"SOME FACTORS REGULATING ALBUMIN CATABOLISM AND SYNTHESIS".

By

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1968.

To Margaret

With love, admiration and respect.

" 'If my guess is right,' someone in a laboratory will say, 'if the hypothesis I am working on is a sound one, then it follows ....' "

Sir Peter Medawar

The Listener. 12 October 1967.

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LIST OF ABBREVIATIONS.

IV	=	Intravascular
IVP	=	Intravascular pool (albumin)
EV	=	Extravascular
EVP	=	Extravascular pool (albumin)
SR	=	Synthesis rate
CR	=	Catabolic rate
S + T	=	Synthesis + transfer rate
FCR	=	Fractional catabolic rate
SA	=	Specific activity
NPD	=	Normal protein diet
LPD	=	Low protein diet
ALB.	=	Albumin

INTRODUCTION.

Hypoalbuminaemia is an essential accompaniment of protein malnutrition[1-13]. Yet the level of plasma albumin is usually regarded as a relatively crude reflection of a patient's nutritional status, reduction occurring only after prolonged or severe inadequacy of dietary protein. Brock[11] recognized that a marginal degree of hypoalbuminaemia might be evidence of impending or early deficiency, and suggested that minor grades of "protein subnutrition" could conceivably exist with serum albumin levels still within the normal range.

The work reported in this thesis developed originally out of an attempt to explore this possibility, and to characterize some of the changes in albumin metabolism that followed mild or early experimental protein deprivation in man and rabbits. Dynamic studies using albumin labelled with radioactive iodine revealed evidence of early adaptational changes, possibly occurring before alteration in the plasma albumin levels[14,15]. While these studies shed some light on the response of albumin metabolism to experimental depletion, they failed to provide the hoped-for means by which subclinical protein malnutrition could be detected. From this work, however, it was but a short conceptual jump to the general problem of albumin homeostasis, a consideration of which forms the basis of this thesis.

The first approach was a study of changes in albumin synthesis and catabolism in rabbits following limitation of dietary protein intake. Adaptive responses were then investigated in animals, provided with normal protein diets, after protein depletion induced by an alternative method - plasmapheresis - and, finally, after intravenous infusion of albumin solutions. Based on these and reported results, a tentative hypothesis has been adduced to account for the body's adaptation to variation in the plasma albumin pool, brought about by experimental manipulation or occurring spontaneously in disease.

CHAPTER I.

GENERAL HISTORICAL BACKGROUND.

At the turn of the century interest had already been taken in the pattern of events that followed alteration of dietary protein intake. Argument centred around the source of urinary nitrogen, which had been shown to fluctuate in amount according to the protein content of the diet[16], yet which persisted as a small, constant loss, even when protein was entirely excluded - an essential loss attributed to "wear and tear" and arising from breakdown of endogenous tissue proteins[17,18].

The concept developed of two "pools" of protein which could be drawn upon to supply nitrogen needs - one exogenous, derived from the diet, the other endogenous, from the protein-containing tissues of the body[19]. Borsook and Keighley[20] laid the foundations of modern concepts by introducing a new term, "the continuing nitrogen metabolism", to indicate nitrogen metabolized on any one day which was already present in the tissues. The idea of separate protein pools with independent metabolic functions and fate was abandoned, as it came to be realised that amino-acids for protein synthesis could be derived - randomly - from digested food protein or from the end-products of breakdown of endogenous proteins.

The dynamic state of tissue proteins came to be accepted.

Yet, common-sense forced the recognition of some sort of "protein-reserve" - a labile pool of protein which could be called on in times of need, and which could accumulate in times of plenty. The nature and extent of this "reserve pool" has never been defined and, in some ways, the concept is artificial, since protein is not laid down separately as an inert store. Indeed, as shown by Borsook and Keighley[20], "reserve" protein participates actively in metabolic processes as part of different tissues. Its lability is best demonstrated when dietary protein is insufficient for the needs of the body - indirectly, by a study of nitrogen excretion patterns, or, directly, by measurement of protein loss from all or some of the organs of the body. Voit[21] and Rubner[17] had shown that adaptation of urinary nitrogen to dietary deprivation was preceded by a lag in reaching equilibrium. This was confirmed by Borsook and Keighley[20] who showed further that the duration of the lag was directly proportional to the level of dietary nitrogen which the animals had previously been fed, i.e. to available stores of metabolizable protein.

The concept of a "protein reserve" is further complicated by variations in the response of different tissues to alterations in the quantity and quality of dietary protein. Some tissue proteins are rapidly depleted when intake is deficient, others more slowly, so that an imbalance in protein reserves

may be created. Over 100 years ago Voit[22] showed the major loss in protein-deficient rats to be reflected in the liver; skeletal muscle was less affected, brain and heart hardly at all - a pattern which has been confirmed or slightly modified by many authors[23-29], although muscle seems to play a more important role in chronic depletion than was previously recognized[30-33]. Waterlow et al.[10] separated "fixed" and "mobile" categories of body proteins according to the facility with which they were reduced during protein depletion. In a later paper Waterlow[32] suggested three divisions, but admitted that a sharp dividing line probably does not exist between proteins which are very labile and those less readily metabolized.

Muscle protein can obviously be regarded as relatively labile from the way in which wasting occurs in conditions of chronic deprivation or loss. This clinical observation is well-supported by experiment, which suggests that skeletal muscle may be sacrificed during protracted protein deficiency to ensure maintenance of other more "essential" body proteins[29, 34, 35].

