal molecular albumin found with them in the urine.

4. The minialbumins of the various species differ antigenically from one another, as do their normal prototypes: Coombs' antihuman serum will not cause precipitation with monkey albumins, and a rabbit anti monkey albumin preparation likewise is inactive against human albumin. No cross reactions have been observed.

5. Monkey minialbumin has a somewhat higher metabolic turnover than its normal serum counterpart as shown by incorporation of C^{14}-lysine and radioactive decay of the serum and urinary albumin molecules.

6. C^{14}-labelled minialbumin, when given as a large single intravenous injection into a normal monkey, is very rapidly excreted through the kidneys.

7. Infusions of homologous haemoglobin into the monkey caused increased excretion of minialbumin. The mechanism is not understood but maybe due to saturation of renal tubular systems which are responsible for cellular reabsorption of proteins from the glomerular filtrate.

8. Minialbumin was found in the peritoneal dialysate of a cadmium poisoned monkey whose kidneys had been removed 6 days previously, and the quantity of the protein which appeared in the dialysate was increased following an intravenous injection of cadmium chloride. No minialbumin can be detected in the

peritoneal dialysate of untreated monkeys.

Minialbumin was formed, therefore, in some tissue other than in the renal tubular cells, and its rapid synthesis and excretion in the poisoned monkey, suggest that it was produced in the liver and its urinary clearance expedited by renal tubular damage. These events have not yet been properly timed in relation to one another.

9. If it is assumed that all the minialbumin, found in the cadmium-poisoned monkey, is excreted in the urine, then approximately 7% of all albumin molecules synthesized in the liver will be diminutive in size. In cadmium workers, the corresponding figure is approximately 20%.

10. The overall data support the view that cadmium ions will provide a valuable tool in the study of the metabolism of serum albumin and perhaps of other proteins in man and animals.

PART V

SUMMARY.

1. Rabbits, monkeys and dogs were brought to and maintained in a state of chronic intoxication by intravenous injection of cadmium chloride. Definite clinical features were observed and a proteinuric state developed.

2. The urinary proteins were separated and a low molecular albumin as well as a normal albumin were isolated.

3. The low molecular albumin was characterised and found to behave like a true serum albumin with respect to electrophoretic mobility on paper and on acetylated cellulose columns, as well as titration curves and amino acid composition.

4. Diffusion constant determinations and ultracentrifugation for the sedimentation constant proved the molecular weight to be $\pm$ 20,000.

5. The urinary albumins, both low and normal molecular weight, and low molecular albumins found in the peritoneal dialysates in later experiments were all immunologically identical with normal monkey serum albumin.

6. C^{14}-lysine, generally labelled, was injected into a proteinuric cadmium poisoned monkey, in order to follow the metabolic turnover of the low and normal molecular species of albumin. These were separated from one another, and it was observed that minialbumin has a markedly greater turnover rate

than had normal serum albumin.

7. Infusion of homologous haemoglobin augmented the excretion of minialbumin in a unilaterally nephrectomised, cadmium-poisoned monkey.

8. Minialbumin appeared in the peritoneal dialysis fluid of a completely nephrectomised monkey 6 days following removal of the kidneys. The concentration of this protein was raised soon after an intravenous injection of cadmium.

9. These observations are discussed from the viewpoint of the biochemical effects of cadmium and the nature and origin of minialbumin in cadmium-poisoned animals.

PART VI

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PART VII

ADDENDA.

Liver and renal biopsies were performed 15 days before the death of the animal. Thin slices of tissues were immediately fixed in Carnoy's Solution.

In the kidney, the glomeruli themselves showed no definite lesions but in a few instances the epithelium of Bowman's capsule had become cuboidal and resembled that of the proximal tubule. Definite degenerative changes were evident in the proximal convoluted tubules. The cells often were pale, swollen and vacuolated. Occasionally this had progressed to frank necrosis of small groups of tubular cells, which appeared shrunken and brightly eosinophilic while the nuclei were fragmented and pyknotic. The necrotic cells were then desquamated into the lumen. Attempts at regeneration on the part of the epithelial cells were minimal. Hyaline droplet degeneration was seen in a few tubules.

The liver biopsy showed no evidence of cirrhosis. The cytoplasm of the parenchymal cells appeared pale and finely vacuolated. Occasional areas of focal necrosis were present.

