THE HETEROGENEITY OF ALBUMIN. A STUDY OF COMPARATIVE ALBUMIN TURNOVERS IN NORMAL PEOPLE, CANCER OF THE LIVER AND THE NEPHROTIC SYNDROME.

BY

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I hereby certify that this dissertation is my own work and that it has not been presented in fulfillment of the requirements for other degrees at any University.

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THE HETEROGENEITY OF ALBUMIN.

The abundance and easy preparation of serum albumin has made it uniquely attractive as an object of research by physiologists, chemists and physicists interested in protein metabolism. There is an extensive literature and many superb reviews of the physiology (McFarlane 1964) and physical-chemistry (Foster 1960) of albumin. Briefly, albumin comprises over 50% of the serum proteins of most animals. It is one of the smallest proteins of serum and has a molecular weight, in man, of 65,000 (Charlwood 1961) yet it is one of the largest single chain polypeptides in the body (Foster 1960). The amino-acid composition (Spahr 1964) seems to be well-defined and the only notable variability in amino-acids is the finding that 60 to 70% of albumin molecules have a highly reactive sulphydryl group capable of forming complexes with metals like mercury (Hughes 1964).

Heterogeneity of albumin was first reported by Luetscher (1939) who described multiple boundaries of serum albumin with moving boundary electrophoresis near pH 4. From this observation grew the concept of the pH-dependent isomerisation of albumin due to the presence of two major forms of albumin, at any one pH value, designated the N and F forms. This concept was developed notably by Foster (1965) and differences were found in a number of the physical properties of the albumin during the isomerisation reaction. Recently Foster et al.(1965) have proposed a model to explain this phenomenon on the basis that albumin exists as a number of stable, non-interacting components each having its own particular reactivity at acid pH. They have also succeeded in preparing subfractions of albumin having constant properties (Foster 1965). These authors were confident that the source of microheterogeneity did not lie in the fact that pooled albumins had

been used for their experiments,since even albumin from a single individual showed the same properties. They felt that the most plausible explanation was a degree of randomisation of the disulphide cross-linkages,although variation in amino-acid composition could not be rigorously excluded, nor varying interactions of the plentiful amide groups,nor some subtle "freezing" or moulding of tertiary structure.

Apart from reports of rare genetically-determined albumin variations (Gitlin 1961,Bennhold 1962) there have been singularly few reports of variations in the physiological properties of albumin. Certainly the bulk of the work on the physico-chemical structure of albumin has contributed little to our understanding of normal albumin catabolism.

A recent report (Glazer 1965) suggests that fatty acids may cause some unfolding of the peptide chain with exposure of extra tyrosyl residues. Albumin,being a highly-charged molecule, is capable of reacting with and binding a wide variety of natural and exogenous substances (Bennhold 1962).

Differences have been found in the electro-phoretic mobility of albumin from rats bearing a Walker tumour (Dufour 1959).The differences were attributed to acidic polysaccharides bound to the albumin.

Charlwood (1952) has suggested that the mean molecular weights of human nephrotic serum and urinary albumins are respectively higher and lower than normal.

Human urinary albumin occurring in normal people has been found to be smaller than serum albumin (Merler 1962).

Poulik (1960) has described proteins having the same electrophoretic mobilities on paper as the bulk of serum albumin but which run slower in starch gels. Some of these proteins are related immunologically to albumin.These proteins are absent from the serum of nephrotic children but appear in the urine.However, although not quantitated in their paper, the amounts appear to be very small, of the order of a few percent of the total albumin.

An intriguing finding by Kench and Gain (1965) of an albumin "fragment" in the urine of animals and man poisoned by cadmium is not explained by any of the known physico-chemical properties of albumin or for that matter by any of the known mechanisms of ribosomal protein synthesis.

In contrast to the meagre literature concerning the physiological heterogeneity of serum albumin,there exists a large volume of work on the metabolism of albumin which would seem to deny significant differences in the physiological properties of albumin. Indeed an almost invariable tacit assumption is made that albumin does not vary and that pooled albumin or individual albumin can be used in metabolic studies and show equivalent results.Pooled albumin, labelled with iodine isotopes or radio-active aminoacids, has been used to test albumin catabolic rates in most diseases of man (reviewed by McFarlane 1964). It has been shown that iodoalbumin does not differ significantly from biosynthetically-labelled albumin in its catabolic propensity (Cohen 1956,Volwiler 1955).

Freeman (1959) has shown that albumin,prepared by the CM-cellulose method or by Porath column electrophoresis,behaves like native albumin when tested in rabbits rendered immunologically tolerant to human albumin,whereas salt-precipitated and trichloracetic-ethanol-prepared albumin,or albumins labelled with more than 2.7 atoms of iodine to one molecule of albumin,show a denatured component which is rapidly removed.

Recently synthesised and older albumin biosynthetically labelled (in the rat) does not show differences in catabolism (*).

* This reference has been mislaid and will be inserted as soon as found (from Science)

respect.

The purpose of this dissertation is to examine the possibility that albumins from individuals may vary in their physiological properties.

The use of albumin labelled with radioactive iodine is a well-established method of estimating albumin catabolic rates (McFarlane 1964).

In adapting this technique the first requirement was to make it sufficiently sensitive to detect differences in albumin catabolic rates by eliminating as many factors as possible which would tend to cause differences in apparent catabolism irrespective of the albumin status. The range of albumin catabolic rates in normal people is fairly wide (McFarlane 1964,Hoffenberg 1966) so that comparing two albumins in different subjects would be unreliable if the differences in true catabolism were small. This objection could be met by performing two albumin turnovers simultaneously in a single individual. To make all the studies comparable the same control albumin was used (the author's serum). In order that the albumins used should be subjected to identical preparative,labelling and storage procedures,two albumins,one of which was the control, were always prepared simultaneously. The only differences in these two albumins would then be the source and the iodine label;either 125-I or 131-I.This latter difference

was partly nullified by alternating the isotope in successive experiments.

Since it is the ratio of the two catabolic rates that is important and not the absolute values, errors due to incomplete urine collection, inadequate thyroid blockage and fluctuations in iodine excretion do not cause major difficulties in the interpretation of the results.

Three groups of subjects were used in the experiments to be reported; normal people, patients with the nephrotic syndrome and patients with malignant hepatoma. The basis for selecting these categories of patients for study was the following. The albumin of normal patients, i.e. with no obvious defect in albumin metabolism, had to be compared with the control albumin to show that only small differences existed in catabolic rates between normals. The nephrotic patients were selected as an example of situation where albumin is synthesised rapidly (Gitlin 1956, Kaitz 1959) by an unaffected liver due to the urinary leakage of albumin. The hepatoma cases were selected since it is possible that the tumour might be producing an albumin (Campbell 1957) which is abnormal; a situation perhaps analogous to multiple myelomatosis where abnormal gamma-globulins are produced by malignant plasma cells.

Methods and materials.

1. Protein concentrations.

Serum proteins and the albumin content of the preparations were determined by the biuret method (Wooton 1964) using a standard of commercial albumin accurately determined by Kjeldahl nitrogen analysis (Wooton 1964).

The albumin content of serum was determined by microzone electrophoresis using the Beckman System (Model R 101).

2. Preparation of pure radioactive iodoalbumin.

A number of different techniques, including Porath column electrophoresis, CM- and DEAE-cellulose columns, starch block, agar block, Sephadex block and Cohn fractionation were used but eventually electrophoresis of whole serum in agar columns was found to be the most suitable technique. This method was suggested to the author by Dr.Polson (U.C.T. Virus Research Unit).