Plasma albumin must be regarded as one of the major and most labile of body proteins. In kwashiorkor of infants and adult protein-deficiency states, a reduced concentration is an essential feature[1-13]. Loss of protein in the nephrotic syndrome or via the gut is also associated with lowering of

the plasma albumin level, and, experimentally, it can readily be modified by variation of dietary protein content, removal of plasma by plasmapheresis or addition by infusion[12,15,23-25,35-45].

The ease with which plasma albumin may be manipulated makes the investigative use of this protein attractive. Added features which encourage its use are its ready accessibility by venepuncture, simple and precise methods of fractionation in pure form, and ease of labelling with radioactive markers (usually isotopes of iodine) for tracer experiments. Despite these many advantages which have led to intensive research into all aspects of albumin metabolism, much remains to be learnt about the control of its plasma concentration. In health a remarkable constancy is maintained, suggesting the operation of a delicate and sensitive homeostatic mechanism. Further exploration of the nature of this mechanism forms the basis of this thesis.

CHAPTER II.

THE BACKGROUND TO TRACER STUDIES WITH PARTICULAR REFERENCE TO ALBUMIN METABOLISM.

The development of the concept of continuous synthesis and breakdown of body proteins coincided with the discovery of a new and exciting tool for the investigation of dynamic biological changes - the application of isotopic tracer techniques.

Because they are detectable in minute quantities, radionuclides provide an elegant tool for following or "tracing" the metabolism of biologically interesting compounds or estimating the size of various body compartments. The principle underlying their use is that they may be introduced into the body in amounts so small that they do not interfere with normal metabolic processes; they participate in these processes in precisely the same manner as their stable non-radioactive counterparts, without "discrimination" by the biological system; and they may be detected by suitable radioactivity counting equipment with great sensitivity.

Hevesy (1923) pioneered the tracer field by studying the absorption and distribution of thorium B in the broad

bean[46]. Schoenheimer, Rittenberg and their colleagues[47-51] applied similar techniques to dynamic studies of lipid and protein metabolism and, in a series of experiments reported through the decade 1940 - 1950, they brilliantly exploited the stable isotopes, deuterium and ^{15}nitrogen, to provide a new, exciting approach to an understanding of the "continuing" metabolic processes of the body. In the course of their work they showed that administered ^{15}N was partly excreted as urinary urea nitrogen, but a large fraction was retained in the body, appearing predominantly in the proteins of plasma, intestinal mucosa, liver, spleen and bone-marrow, to a lesser extent in bone, skin and muscle. Their work provided firm evidence of one of the main characteristics of living tissue - its ability constantly to synthesize and degrade protein - and confirmed Borsook and Keighley's proposal that amino-acids from exogenous and endogenous sources were inextricably mixed in the "metabolic pool" of the body.

The next decade witnessed capitalization on their experience, as many new radioactive isotopes were produced, techniques were developed for labelling stable compounds with radioactive elements, and increasingly sophisticated means of assay and analysis were introduced. As this new science unfolded, there arose a new terminology, some terms of which might require elucidation.

The realisation that most body constituents are in a

state of flux produced the term "turnover", by which is meant "the replacement of an amount of protein (or any other substance) by an equal quantity of the same material newly synthesized from metabolic precursors or transported into the system from outside"(52). "Turnover rate" is a measurement of this process and may be expressed as grams per day, micromoles per hour, or any other mass-unit per unit-time. "Degradation (catabolic) rate" and "formation (synthesis) rate" are equivalent to turnover rate, if the system under observation is in a "steady state", i.e. where the total amount of a substance in the system does not change during the period of observation, and loss due to degradation is replaced exactly by synthesis. In most circumstances degradation and synthesis are independent processes under different forms of control.

The term "specific activity" has acquired a looseness of meaning, but, in its strict and proper sense, refers to the ratio of radioactive to total atoms of an element in a sample. For instance, a solution of radioactive ^{131}I with carrier stable ^{127}I may be said to have a specific activity of x microcuries (μCi) per microgram (μg) of iodine. (In this thesis, I accept the imprimatur of many senior and experienced authors and refer to the specific activity of an iodine-labelled albumin preparation as μCi iodine per mg. albumin).

If one injects radio-actively labelled albumin

intravenously, it rapidly disperses or "equilibrates" throughout the plasma. The extent to which it is diluted, after complete mixing has occurred, gives an accurate measurement of the volume of plasma in the body. This technique of measuring volumes of distribution is known as "isotope dilution"; the theoretical basis of the technique is well-discussed by Bergner(53). The product of plasma volume and plasma concentration of albumin gives the total mass of albumin present in plasma - the "plasma albumin pool". By different techniques it is possible to show that injected labelled albumin will shortly begin to leave the plasma to mix or equilibrate with one or more extravascular pools, the precise size of which, in certain circumstances, may also be measured. The recognition of pools or "compartments"* of albumin in the body leads to an acceptance that exchange or flux occurs between them, and "rate constants" measuring the rates of this exchange may be calculated.

As a consequence of tracer experiments, a branch of biomathematics has developed, which is concerned with "compartment analysis", seeking to provide information

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* Again I use the terms "pool" and "compartment" in a generally-accepted, albeit loose, sense. Brownell and Ashare(54) have set out coherent definitions of these and other terms to be used in tracer kinetics, but their recommendations have not yet been widely adopted.

about pool-sizes and distribution, rates of exchange, synthesis and breakdown of different body constituents. In this thesis tracer methodology is applied to investigate some aspects of the regulation of albumin metabolism.

Labelled Albumin Tracer Studies.