The histology of the liver as examined post-mortem differed from that seen in the biopsy. Here there was often a clear distinction between the central and peripheral parts of the lobules, the cells of the former being smaller and more eosinophilic while the nuclei were shrunken. In no area, however, did this amount to an outspoken centrilobular necrosis.

Both the liver and kidney, therefore, showed evidence of damage which may be attributed to the cadmium intoxication. In the kidney the changes were unmistakable but those in the liver were less impressive and their significance much less certain. The fact that the post-mortem specimen of liver showed more severe changes than seen in the biopsy may be due to the metabolic disturbances following the nephrectomies.

LEGEND TO FIGURES 40, 41, 42, 43, 44 & 45.

Fig. 40 Kidney glomeruli are normal. Tubules show vacuolation of the lining cells and some necrotic cells present in the lining (magnification - 16 x 3.2 x 1.25 x 8).

Fig. 41 Kidney: Vacuolation of the tubular cells (Mag. 40x 3.2 x 1.25 x 8).

Fig. 42 Liver biopsy: there is no cirrhosis. Liver cells show some vacuolation. (Mag. 1.6 x 3.2 x 1.25 x 8).

Fig. 43 Liver biopsy: same field as Figure 42. (High power).

Fig. 44 Liver: Post-mortem specimen. The cells in the centrilobular region, central portion of micrograph are small and nuclei pyknotic. (low power).

Fig. 45 Liver: Post mortem specimen. The liver cells in the upper half of the field are peri-portal and those in the lower half are centrilobular. (high power).