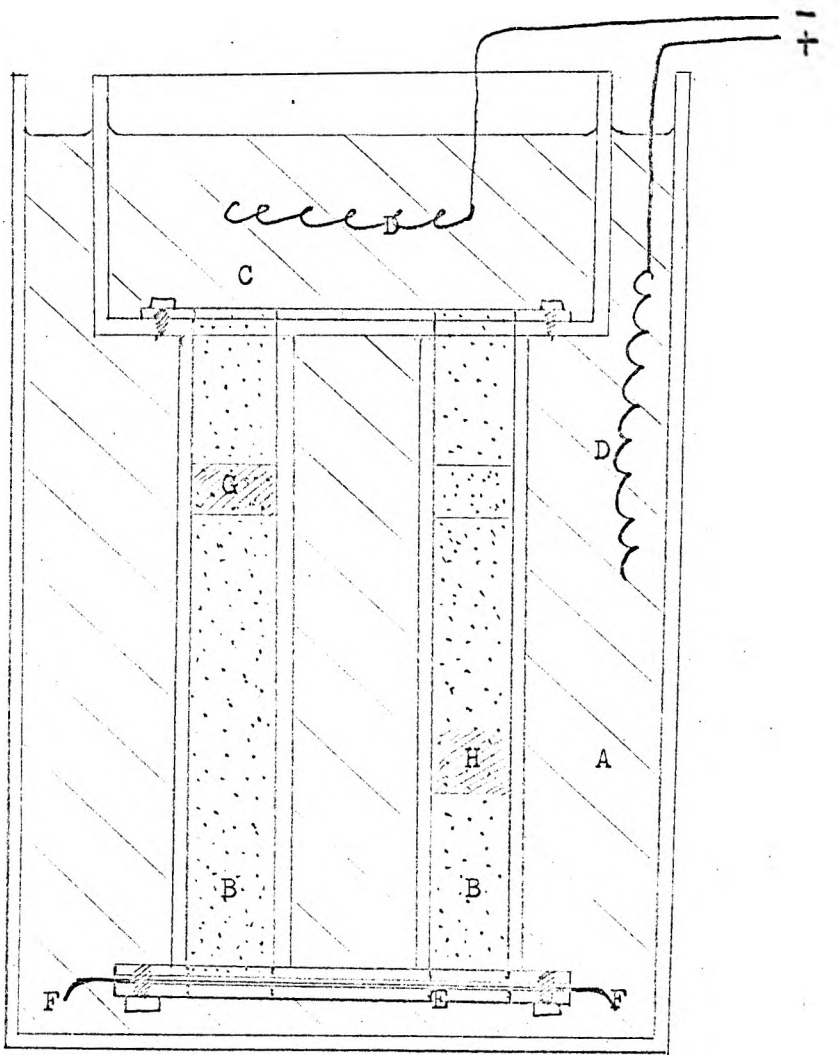
Using this technique two albumins could be prepared simultaneously at 4°C without using elaborate apparatus. In addition there is no artificial selection of albumin zones and all the albumin is represented. It has been shown that using DEAE-cellulose columns (Stokrova 1963) physico-chemical differences occur in early- and late-eluted albumin.

a) Serum. Blood from the control (the author) and the test subject were obtained at the same time and the serum separated without delay. Thereafter the serum was kept frozen at -20°C or kept at 4°C prior to use.

b) Electrophoretic apparatus (Figure 1). The apparatus consisted of two perspex tubes 1" in diameter and 9" long joined to the base of a small reservoir and open at the other end into another lower reservoir. To prevent hydrodynamic flow the lower reservoir was made large enough to enclose the upper reservoir so that the fluid levels in the two could be equalised. Prior to use the upper reservoir with its two protruding tubes was clamped to a retort stand and the lower apertures of the tubes closed with a piece of filter paper and a perforated perspex plate.

Oxoid Ionagar No.2 was autoclaved to 10 lbs/sq.inch for 15 minutes in sufficient buffer (glycine 0.1M, sodium chloride 0.04M, pH 8.3) to give a concentration of 0.9%. The clear solution was allowed

Figure 1. DIAGRAM OF THE AGAR ELECTROPHORESIS APPARATUS.



A Outer or lower reservoir containing buffer

B Tubes containing agar (0.9 %) connecting upper to lower reservours

C Upper reservoir containing buffer

D Platinum wire electrodes

E Base plate

F Filter paper

G Sample in agar

H Yellow albumin band at end of run

to cool to about 45°C and then poured into the two columns until two inches from the top. The agar solidified when cooled to 25°C. A small amount (30 ccs) of agar solution was allowed to evaporate down to 1/3 of its former volume in an open beaker on a hot-plate and was then cooled to 38°C (final concentration 2.7%). Two or four millilitres of serum were warmed to 38°C momentarily and then rapidly mixed with exactly half its volume of the concentrated agar and poured onto the column where it rapidly solidified. This operation must be carried out rapidly, as once solidified, the mixture could not be reliquified without heating to 100°C. More 0.9% agar at 37°C was poured on top of the serum agar and when solidified the whole apparatus was assembled and filled with buffer (same as above) at 4°C. The apparatus was then placed in a constant temperature cupboard at 4°C and the electrodes connected with the anode in the outer or lower reservoir. Albumin then migrated downward against the cathodic hydronium ion flow. A current of 50 mA (50 to 100V) was then applied for 18 to 24 hours. The yellow-coloured albumin migrated as a band not much wider than the zone of application and was easily seen even if only 20 mg.of albumin was being run. At the end of the run the apparatus was disconnected and the agar tube slid out of its column. The yellow albumin band was excised and then frozen in a test tube in an acetone/dry ice mixture. It was then thawed and the denatured gel spun down in a cold centrifuge at 2°C at 15,000 r.p.m. for 30 minutes. The albumin was recovered in the clear yellow supernatant. The yield of albumin was approximately 50 to 80% of that expected when serum was used, but with bromphenol-blue-stained, pure commercial albumin, the recovery was over 95%. The difference was probably due to an albumin "tail" produced when other serum proteins were simultaneously electrophoresed.

This albumin was then quantitated and 10 or 20 mg. used for labelling. Cellulose acetate electrophoresis was performed on all samples and showed that normal and "hepatoma" albumin was over 95% pure and that "nephrotic" albumin contained 5 to 10% of alpha-1 and alpha-2 globulins.

c) Labelling with radioactive iodine.

The iodine monochloride method of McFarlane (1963) was used to label the albumin. 125-I and 131-I samples of high specific activity were obtained from Amersham. A constant labelling ratio of one atom of iodine to one molecule of albumin was used. As the efficiency of labelling ranged from 40 to 80% this means that the actual labelling ratio was 0.4 to 0.8 atoms of iodine to one molecule of albumin. It was found to be unnecessary to pre-oxidise the albumin at pH 4.5 with iodine to obtain this high labelling yield. Free iodide was removed either by passage through a short column of Sephadex G 25 (Pharmacia, Uppsala,Sweden) or by electrophoresis (see below). Several samples of iodoalbumin contained more than an acceptable amount of free iodine when only electrophoresis was used, nevertheless these samples were used as the problem of not introducing pyrogens by further procedures was almost insuperable. It was felt that, as the free iodide would be almost completely excreted by the third day, it would not affect the results especially if comparable amounts of free iodide were present in both test and control iodoalbumins and it can be seen (Table 1) that high initial (days 1 and 2) excretions usually ran parallel in both test and control. The problem of deionising albumin efficiently without introducing pyrogens remains a formidable one.

d) Purification of the iodoalbumin. The iodoalbumin was again electrophoresed in the agar column apparatus as described previously. Occasionally it was necessary to concentrate the specimens slightly by partial freeze-drying. The only modification necessary was to mix the serum with 2.7% agar in distilled water as the albumin was already in buffered solution. Failure to do this resulted in wide albumin bands. After the run the iodoalbumin was located visually or by a hand-held geiger-counter. Electrophoresis also served to remove the free iodine which migrates to the anode at a faster rate than albumin. When these purified preparations were electrophoresed on cellulose acetate and auto-radiographed on X-ray film the normal and hepatoma albumins were almost free of other proteins i.e. over 98% pure and the nephrotic albumins contained 5% or less of a contaminant. With prolonged exposure most of the other proteins of serum could be detected in all the samples. Representative densometric scans of an auto-radiographed cellulose acetate strip are shown in Figure 2.

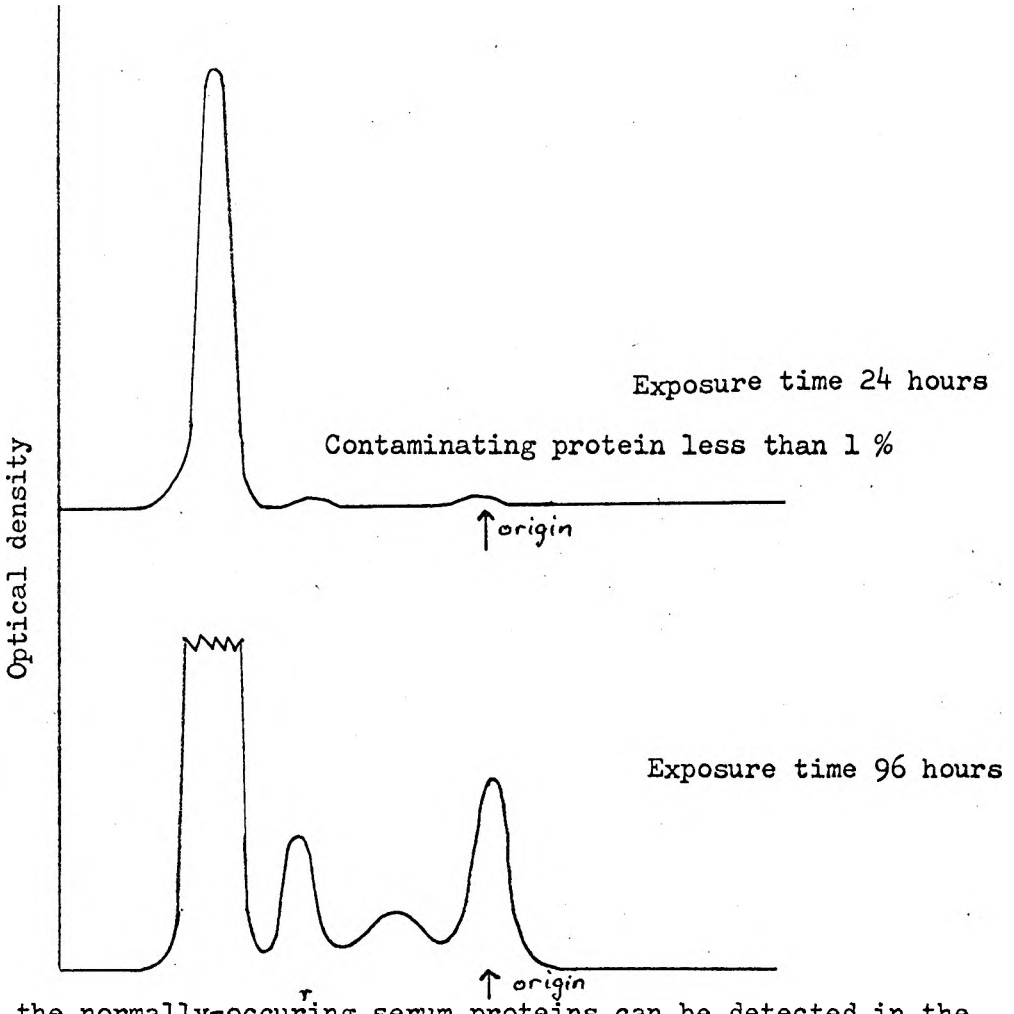
Two of the nephrotic iodo-albumins were "defatted" (Goodman 1957) and the fat extract was found to contain less than 0.01% of the iodine label.