Sterling[55] was the first to apply ^{131}I-labelled albumin tracers to the study of plasma albumin metabolism in humans. Since then the technique has been widely and fruitfully employed using molecules labelled with ^{131}I or the other radioactive isotopes of iodine, ^{125}I and ^{132}I. Excellent reviews by Rescigno[56], Matthews[57], Cohen et al.[58], McFarlane[59], Jarnum[60], Matthews and Freeman[61] and others have set forth modern views of the distribution and metabolism of body albumin, and have discussed at length many of the difficulties inherent in measurement by this technique. Andersen's monograph contains detailed consideration of methods of calculating turnover data from iodine-labelled protein studies[62].

At the risk of oversimplification, albumin may be considered to exist in the body in two communicating pools - one intra-, the other extra-vascular (IVP and EVP). Matthews[57] and Matthews and Freeman[61] have discussed more complex mathematical models, including that of Reeve and Bailey[62a] which dispenses with finite compartmental boundaries. On the basis of analogue computer studies,

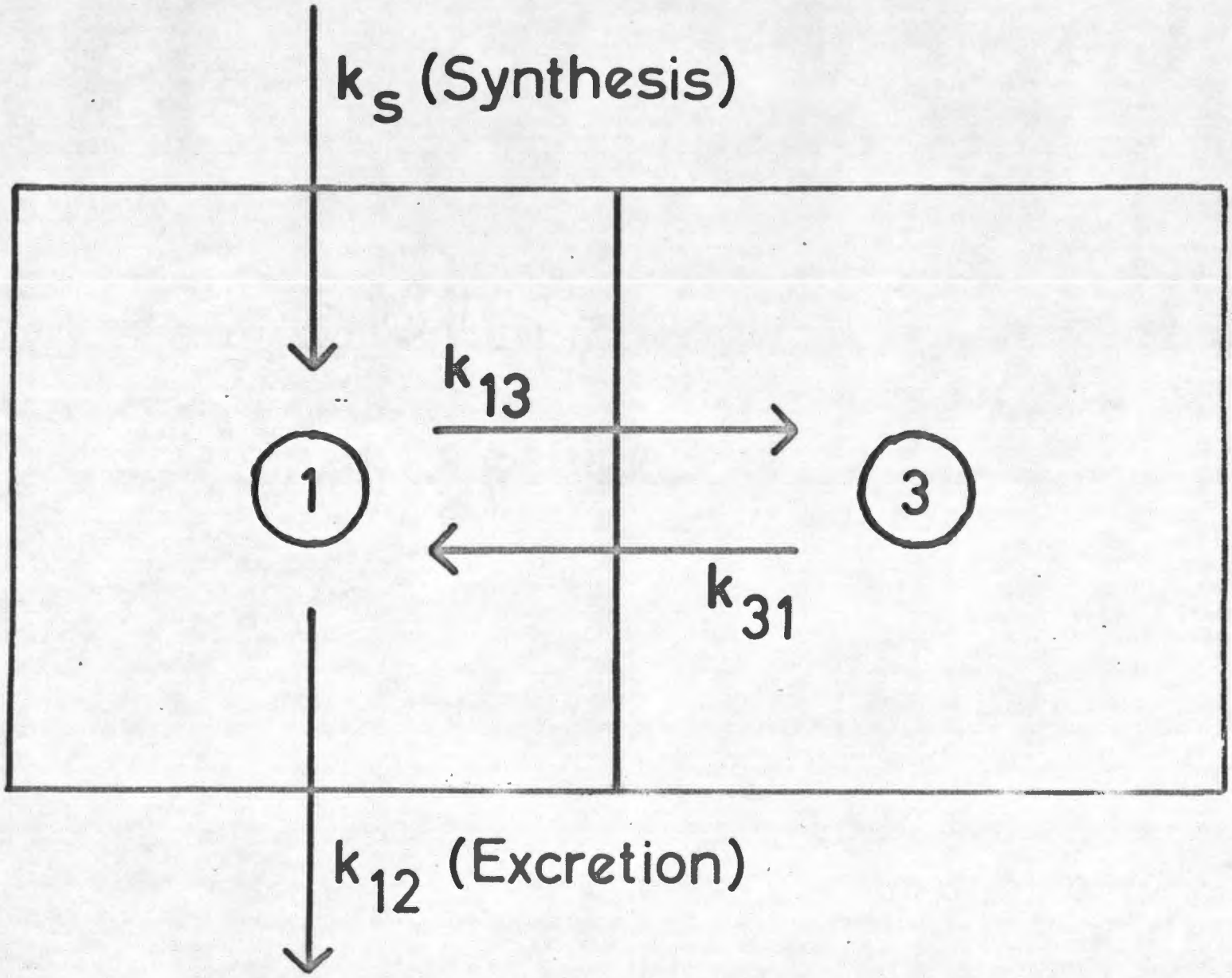

Figure 1: Metabolic behaviour of albumin. (1) = Intravascular (plasma) pool. (3) = Extravascular pool(s). K_S = Rate of synthesis of new molecules delivered to (1). K_{13} and K_{31} = Rates of exchange between (1) and (3). K_{12} = Rate of loss of molecules from (1) by excretion.

Matthews favoured a model with three extravascular pools as the most satisfactory fit for known human and animal data. Whether the extravascular albumin pool is regarded as single or consisting of several pools does not materially affect the general consideration.

Synthesis delivers newly-formed albumin molecules into the IVP; breakdown or loss, e.g. in the nephrotic syndrome, removes albumin molecules; constant flux or exchange takes place between the two pools. This simple state of affairs is described by the mathematical model of an open, two-compartment, mammillary system (Figure 1).