FIG
40

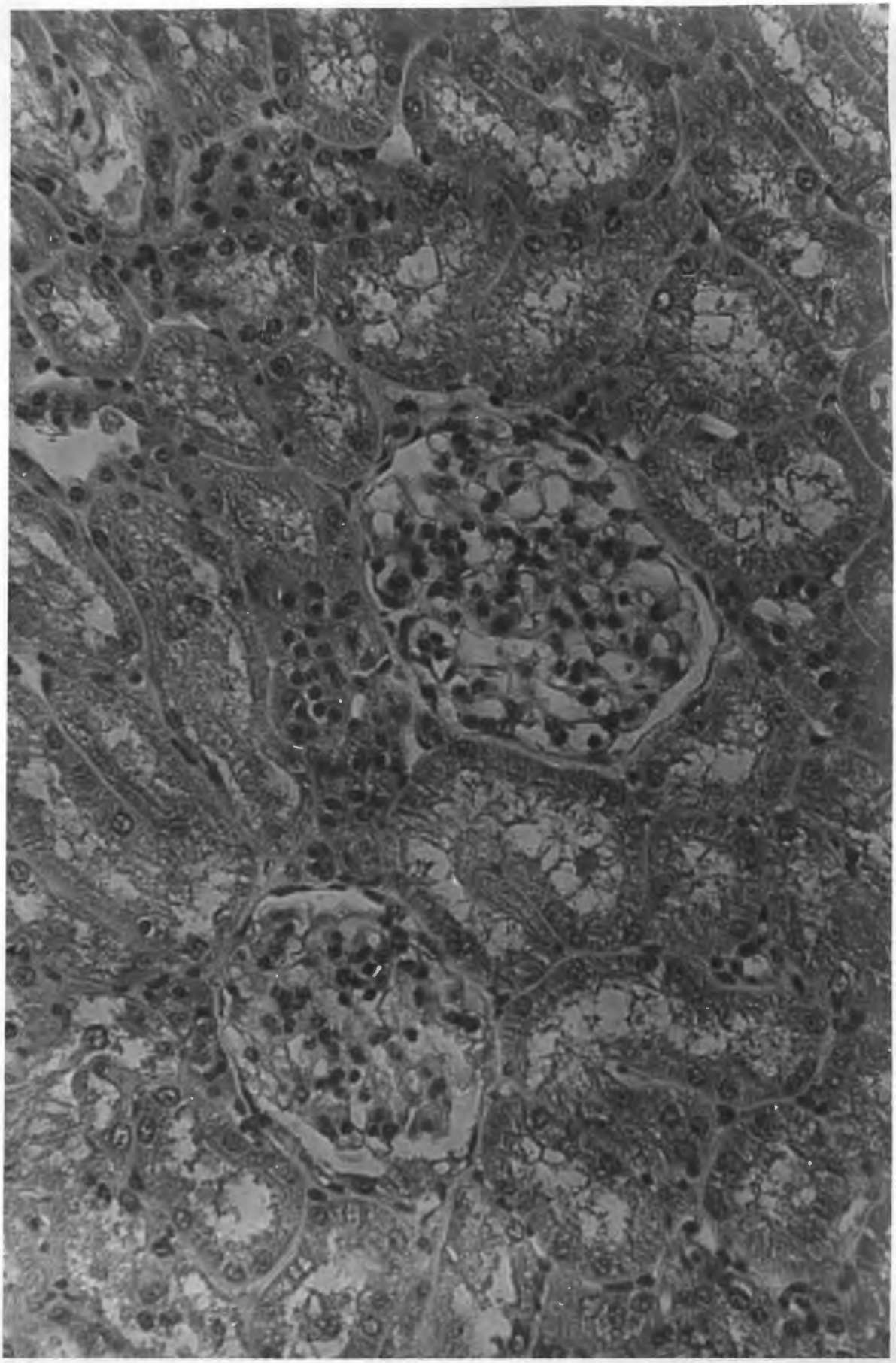

FIG
41