3. Sterilisation of Albumin.

As an added precaution against pyrogens most of the latter preparations were made using only pyrogen-free water (Saphar Laboratories) in all the reagents.

Prior to utilisation a sufficient volume of each albumin was mixed and made up to about 10 ml. with pyrogen-free saline and then filtered through a Millipore filter into a sterilized container. The sterility of the sample was checked by culture.

Figure 2.DENSOMETRIC SCANS OF AN AUTORADIOGRAPHED CELLULOSE ACETATE ELECTROPHORETOGRAM OF A NEPHROTIC IODOALBUMIN PREPARATION.



Most of the normally-occurring serum proteins can be detected in the contaminating protein. All other preparations showed similar patterns. The iodoalbumin was run on inch wide cellulose acetate strips at 150 volts for 1½ hours. The buffer was veronal pH 8.6 , ionic strength 0.1.Ordinary Kodak X-Ray film in a cassette was used for the autoradiogram with the cellulose acetate strip applied directly on the film.

A tenth or more of the sample was injected into one or more rabbits specially prepared for pyrogen testing. (Mr.Kundig of Pharmacology Department and Dr.Janovicz of Saphar Laboratories provided the facilities). A number of samples containing pyrogens had to be discarded and reprepared. It was found however that rabbits commonly developed minor reactions e.g. a 1°C temp.rise, falling after 2 hours, and when these samples were injected into humans no adverse effects could be detected.

4. Dosage of isotope.

It was aimed to give each patient 25 microcuries of both 125-I and 131-I. This was achieved by counting aliquots in an Ekco scintillation crystal with a well, which had been previously calibrated using known standards of both isotopes.

5. Test procedure.

The subject was given half a teaspoon of Mist.Pot. Iodide (B.P.) twice a day for at least several days before and during the test to block thyroid uptake of radioiodine. The iodoalbumin was injected into a vein on one arm and all bloods taken from the opposite arm. Blood (5 ml.) was taken after 10 minutes and at regular 24 or 48 hour intervals thereafter. Complete 24 hour urine collections were made for the duration of the test. The amount of iodoalbumin solution injected was determined by weighing the syringe and a weighed amount of iodoalbumin was also kept as a standard in a volumetric flask.

6. Scintillation Counting.

The samples were counted in a Packard Autogamma Scintillation Counter for sufficient lengths of time to ensure statistical reliability. 4 ml.Aliquots of urine, 2 ml.amounts of serum, 2 ml. and 4 ml. aliquots of the standard, a sample of 131-I and 125-I and a number of blanks were counted. The two isotopes could

easily be separated by discrimination. 131-I Could be counted without 125-I contribution with only a 30% loss of efficiency.

7. Calculation of Turnover Data.

The equilibrium time method of Veal and Vetter (1958) was used to calculate turnover rates and pool sizes in the hepatoma cases and for the computer-calculated cases. Fractional catabolic rates, i.e. the percentage of the plasma albumin pool catabolised per day, were calculated (McFarlane 1964) for all cases. However, for practical purposes since the ratio of the fractional catabolic rates of the two compared albumins always paralled closely the more reliable and more-easily calculated ratios of daily urinary excretions, the latter was more extensively used.

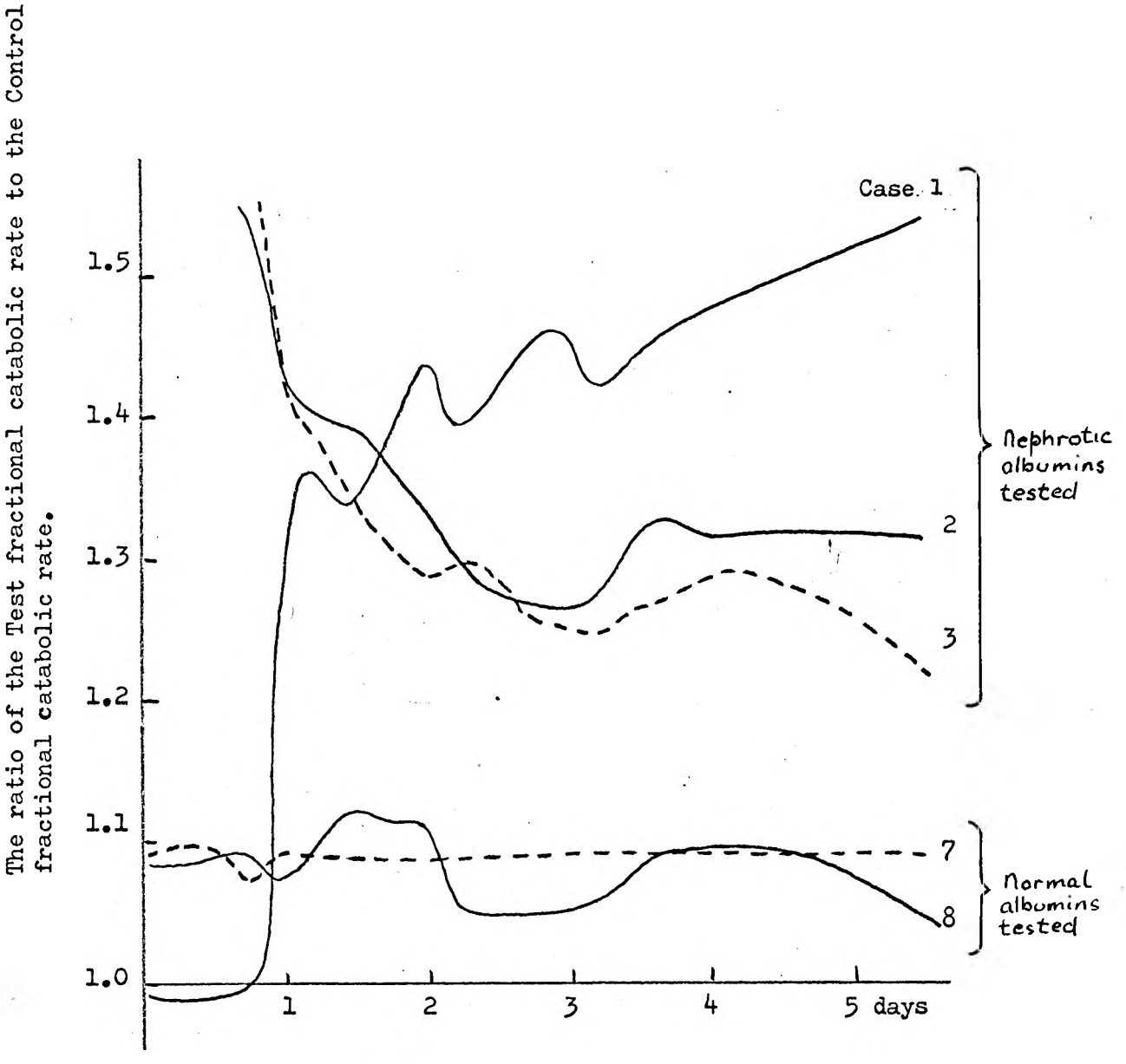
The computer program was devised (Mr.Horn, Electrical Engineering Department,Witwatersrand University), to compare fractional catabolic rates when the curves of plasma and excretory activity were smoothed. This technique was applied to several normals and to those nephrotics in whom sufficient data was available (Figure 3).