In terms of this model, newly-synthesized albumin molecules are introduced into (1), the IVP, at a rate K_S. These molecules diffuse across to the EVP (3) at rate K_{13} and return at rate K_{31}. Initially the new molecules must exist in higher concentration in (1) than in (3) so that K_{13} must greatly exceed K_{31}. After a variable time the concentration of new molecules in the two pools will become equal, i.e. equilibration will have occurred, so that net loss of new molecules from (1) will ensue only as a result of breakdown, at rate K_{12}*. If a steady state exists, K_{12}

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* Most authors are agreed that degradation of albumin occurs in a pool which exchanges rapidly with plasma and which may be regarded, mathematically, as part of the intravascular albumin pool (Jarnum[60], Beekin et al.[63], McFarlane[64]).

should equal K_S, so that pools (1) and (3) remain constant. If we consider the behaviour of trace amounts of labelled albumin introduced directly into pool (1), we find it simulates precisely that of the new molecules discussed above. If radioactive isotopes of iodine are used to label albumin, breakdown of protein molecules releases iodine, which is rapidly excreted in the urine, provided blockade of thyroidal uptake has been achieved through administration of stable iodide in sufficiently large quantities. By serial measurement of plasma and urine radioactivity after intravenous injection of radio-iodinated albumin, it is possible to define several aspects of the distribution and metabolism of the protein. The general theory, methods and interpretation will be discussed in brief.

Measurement of Albumin Catabolism.

As mentioned earlier, the principle of isotope dilution readily establishes the size of the plasma albumin pool. If one follows the fate of injected labelled protein molecules over the next 7 - 10 days, they are found to disappear rapidly from the plasma over the first 36 - 48 hours, after which activity falls at a slower and steadier rate[55]. If activity is plotted on a log-linear scale, the curve so obtained may be resolved into two linear components (Figure 2), indicating that the overall rate of fall of labelled molecules results from two simultaneous exponential rates of removal, the slower of which finally obtains.

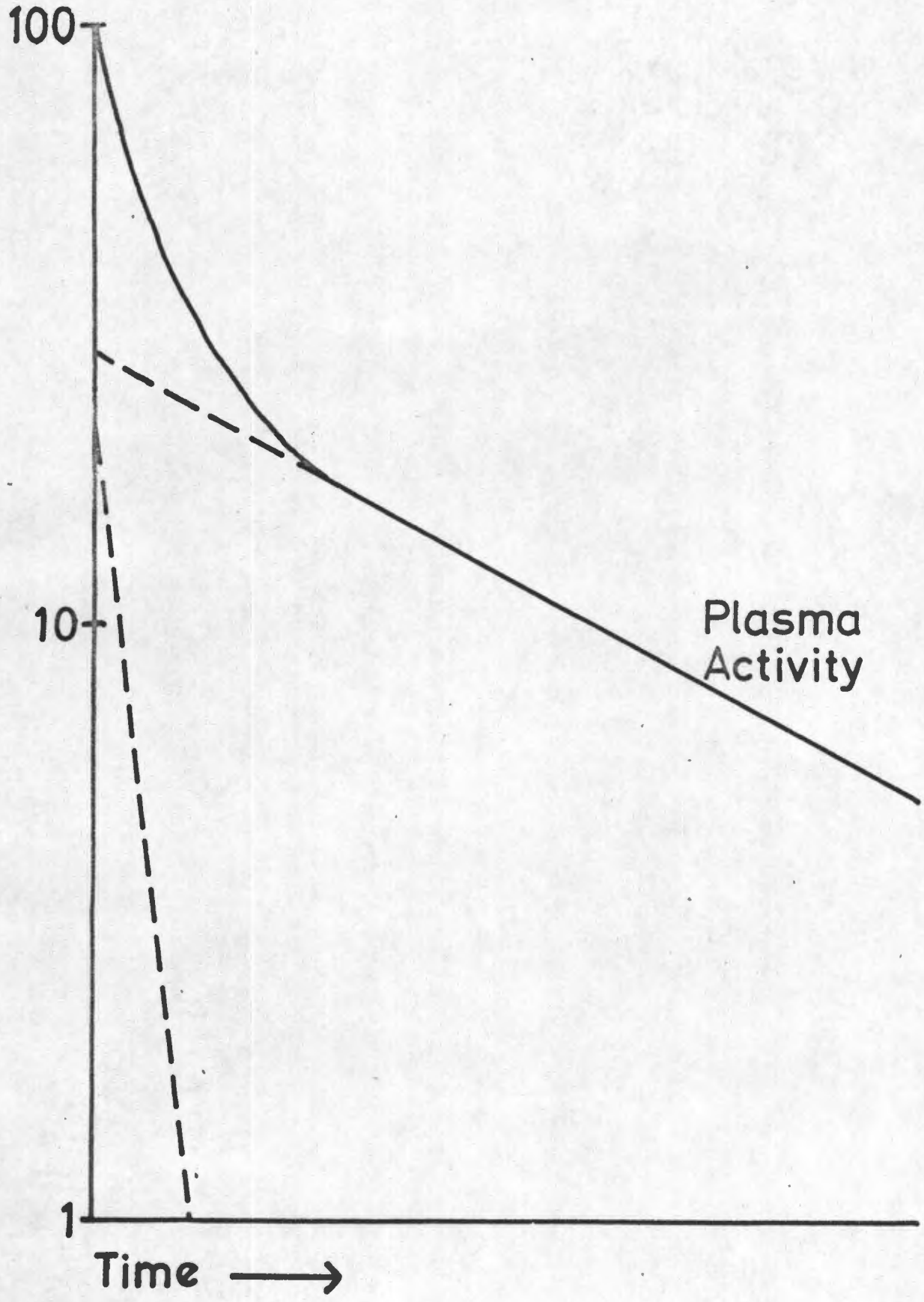

Figure 2: Plasma activity curve (logarithmic) after intravenous injection of labelled albumin. Unbroken line can be resolved into 2 linear components (broken lines).