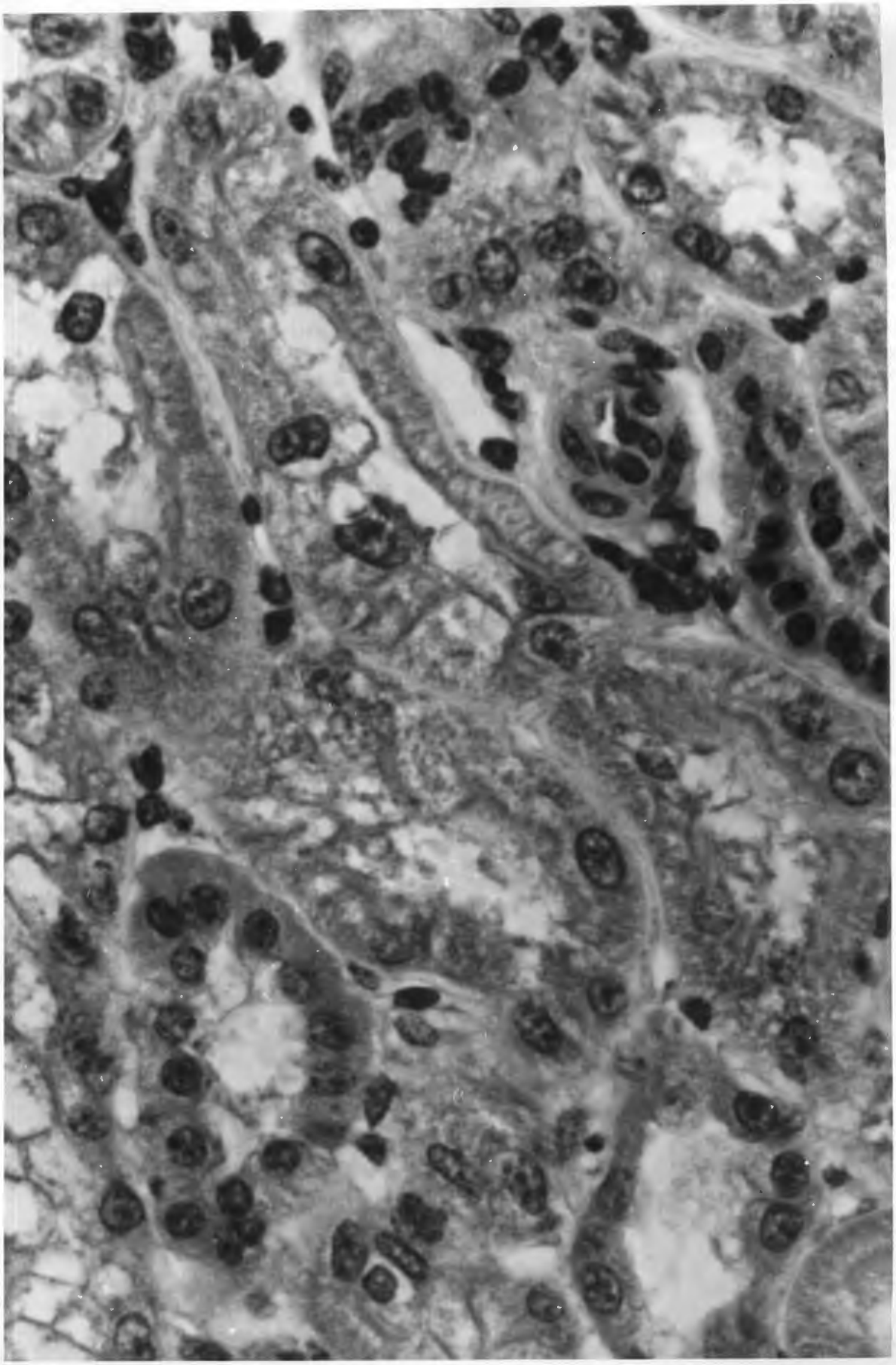

FIG
42