8. Selection of Cases as Albumin Sources.

a) Normals. Blood from 3 non-proteinuric adults,including the author, was used.

b) Nephrotics. Two cases of the nephrotic syndrome occurring in children and two cases occurring in adults were used. These were all clear-cut clinical and biochemical cases of subacute glomerulo-nephritis. All showed massive proteinuria at the time the blood was taken and they were all past the acute phase of the illness.Three of the cases had also been subjected to confirmatory renal biopsy.

Figure 3. THE RATIOS OF THE FRACTIONAL CATABOLIC RATES OF TEST AND CONTROL ALBUMINS USING COMPUTER-SMOOTHED ACTIVITY CURVES. (THREE NEPHROTIC AND TWO NORMAL ALBUMINS.)



Computer-smoothing of the plasma and excretory curves enabled values of the fractional catabolic rate to be calculated at 5 hour intervals.

The numbers on the graph refer to case numbers in Table 1.

c) Malignant hepatomas. Five patients with biopsy-proven malignancy who were participating as controls in a cancer of the liver treatment survey at City Deep Hospital, Johannesburg, were used.

No cases in category a) or b) had a history of jaundice or any communicable illness.

9. Selection of Cases for Turnover Studies.

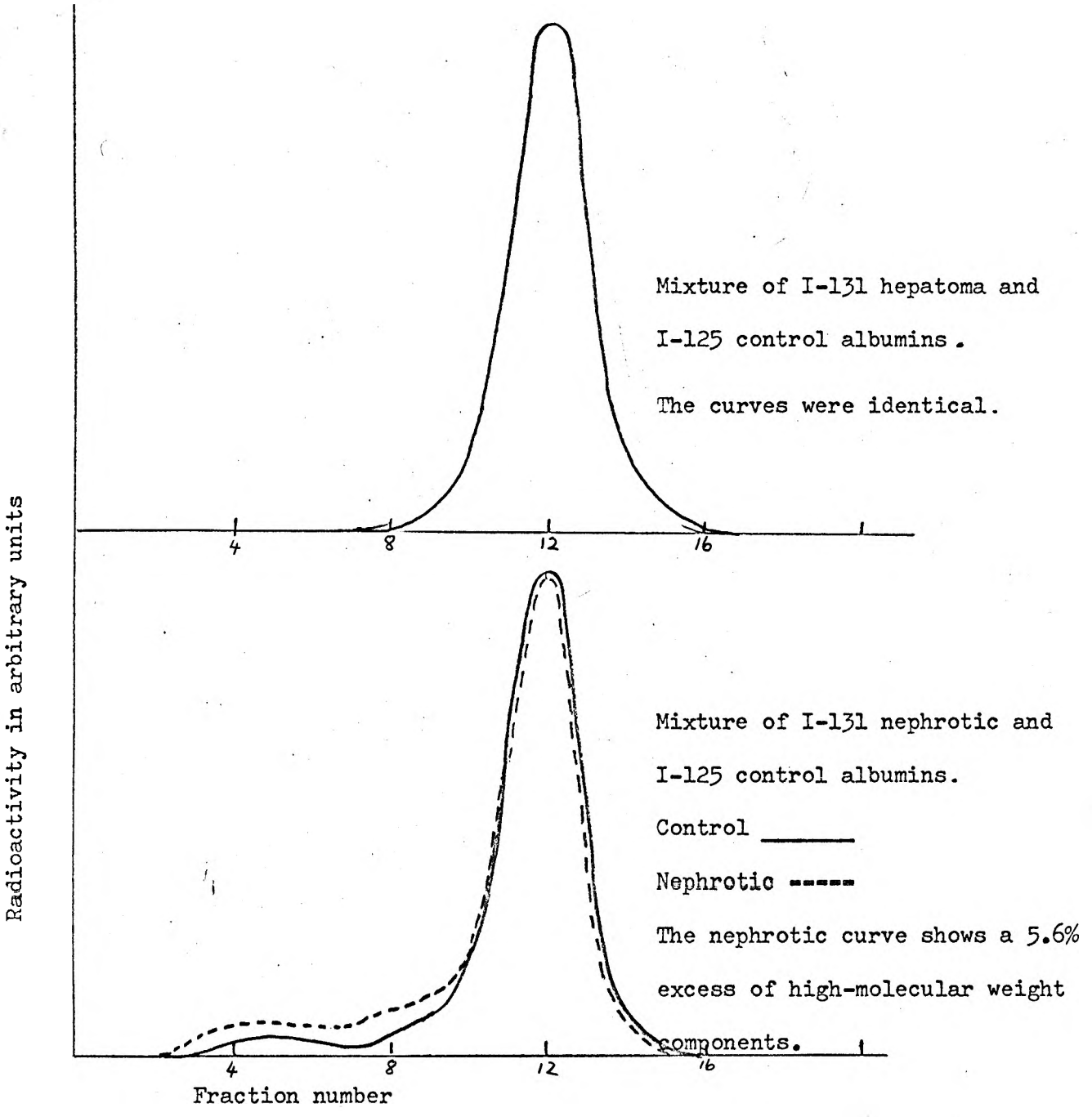
a) The double-isotope experiments, using normal and nephrotic albumins, were performed on hospital patients who were recovering from illnesses not obviously affecting albumin metabolism and who were non-proteinuric. They were all adults and volunteered to co-operate, after the test and possible hazards had been explained to them. Two cases developed pyrogen reactions early in the series but steps taken to avoid this prevented further incidents. Those two cases were excluded from the results.

b) The "hepatoma" albumins could only be tested on the donors themselves in view of the presence of jaundice and the possible viral aetiology of the disease.

10. Molecular Weight Studies on Iodoalbumin.

a) Molecular sieving. The molecular weights of several of the nephrotic and hepatoma cases were compared with the control by passing a mixture of the two iodoalbumins through a 100 x 1 cm. Sephadex G200 column (Pharmacia, Uppsala, Sweden) and counting fractions of the effluent in a Packard Autogamma Counter. The counts in each fraction were expressed as a percentage of the highest value (see Figure 4). Normal, control and hepatoma albumins showed only traces of high molecular weight components. Nephrotic albumin, the worst example of which is shown in Figure 4, contained a 5.6% excess of high molecular weight components

Figure 4. MOLECULAR-SIEVING OF MIXTURES OF IODOALBUMINS FROM THE CONTROL, A HEPATOMA AND A NEPHROTIC CASE (CASES 13,2 and 3. See Table 1)



Fractions, 5 ml., were collected from a 100 x 1 cm. column containing Sephadex G 200, using 0.15 M saline buffered to pH 8.6 with glycine buffer 0.05 M. A 2 ml. sample was applied. Radioactivity was analysed in a Packard Autogamma counter and the I-125 and I-131 separated. The arbitrary radioactivity units were obtained by expressing all counts as a % of the most active tube.

b) Ultracentrifugation. Several samples of control, nephrotic and normal albumin were analysed in a Spinco Analytical Ultracentrifuge. In no case was more than one peak seen which suggests that the technique is a good deal less sensitive than molecular sieving. Sedimentation coefficients were not determined.

RESULTS.

Table 1 lists the daily urinary excretion of radioiodine, the ratio of the test to the control excretion and the average ratios, excluding the first and second day.

The first and second days were excluded from the calculations of averages as the results on those days are disturbed by the rapid elimination of any denatured albumin or any free iodide. By the third day these processes are largely completed. Examples of this were the rapid excretions on the first two days in Cases 9 and 10 where the mixture of iodoalbumins was accidentally partially denatured by copper salts present as verdigris in the sterilization apparatus. All glass apparatus was subsequently used.

Table 2 gives details of the turnover of albumin calculated by the equilibrium time method (Veal and Vetter 1958) for the five patients with malignant hepatomas. The difference in catabolic rate of the hepatoma albumins compared to the control albumin is expressed as a percentage.

Figure 3. The ratios of the fractional catabolic rates of the test and control albumins derived from computer-smoothed plasma and excretion curves are plotted for those nephrotics and normals in whom sufficient data were available. In addition a factor causing irregularities in the first two days, when catabolic rates are calculated, is the difficulty encountered in extrapolating values on the steep part of the plasma activity curve (see Addendum).

TABLE I. THE DAILY URINARY EXCRETIONS OF IODINE ISOTOPE EXPRESSED AS A PERCENTAGE OF THE ADMINISTERED DOSE AND THE RATIOS OF THE TEST AND CONTROL EXCRETIONS IN EACH CASE.