The early, more rapid component is due to transcapillary exchange between IVP and EVP. The slower component basically represents removal by catabolism, but is complicated by return of labelled molecules from the EVP to plasma; to this extent, the observed fall in plasma radioactivity will fail to reflect the true catabolic rate. This plasma activity curve, consisting as it does of two exponential components, is described by the equation

$$x = c_1 e^{-b_1 t} + c_2 e^{-b_2 t}$$

where x is the isotope concentration in the plasma at time, t, and c and b values represent the Y intercepts and slopes of the two components. Matthews[57] has elaborated a mathematical expression based on the plasma curve, from which the catabolic rate of albumin can be derived, but possible fluctuations in concentration during the period of observation make her method difficult to interpret. Albumin catabolic rate may, however, be measured reliably under almost any circumstances by expressing urinary excretion of radioactive iodine label as a function of plasma specific activity (radioactivity per gram of albumin). The assumption made here is that iodide, liberated from the catabolism of labelled albumin molecules, is completely excreted in the urine, as would be anticipated if thyroidal uptake were completely blocked by large doses of stable iodide. In this case catabolic rate (in grams per day) is given by the expression -

$$\frac{\text{Urinary radioactivity (}\mu\text{Ci per day)}}{\text{Plasma albumin specific activity (}\mu\text{Ci/gram albumin)}}.$$

This analytical method, or modifications of it, was first successfully applied by Berson and his group[65-67] and Campbell et al.[68] and has been widely accepted as valid, even where steady state conditions cannot be assumed (Matthews[38]; Franks[69]).

The Distribution of Albumin.

The distribution of albumin between the two pools (IVP and EVP) can be resolved mathematically only if certain assumptions are made:-

(1) total protein in each pool is constant during the period of measurement;

(2) rate of breakdown is equal to rate of synthesis, and these and rates of transfer from one compartment to another are constant;

(3) labelled protein is uniformly distributed throughout each pool (Matthews[57], and Campbell et al.[68]).

If these assumptions are made, it is possible to derive a figure for EV/IV ratio and distribution of albumin. In order to do this, total body radioactivity must be serially determined. Since intravascular activity is known, extravascular activity can be obtained by difference. In theory, EV activity should reach a maximum when equilibrium has occurred between IVP and EVP, after which decline will be

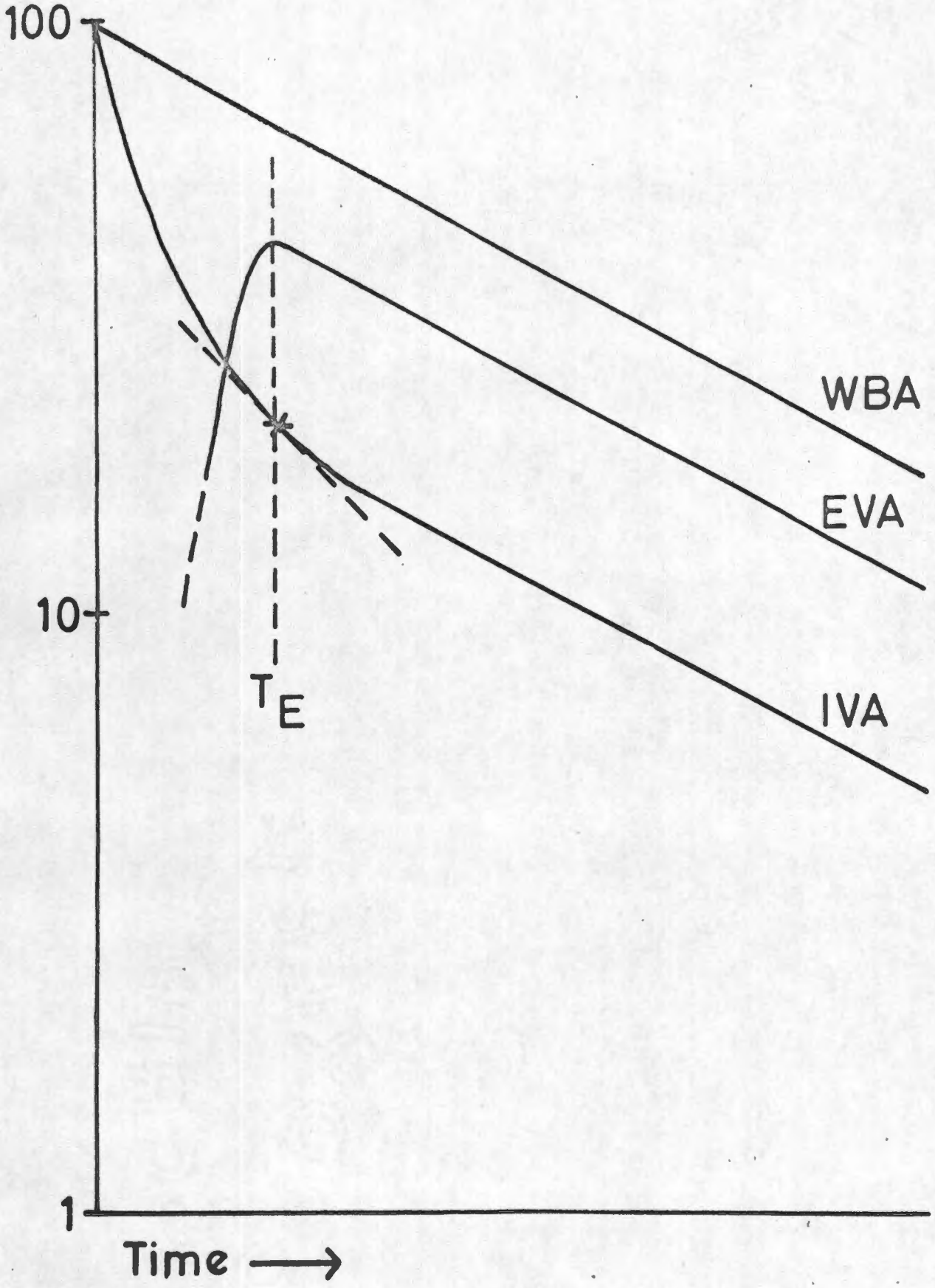

Figure 3: Whole-body (WBA), extravascular (EVA) and intravascular (IVA) activities after intravenous injection of labelled albumin. T_E = Equilibrium time.