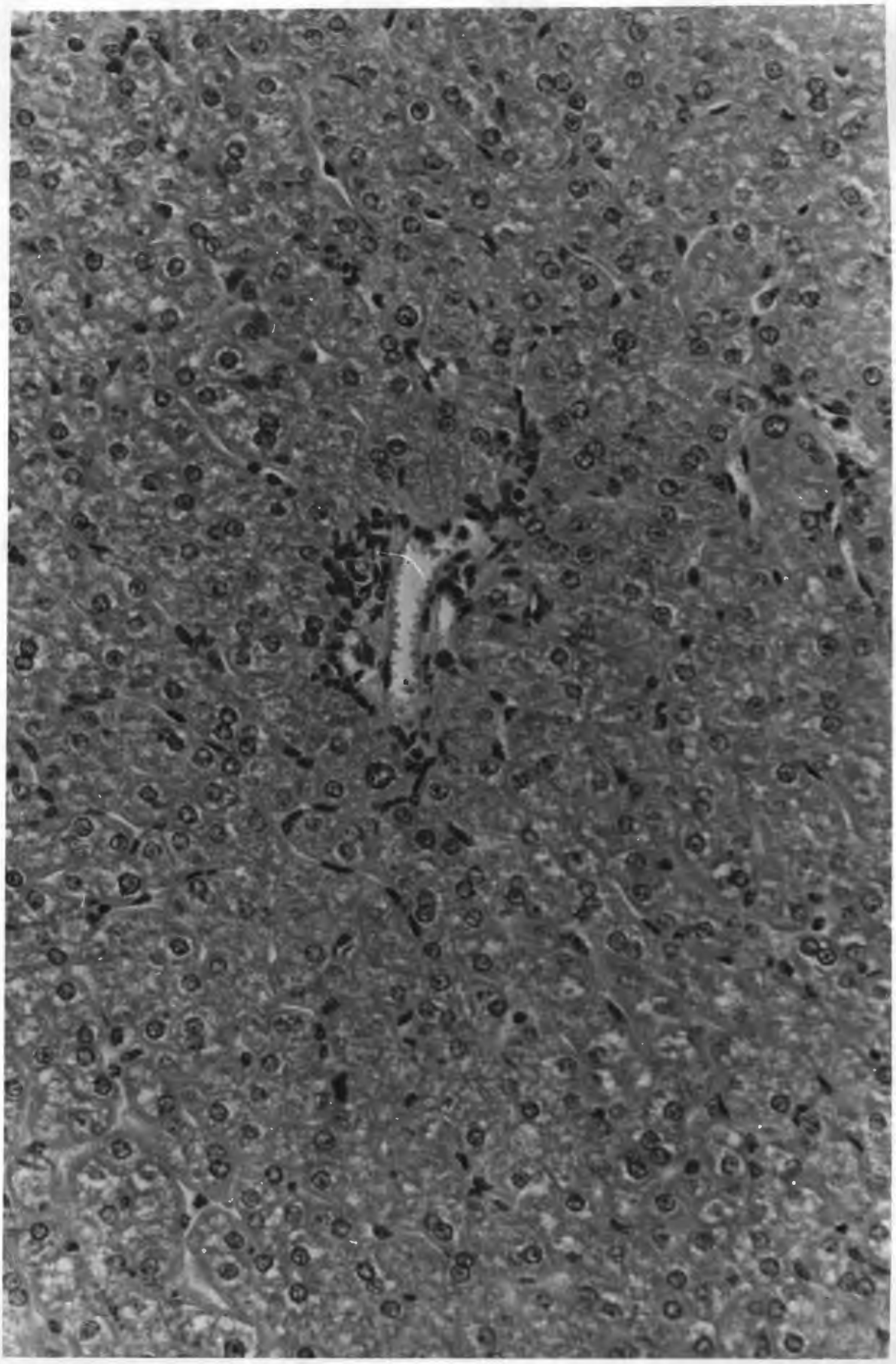

FIG
43

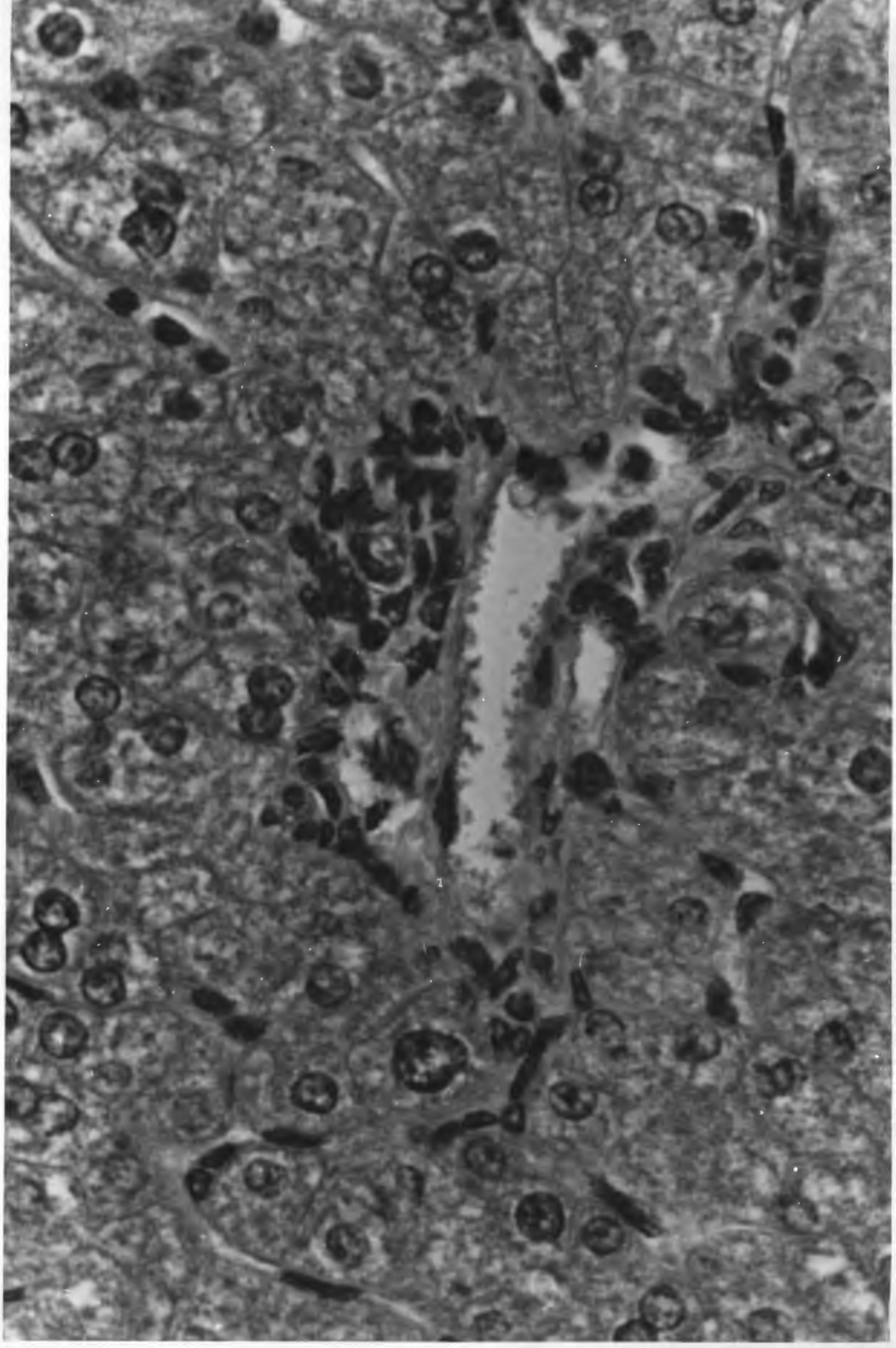