	Test subject.	Source of albumin tested.		Day 1.	Day 2.	Day 3.	Day4.	Day 5.	Day 6.	Day 7.	Day 8.	Day 9.	Day 10.	Ratio average.
1.	Altman	Juvenile nephrotic	Test.	3.77	3.44	2.92	2.40							
			Control.	3.86	2.30	2.04	1.59							
			Ratio.	0.9767	1.4957	1.4313	1.[illegible]34							1.4703
2.	Brynius	Adult nephrotic	Test.	4.37	3.26	3.00	2.23	2.79						1.2324
			Control.	2.74	2.37	2.48	1.78	2.26						
			Ratio.	1.5948	1.3756	1.2096	1.2529	1.2346						
3.	Lee	Adult nephrotic	Test.	5.14	5.25	4.03	3.44	3.22						
			Control.	2.89	4.09	3.32	2.84	2.77						
			Ratio.	1.7785	1.2836	1.2139	1.21[illegible]2	1.1624						1.1958
4.	Jarvis	Juvenile nephrotic	Test.	6.56	4.75	2.91	2.58	2.38						
			Control.	5.24	4.22	2.70	2.31	1.76						
			Ratio.	1.2520	1.1256	1.0777	1.1168	1.3522						1.1822
5.	Pretorius	Juvenile nephrotic	Test.	3.13		6.51	4.51							
			Control.	1.91		4.31	3.24							
			Ratio.	1.6384		1.5104	1.3919							1.4511
6.	Bork	Adult nephrotic	Test.	10.57	7.48	3.68	4.28	3.45						
			Control.	12.24	7.32	3.14	3.69	2.89						
			Ratio.	0.8634	1.0221	1.1722	1.1594	1.1901						1.1739
7.	Botes	Control	Test.	4.54	5.14	4.74	3.95	3.88						
			Control.	5.04	4.95	4.57	3.76	3.74						
			Ratio.	0.9007	1.0384	1.0372	1.0505	1.0374						1.0251
8.	Signoratto	Control	Test.	2.63	3.52	4.21	4.45	2.39						
			Control.	2.90	3.26	4.09	4.14	2.27						
			Ratio.	0.9069	1.0797	1.0294	1.0749	1.0520						1.0521

TABLE 1 Continued.

	Test subject.	Source of albumin tested.		Day 1.	Day 2.	Day 3.	Day 4.	Day 5.	Day 6.	Day 7.	Day 8.	Day 9.	Day 10.	Ratio Averages
9.	Murly	Normal	Test.	16.52	9.07	3.90	2.60							
			Control.	14.98	8.55	3.64	2.50							
			Ratio.	1.1029	1.0608	1.0714	1.0400							1.0557
10.	Van Andel	Normal	Test.	35.80	12.19	5.46	3.66							
			Control.	32.07	11.51	5.15	3.50							
			Ratio.	1.1163	1.0591	1.0602	1.0457							1.0529
11.	Catto	Normal	Test.	16.90	10.14	4.87	3.87	3.37						
			Control.	15.50	10.27	5.09	4.04	3.46						
			Ratio.	0.9171	1.0129	1.0452	1.0440	1.0267						1.0386
12.	Vermeulen	Normal	Test.	6.22	9.59	4.47								
			Control.	7.09	10.27	4.55								
			Ratio.	1.1139	1.0709	1.0179								1.0179
13.	Enacio	Enacio	Test.	4.51	5.63	4.27	3.56	1.76	1.92	1.45	1.26	0.96	0.89	
			Control.	3.75	4.69	3.69	3.20	1.52	1.67	1.27	1.10	0.81	0.77	
			Ratio.	1.2027	1.2005	1.1572	1.1126	1.1579	1.1497	1.1418	1.1454	1.1852	1.1558	1.1567
14.	Phineas	Phineas	Test.	2.452	1.971	2.957	2.539	2.247	1.330					
			Control.	3.020	2.269	3.066	2.544	2.115	1.308					
			Ratio.	0.8119	0.8686	0.9645	0.9981	1.0624	1.0160					1.0102
15.	Mucinda	Mucinda	Test.	11.13	2.33	2.81	3.35	2.31	2.57	2.43	2.58	1.89		
			Control.	8.41	2.00	2.65	2.95	2.20	2.38	2.43	2.49	1.78		
			Ratio.	1.3230	1.1636	1.0589	1.1356	1.0501	1.0811	1.0014	1.0353	1.0641		1.0609
16.	Matakati	Matakati	Test.	17.13	9.45	5.31	3.99	3.66	1.24	1.78	4.22	3.40	3.22	
			Control.	16.94	9.64	5.41	4.06	3.82	1.37	1.82	4.30	3.47	3.38	
			Ratio.	1.0112	0.9803	0.9816	0.9827	0.9581	0.9051	0.9780	0.9814	0.9798	0.9526	0.9649
17.	Michael	Michael	Test.	4.93	2.37	2.61	2.48	3.07	2.98	2.09	2.81	1.97	2.81	
			Control.	5.02	2.45	2.55	2.59	3.08	2.98	2.04	2.81	2.00	2.78	
			Ratio.	0.9820	0.9635	1.0236	0.9575	0.9967	1.000	1.0245	1.000	0.9850	1.0108	1.0000

The average ratios were calculated ignoring the results on the first 2 days (see text). The control albumin was obtained from the same source (the author) throughout. It should be noted that in Cases 7 & 8 the ratios could be just as well expressed in the reciprocal form. Cases 13 to 17 had malignant hepatomas.

TABLE 2. PLASMA ALBUMIN CONCENTRATIONS AND POOL SIZES AND CATABOLIC RATES IN FIVE PATIENTS WITH MALIGNANT HEPATOMAS, USING HEPATOMA AND CONTROL ALBUMIN.

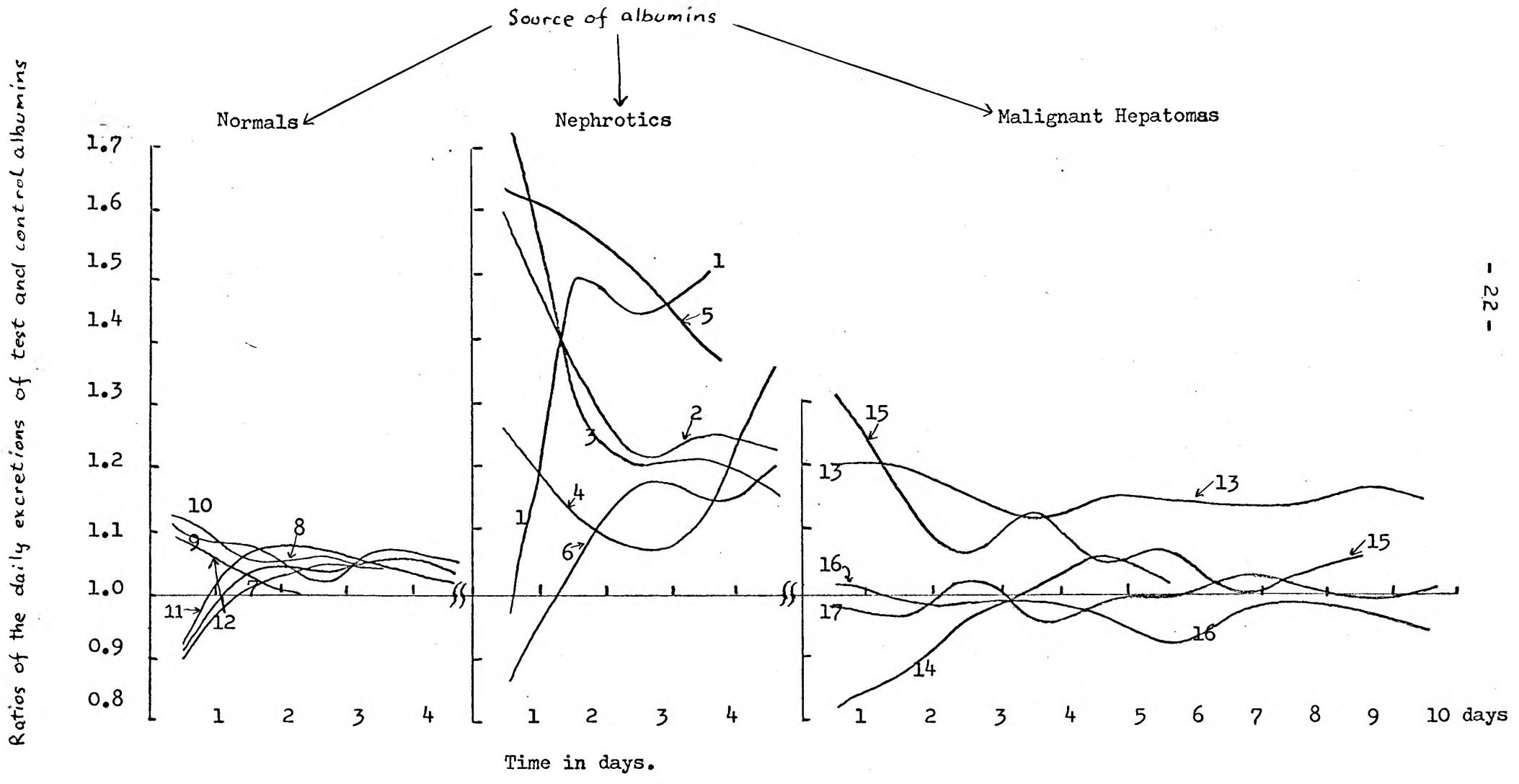
Case.	Name.	Albumin type used.	Body weight. kg.	Plasma albumin g %.	Turnover %/day(1).	Plasma albumin g/kg.	Total albumin g (2).	Daily catabolism (3) g/day	Daily catabolism (3) mg/kg/day.	Plasma volume (4) ml	Plasma volume (4) ml/kg.	Change in catabolic rate (5). %	Urine volume ml/day.
13	Enacio	Hepatoma Control	51.81	3.37	6.53 5.44	1.637	230	5.54 4.61	106.93 88.97	2570 2465	49.61 47.52	+ 20.1%	574
14	Michael	Hepatoma Control	44.54	2.92	8.49 8.49	1.476	172	5.58 5.58	125.16 125.16	2200 2306	49.39 51.71	0.0%	1224
15	Mucinda	Hepatoma Control	50.91	1.76	9.43 8.90	0.879	124	4.22 3.98	82.89 78.18	2570 2520	50.48 49.50	+ 6.0%	508
16	Matakati	Hepatoma Control	54.54	2.66	8.99 9.26	1.870	174	9.17 9.44	168.10 173.00	3781 3889	69.30 71.30	- 2.9%	281
17	Phineas	Hepatoma Control	59.10	3.01	6.22 6.16	1.090	140	4.03 3.99	68.19 67.51	2118 2191	35.83 37.08	+ 1.0%	830
	Average of 41 normals (6)	Pooled normals	61	4.12 ±0.07	9.0 ±0.3	1.64 ±0.04			148 ± 5		40.0 ±0.7		