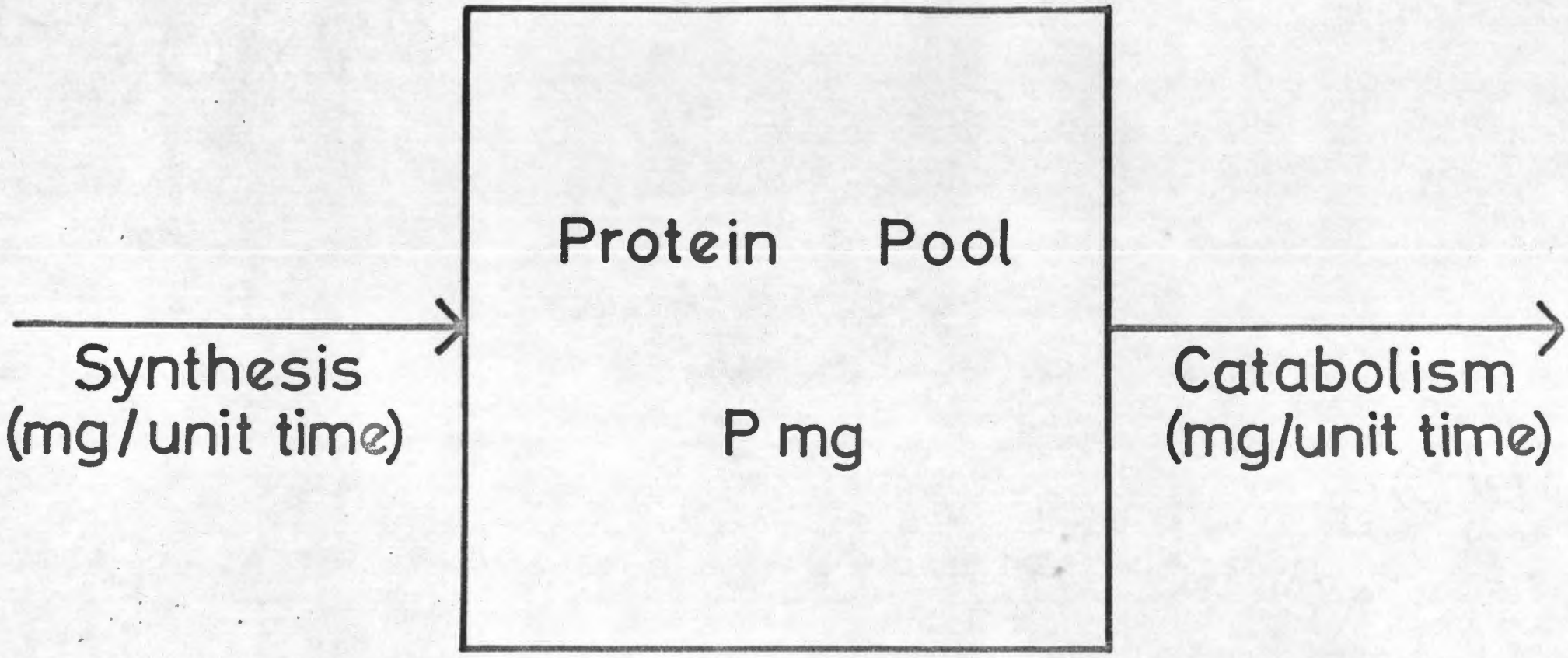

Figure 4: Simple protein system.

observed in both compartments (Figure 3). At this point - the "equilibrium time" - the ratio of EV to IV radioactivity reflects their relative albumin pool-sizes. Since IVP size is known, EVP is easily derived. Unfortunately, this ingenious method has limited value, since it often fails to give clear-cut answers, especially in circumstances which produce "unsteady states", in which the assumptions made above do not apply.

Albumin Synthesis.

Finally, one must consider measurement of albumin synthesis rates. In a simple protein system, as illustrated in Figure 4, the rate of synthesis may be derived from knowledge of the catabolic rate and the change in protein content, P, over the time of measurement (t_o, t_1):

$$S.R.\ (t_o,t_1) = \triangle P + C.R.\ (t_o,t_1)$$

where S.R. (t_o, t_1) = mg. protein synthesized between times t_o and t_1, C.R. (t_o, t_1) = mg. protein catabolized, and $\triangle P$ is the alteration in pool protein content in mg. If P does not change, i.e. $\triangle P = 0$, the system is in equilibrium over the time of measurement, and S.R. (t_o, t_1) is equal to C.R. (t_o, t_1).

Clearly this identity does not obtain under most experimental conditions, which, by design, introduce metabolic

imbalances. In these circumstances S.R. may not differ greatly from C.R., but a more specific evaluation is often needed where equilibrium is significantly disturbed.