FIG
44

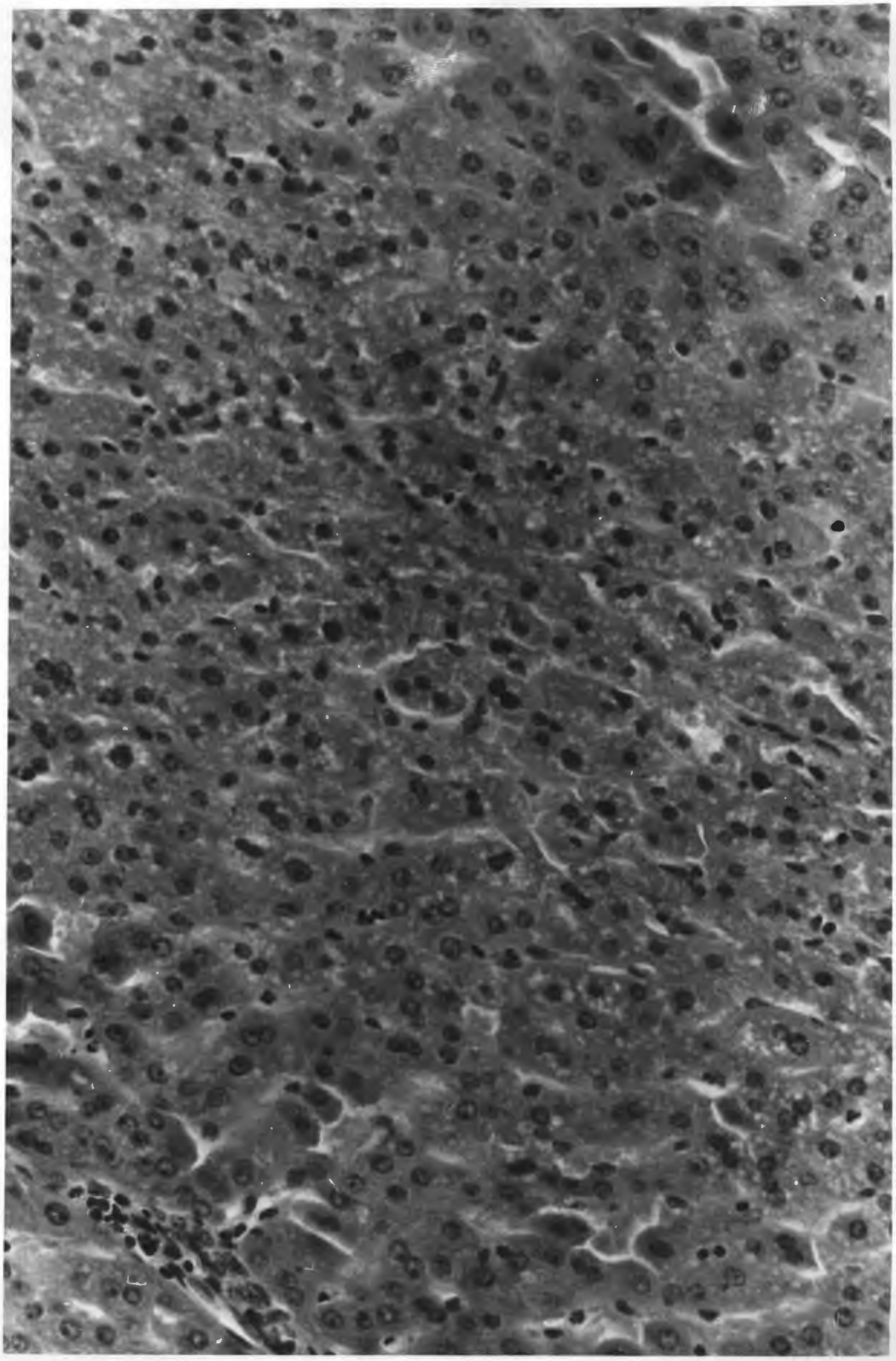

FIG
45