(1) % of the plasma pool catabolised per day.
(2) Total body albumin.
(3) Daily catabolism as g/day and mg/kg body-weight/day.
(4) Plasma volume as ml/kg body-weight.
(5) Percentage change in catabolic rate of hepatoma albumin as compared to the control.
(6) Hoffenberg 1966.

NB. The average plasma volume was used in calculating results in each case. A common plasma activity curve was used in all, except Case 13, as they were almost identical.

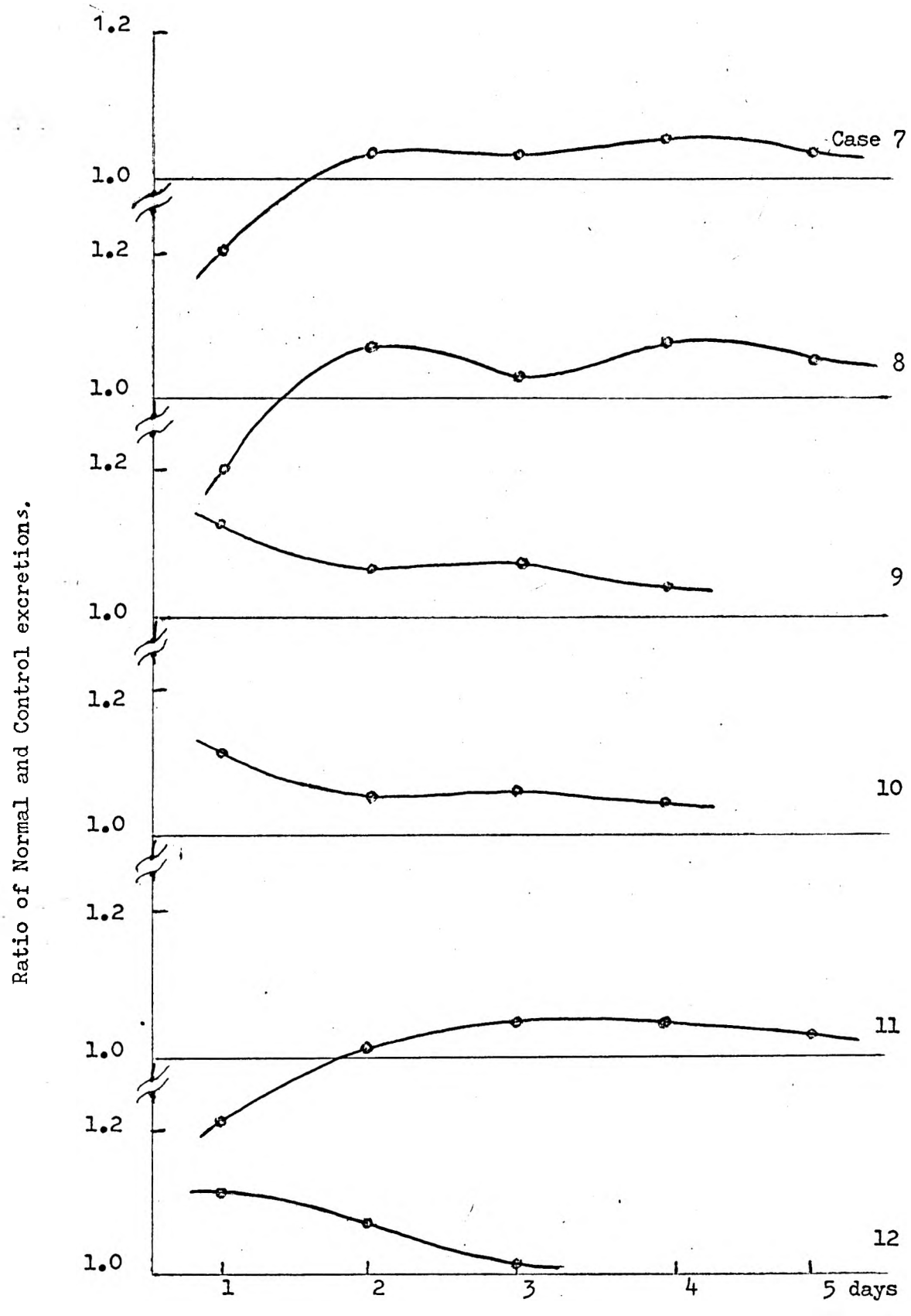
TABLE 2.

Figure 5. THE RATIO OF THE DAILY EXCRETIONS OF TEST AND CONTROL ALBUMINS IN THE THREE GROUPS.



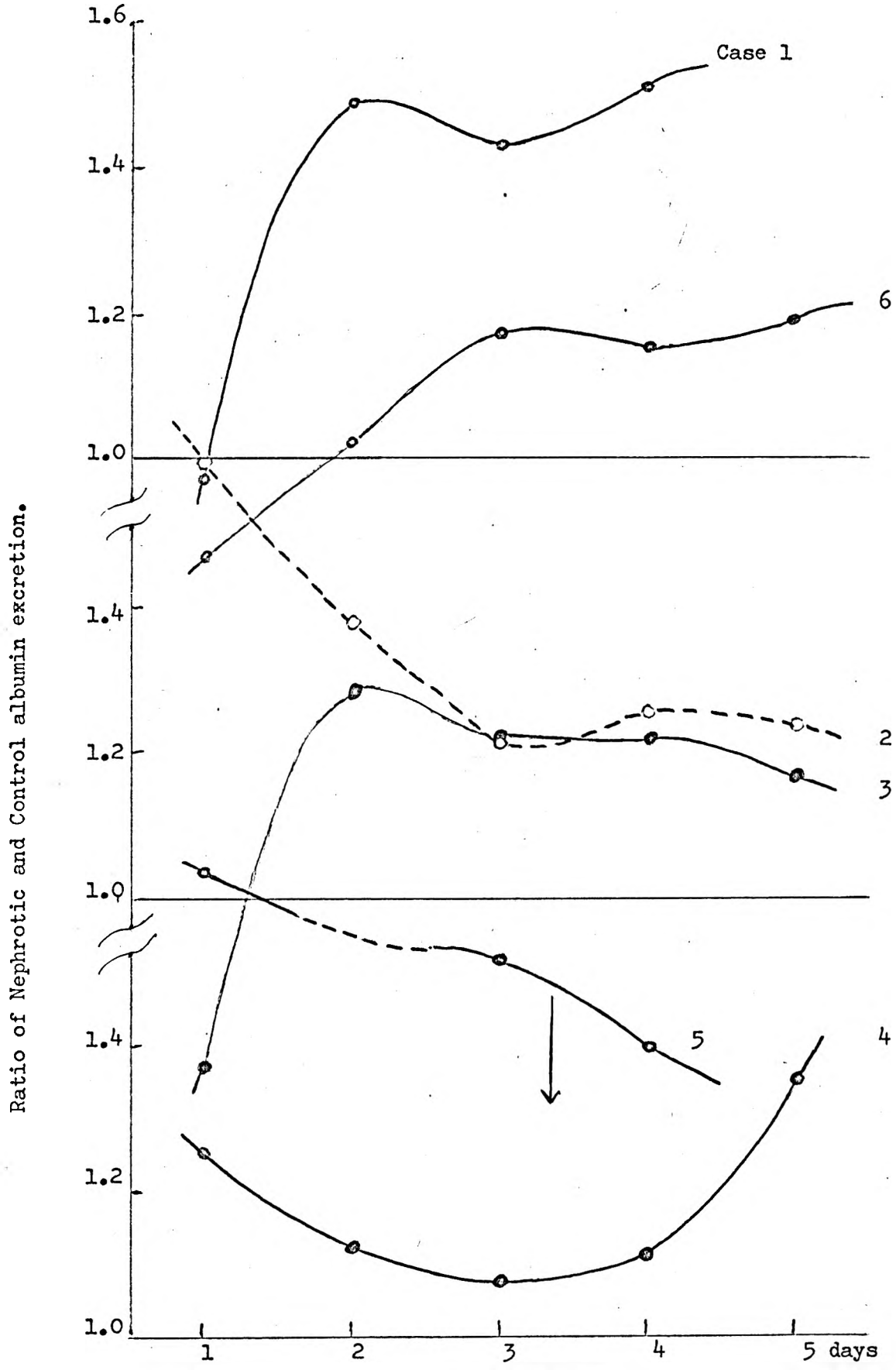
The numbers refer to the case numbers in Table 1.