Matthews[38] was obliged to derive a new index of synthesis rate in her study of the effects of plasmapheresis on rabbits, for, here, considerable inconstancy was introduced by the drastic technique used. She achieved a measurement of a "synthesis plus transfer rate" (S + T) from the equation

$$S + T = b + c. \frac{X_3}{X_1}$$

where b = slope of intravascular radioactivity curve

c = slope of extravascular radioactivity curve

X_3, X_1 = EVP and IVP radioactivities, respectively.

These indices could all readily be measured or derived. Her"S + T" measured the sum of albumin molecules entering the plasma pool from synthesis (S) and net transfer (T) from the EVP. The contribution of each to the total could not be determined, although simulation of the system on an analogue computer gave a reasonable approximation[70].

While this provided useful information in some instances where changes were large[15,71], the need was still felt for a more direct approach to the determination of albumin synthesis rate, which would be valid independent of steady-

state conditions. In 1963 McFarlane[72,73] and Reeve et al.[74] separately reported a technique for direct assay of albumin synthesis. Since the development and application of this technique forms a major part of the work of this thesis, a separate chapter will be devoted to it. (Chapter III).

THE QUALITY OF LABELLED ALBUMIN PREPARATIONS.

This chapter on the application of radioactive tracers of albumin metabolism would not be complete without reference to a major and much-debated problem arising from their use. Acceptance of tracer studies in biological work rests on the assumption that the labelled product is handled by the body in precisely the same way as its stable, endogenous counterpart. As Yalow has observed[75], one does not "do tracer experiments for the purpose of studying tracer turnover" - one does them to infer from the behaviour of the tracer how intrinsic body substances are metabolized. The quality of the labelled product is thus of utmost importance, for it must so closely resemble the native material that the body fails to discriminate; only then can its behaviour be considered a reliable index of endogenous metabolism.

Protein molecules are notoriously susceptible to damage and much has been written about the rigid criteria necessary to ensure biologically sound preparations[59,67,76-84]. Damage may occur during the preparative fractionation from plasma, during iodination, or subsequently as a result of

irradiation or other storage effects. Great care must, therefore, be exercised at all stages of preparation and iodination of albumin for tracer studies. Contact with urea or organic solvents, exposure to excessively high or low pH (above 10 or below 4)[59], and treatment by heat[83] may all cause denaturation. The convenient extraction method of Korner and Debro[85], using trichloroacetic acid, is therefore best avoided, and fractionation by column chromatography (carboxylmethylcellulose)[86] or salt precipitation[73] appears to provide the most reliable products.

Iodination of proteins may be achieved by several techniques. Despite its great value for insulin and growth hormone immuno-assays, the method of Hunter and Greenwood[87], which uses chloramine-T as an oxidising agent, is not suitable for labelling of albumin for biological use, as it appears to damage the molecule. The iodide-iodate technique[57] does not cause protein denaturation, but, for ease and convenience, the iodine monochloride method[88] is preferable, provided certain precautions are observed, e.g. avoidance of excessive substitution with radioiodine. Helmkamp and his associates[89,90] have presented an excellent analysis of the chemical reactions involved in this procedure and have suggested several modifications to promote more efficient labelling. An interesting method of iodinating by constant-current electrolysis has recently been reported by Rosa et al.[80],

who claim that their product shows no ill-effects, even when high levels of substitution are achieved (up to 25 iodine atoms per molecule of albumin). With iodine monochloride, one or two atoms per molecule is considered optimal.

One of the biggest problems in the use of iodine-labelled albumin is how to detect denaturation, as failure to do so may lead to exaggerated values for the extravascular pool and for catabolic rates. The use of biologically-screened albumin overcomes many of these difficulties, but is not practicable in humans. Unsatisfactory preparations may generally be detected by the finding of increased urinary excretion of radioactivity in the first 24 - 48 hours after injection, or of an excessive amount of unbound radioactivity in the plasma during this time. McFarlane[79] has pointed out that two albumins may be physico-chemically identical, "yet when they are iodinated, the additional molecular strain will bring about differences in their metabolic behaviour". This being so, electrophoresis, ultracentrifugation, chromatography and immunological methods may all fail to detect minor degrees of denaturation, the only evidence of which may be a catabolic rate slightly above normal. A good example that illustrates this point is provided by the recent study of Rossing and Jensen[91] comparing two different albumin preparations.

The albumins used in the work of this thesis were

fractionated from rabbit plasma by salt precipitation and labelled by the iodine monochloride method, care being taken to avoid over-iodination. Stable carrier protein was added to reduce radiation damage, iodinated products were always used within 24 - 48 hours, and frequent checks of purity were made by electrophoresis. Wherever possible, the same product was simultaneously injected into experimental and control animals, so that inconsistent behaviour could easily be detected. While none of these safeguards can be regarded as entirely infallible, confidence has been gained from almost 8 years' experience of protein fractionation and labelling. Some justification for this confidence was afforded by a recent comparison with ^{125}I-labelled albumin, prepared and supplied by the International Atomic Energy Agency, Vienna, which confirmed the suitability of the local product[92].

Full details of methods of fractionating and labelling albumin are given in the Appendix.

CHAPTER III.

DIRECT MEASUREMENT OF ALBUMIN SYNTHESIS.

An obvious direct method of measuring the synthesis rate of a protein would be to inject a radioactively-labelled precursor amino-acid, and, after an interval, to determine the amount of radioactivity incorporated into that protein. The fallacies inherent in such simple procedures are fully discussed by Neuberger and Richards[52] who point out that accurate information depends on knowledge of the specific activity of the amino-acids used at the site of protein synthesis. The problem of measuring the synthesis rate of a protein would be solved if the intracellular precursor amino-acid could be isolated and its specific activity measured. But this isolation itself presents technical difficulties of considerable complexity.