Figure 6. THE DAILY RATIO OF THE EXCRETION OF NORMAL AND CONTROL ALBUMIN.



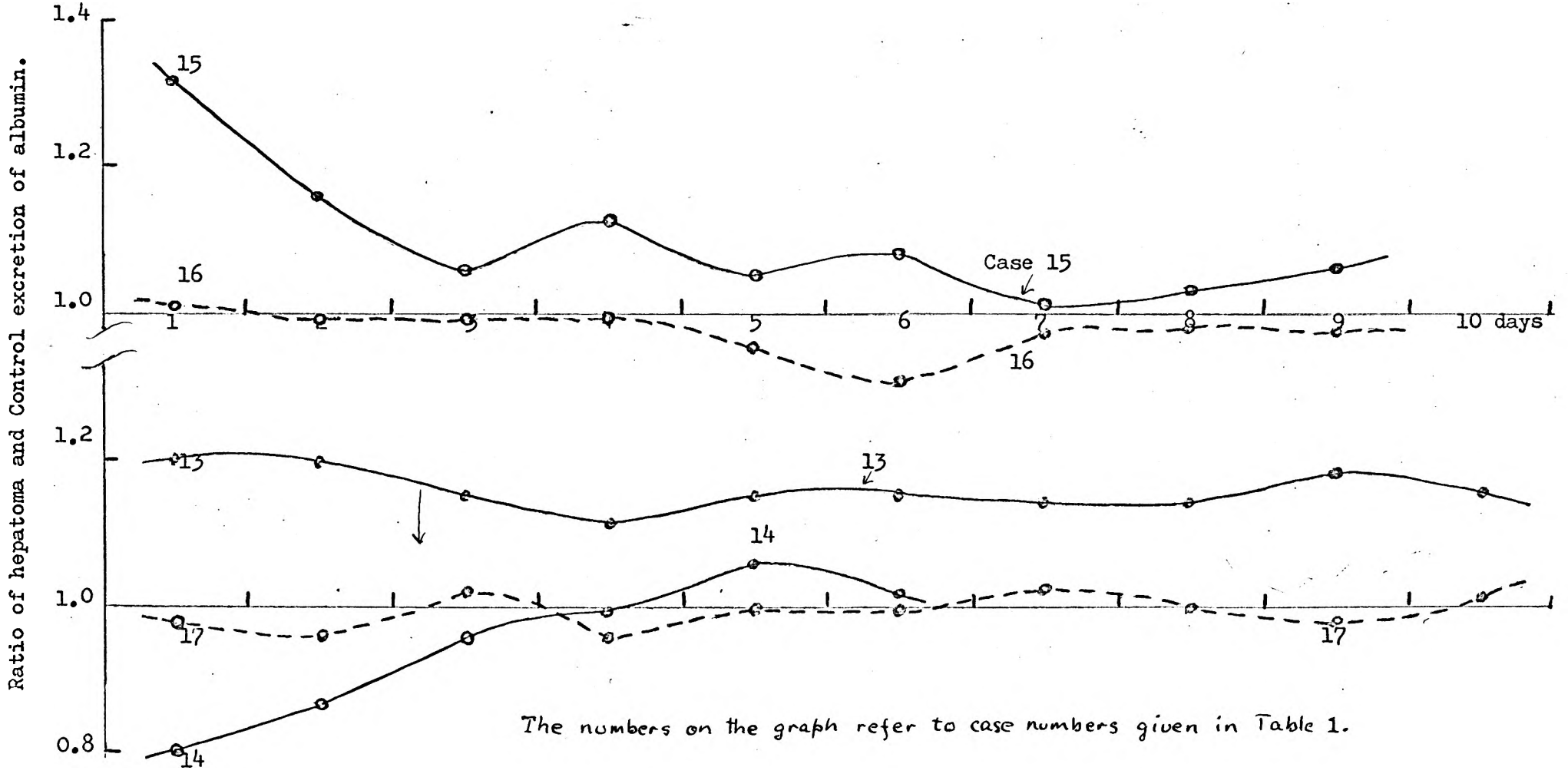
The numbers on the side of the graph refer to case numbers given in Table 1.

Figure 7. THE DAILY RATIO OF THE EXCRETION OF NEPHROTIC AND CONTROL ALBUMIN.



The numbers given at the side of the graph refer to case numbers in Table 1.

Figure 8. THE DAILY RATIO OF EXCRETION OF HEPATOMA AND CONTROL ALBUMIN.



The numbers on the graph refer to case numbers given in Table 1.

Figure 5. The daily urinary excretion ratios from Table 1 have been plotted as three separate groups; normals, nephrotics and malignant hepatomas.

Figures 6,7,8. The ratios of the three groups are plotted separately.

Interpretation of Results.

a) Normal albumins when compared with the normal control albumin (Cases 9,10,11 and 12) showed only slightly faster rates of excretion (1.78 to 5.57%). The ratio of the fractional catabolic rates also showed this similarity (Figure 3).

Control albumin when compared against itself (Cases 7 and 8) showed differences of 2.51 and 5.21%. It was arbitrarily decided to express the ratio as greater than unity (i.e. 1.0251 and not 0.9749). These results show that the technique is reliable and that normal albumins are comparable in catabolic propensity.

b) Nephrotic albumins were excreted from 17.39 to 47.03% faster than the control. This was also reflected in the ratios of the fractional catabolic rates (Figure 3). The ratios in the first two days appeared to bear no definite relation to the rest of the graph and in practice no relation to the isotope used. Towards the end of the experimental series the amounts of denaturation and free iodine were considerably lessened and the early part of the graph was usually closer to unity. This suggests that the explanation offered to account for the results in the first two days (see under Results, Table I) is probably correct.

A possible explanation of the increased catabolism of the nephrotic albumin was that some uniform denaturation

occurred in the protein during preparation. Freeman (1959) has shown that albumin denatured in preparation was rapidly eliminated due to uptake by the reticulo-endothelial system e.g. of the liver and that this process differed from the normal mechanism of catabolism. The liver does not play a major role in normal albumin catabolism (Gordon 1962). If an albumin preparation were denatured then the fractional catabolic rate would gradually revert to normal as the denatured molecules were more rapidly eliminated. This would mean that the ratio of the catabolic rates of a denatured and a normal albumin would gradually approach a figure near unity. The use of computer-smoothed values was an extra attempt to see if this occurred. Certainly it did not appear to be a regular phenomenon although the results were not so clear-cut (see Figure 3). The one hepatoma patient (Case 13) with a 20.1% increase in catabolic rate showed no tendency at all for the graph of the ratio to tend towards unity but remained parallel to the abscissa. These facts did suggest that denaturation was not a factor in determining increased catabolism and that whatever change was responsible for increased catabolism, it was a generalised one and that more-rapidly catabolised components did not exist. This can also be expressed by saying that only one species of albumin molecules, all showing the same change was present.

It can also be shown arithmetically that a 5% contaminant cannot possibly account for the results, in the nephrotic cases, irrespective of its rate of catabolism.

c) Hepatoma albumins did not show uniform changes. Case 13 showed a 20.1% increase in fractional catabolic rate sustained over the 10 day period. Case 15 showed a gradual tendency to become normal

and then the catabolic rate rose again with an average increase of 6.0%. The other cases showed fractional catabolic rates not far removed from the normal (0.%,1.0% and -2.9% increases). The plasma albumin pool sizes and daily albumin catabolism were found to be lower than normal (Hoffenburg 1966) (Table 2) in 4 cases but normal in Case 16. This case was abnormal however in that he was hypervolaemic and oliguric (Table 2). It should be noted that for precise pool size measurements by the equilibrium time method a steady state, with regard to albumin metabolism, should be present. This was probably lacking in these cases as they were all deteriorating steadily clinically. However, this does not affect the validity of the double isotope comparison technique.

DISCUSSION.

It is quite clear that the tacit assumption made by others that albumin, other than the individual's own, can be used to study albumin catabolism, is not valid. Certainly it would not have been valid with the four nephrotic albumins tested and the one abnormal hepatoma albumin. There is no reason to suppose that a nephrotic patient, unlike the normals who were tested, would not be able to recognise his own albumin as defective. This crucial experiment has not been done yet. The one hepatoma case on the other hand was able to recognise his own albumin as being defective. It thus becomes evident that many of the studies on albumin metabolism may be faulty (Gitlin 1952, Kaitz 1959, Volwiler 1955, Purves 1962, Cohen 1962).

It remains to be seen how the albumins behave, from cases with Kwashiorkor where albumin is synthesised in the face of a dearth of amino acids, and from cases of cirrhosis or

hepatitis, where albumin is synthesised by a deranged liver.

No explanation of this "hypercatablic" phenomenon has direct experimental support as yet and no clinical or biochemical correlations with "hypercatabolism" are evident at present as the spectrum of this change has still to be worked out. Teleologically-speaking it is possible that an albumin which is more easily catabolised is produced when the demand for amino-acids is high.

Another possible explanation exists to account for the "hypercatabolism" of albumin which is based on the finding that more tyrosyl residues are available for iodination in "fatted" albumin than in fat-free albumin (Glazer 1965). As the albumin in nephrotics is saturated with fat and no special measures were taken to remove this fat in the preparation of iodoalbumin, it is possible that the sites of iodination are different in the "hypercatabolised" albumins. Catabolism may be faster when certain tyrosyls are iodinated as molecular unfolding may be stabilised by the intruding iodine atoms. This possibility can only be checked effectively by chemical studies and by comparing biosynthetically-labelled nephrotic albumin with iodoalbumin.

The above explanation is not favoured as several of the normal albumins tested were obtained from subjects showing some post-prandial hyperlipaemia and also the one "hypercatabolic" hepatoma case was not hyperlipaemic. This facet could be examined by purposely obtaining albumin from non-proteinuric,hyperlipaemic subjects.

An hypothesis. Since the "hypercatabolic" property appears to be fairly stable and to affect all the albumin molecules in an individual it must be assumed that the molecular shape, i.e. the tertiary structure, is altered - how else could the molecules be recognised as different by the body. It is not a reasonable speculation to suggest that the primary structure (i.e. the amino-acid sequence) of albumin is altered in the nephrotic syndrome since this would entail complete recoding of the albumin cistron in every liver cell, although however, this is feasible in a monoclonal hepatoma. The real explanation may lie in the suggestion by Foster (1965) that the microheterogeneity of albumin is determined by randomisation of disulphide cross-linkages. It is possible that randomisation is directed in certain disease states so that more-easily-catabolised molecules are formed. This might be due to some simple environmental change at the ribosomal surface e.g. an increase in the lipid content of the cell sap. However, this is highly speculative.

The experiments that have been discussed in this dissertation would therefore provide indirect evidence that the tertiary structure of a protein is not wholly determined by the primary structure. Environmental changes causing alteration in molecular shape would provide a novel mechanism for regulating protein catabolism.

SUMMARY.

1. Using albumin labelled with radioactive iodine it can be shown that albumin from some individuals, notably nephrotics, can be catabolised up to 50% faster than albumins from other individuals.

2. The "hypercatabolic" property of some albumins would appear to be stable alteration of the albumin molecule affecting all the subject's albumin.

3. A novel hypothesis for a factor regulating catabolic rates of proteins is proposed.

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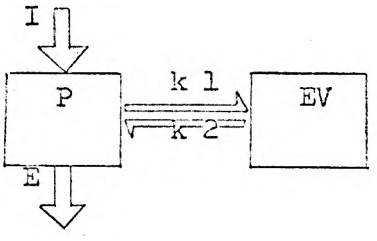
ADDENDUM.

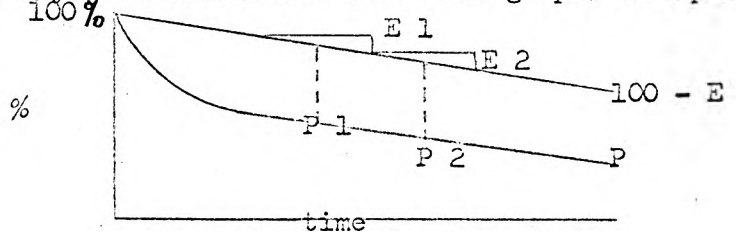
A summary of the principle and technique of the equilibrium time method of calculating turnover rates.

The kinetics of albumin catabolism can be fairly well described on the basis of a two-pool system where P represents the plasma pool and EV the extravascular pool.



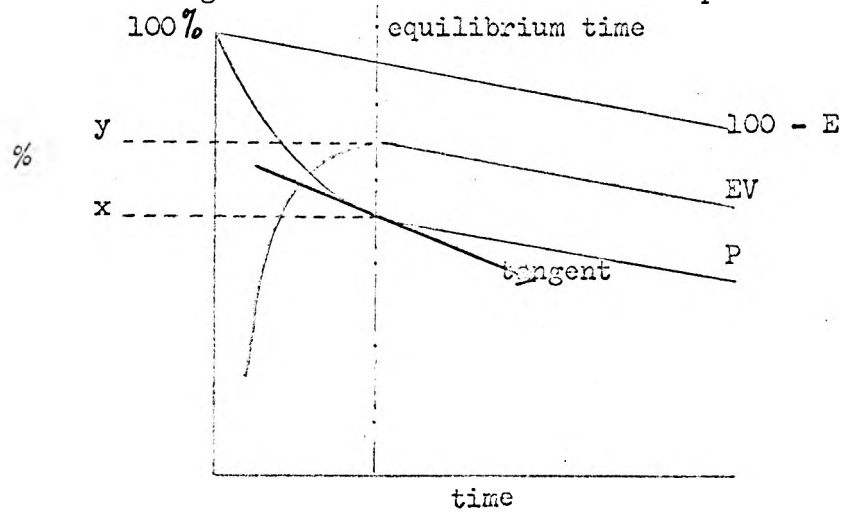
If iodoalbumin, I, is injected into P it equilibrates with EV, where k1 and k2 are respectively the diffusion rates to and from the extravascular pool.

It can be shown mathematically that P and EV are in equilibrium when EV reaches a maximum at which time k1 = k2. Since the concentrations are the same at equilibrium, the size of EV can be estimated as P (the plasma volume) is known and the ratio of P and EV (i.e. x/y) is calculated (from the graph) at equilibrium time.



The excretion of iodine from iodoalbumin breakdown, E, occurs in a pool which is in rapid equilibrium with the plasma, P.

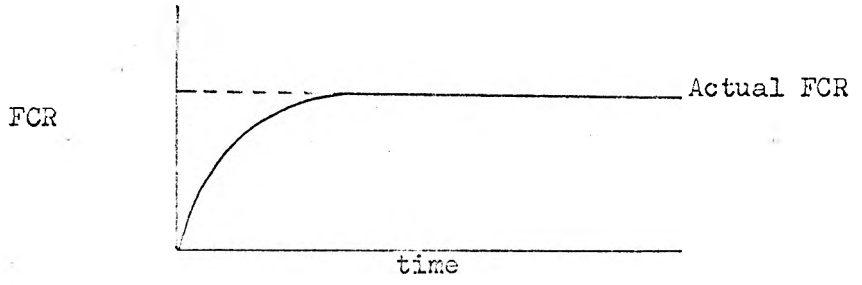
A further check on this calculation stems from the fact that the Fractional catabolic rate, F.C.R., can also be calculated if excretions of iodine are known during fixed intervals and the mean plasma activity during that interval.



F.C.R. = E1/P1 or E2/P2 etc. (% of the plasma pool catabolised per day).

Characteristically the graph of F.C.R. against time shows that the F.C.R. reaches a plateau after a few days and the value on this plateau (the average of a number measurements) is the actual F.C.R.

It is also known that the instantaneous rate of change of the plasma activity curve at equilibrium time is given by the value $\frac{0.693}{F.C.R.}$ and this serves as a useful check as it is also the tangent to the plasma activity curve at equilibrium time.



The advantage of the equilibrium time method of calculating turnover rates is that only a short period of metabolic collection is required. Actual turnover rates are calculated:-

Plasma albumin pool = P grams (derived from plasma volume and albumin concentration of the plasma).

Fractional catabolic rate i.e. % of the plasma pool catabolised per day = F % / day

Turnover of albumin = $\frac{P \times F}{100}$ grams of albumin per day.