McFarlane[73] proposed an ingenious method of resolving the problem, which took advantage of the fact that radioactivity appearing in a protein, or in any other direct product of a labelled amino-acid, is a function of the integral of precursor amino-acid specific activity over the time interval under consideration. If there are two products of a single precursor, radioactivity appearing in each is related in this way to precursor specific activity. This

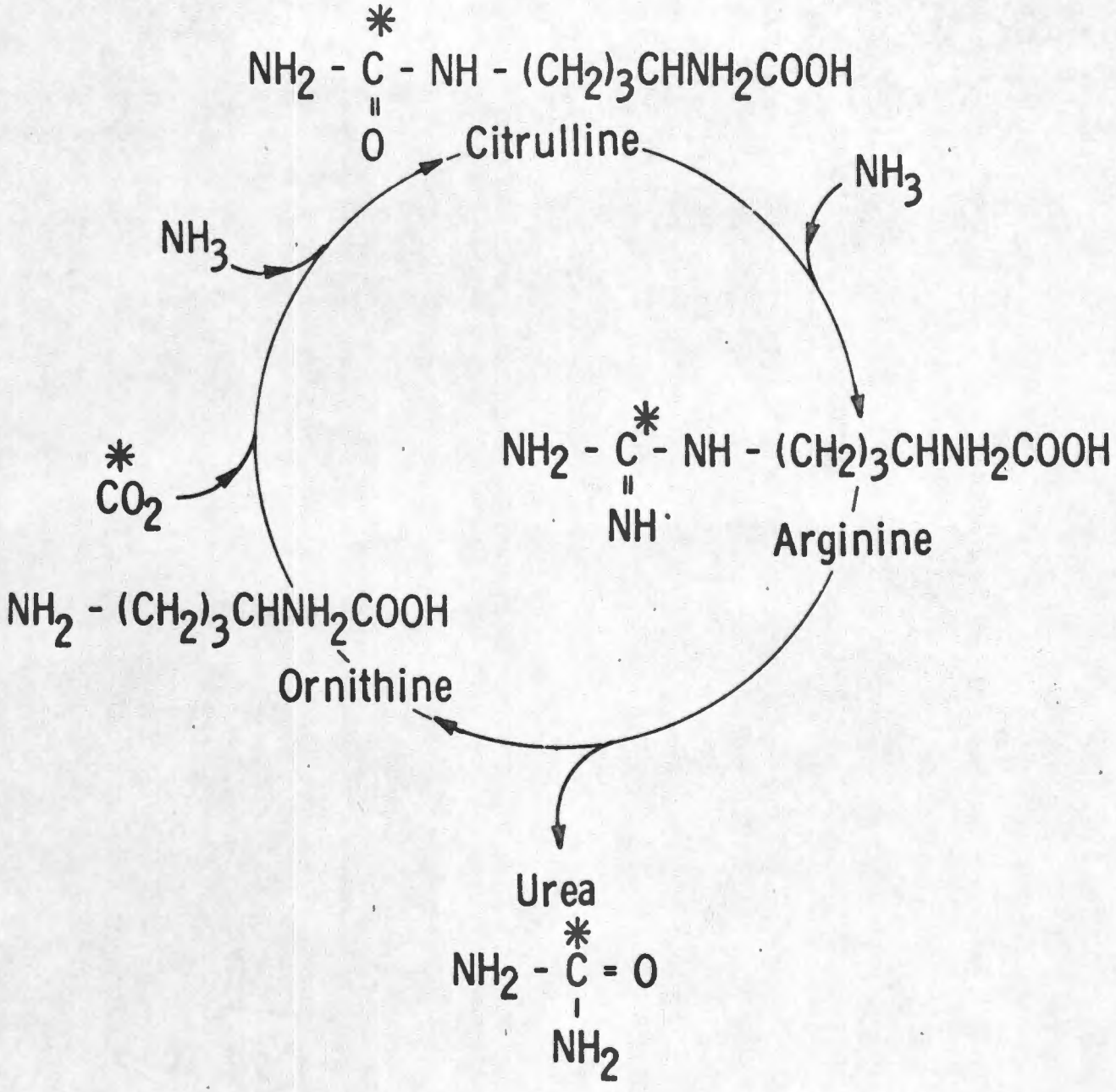

Figure 5: Simplified version of Krebs-Henseleit cycle to show production of urea from arginine, and the pathway of the ^{14}C label(*)

common relationship obviates the need for direct measurement of intracellular amino-acid specific activity, since comparison of the rates of incorporation of radioactivity into these two products reflects their relative rates of synthesis. The known metabolic fate of the guanidine-carbon of liver arginine was exploited by McFarlane to provide a basis for measuring the synthesis rate of albumin, or, for that matter, of any other liver-produced protein.

In earlier studies Delluva and Wilson[93] had conclusively demonstrated that $^{14}CO_2$ combined with endogenously-produced ammonia and ornithine to form arginine, labelled with ^{14}C only in the guanidine-carbon, as outlined in the Krebs-Henseleit cycle (Figure 5). This labelled amino-acid is subsequently used by the liver in the biosynthesis of protein, including albumin[94], and its guanidine-^{14}C is split off by the action of arginase to be incorporated into urea, production of which is virtually all accounted for by this metabolic pathway[95]. We thus have two labelled products - albumin and urea - of a single precursor amino-acid - arginine. By measuring incorporation of radio-activity into these two products over an interval of time and combining this measurement with an estimate of the amount of stable urea produced, it is possible to calculate the amount of albumin synthesized.

Mathematical justification of this argument has been concisely presented by Reeve et al.[74] and Reeve[95]. The

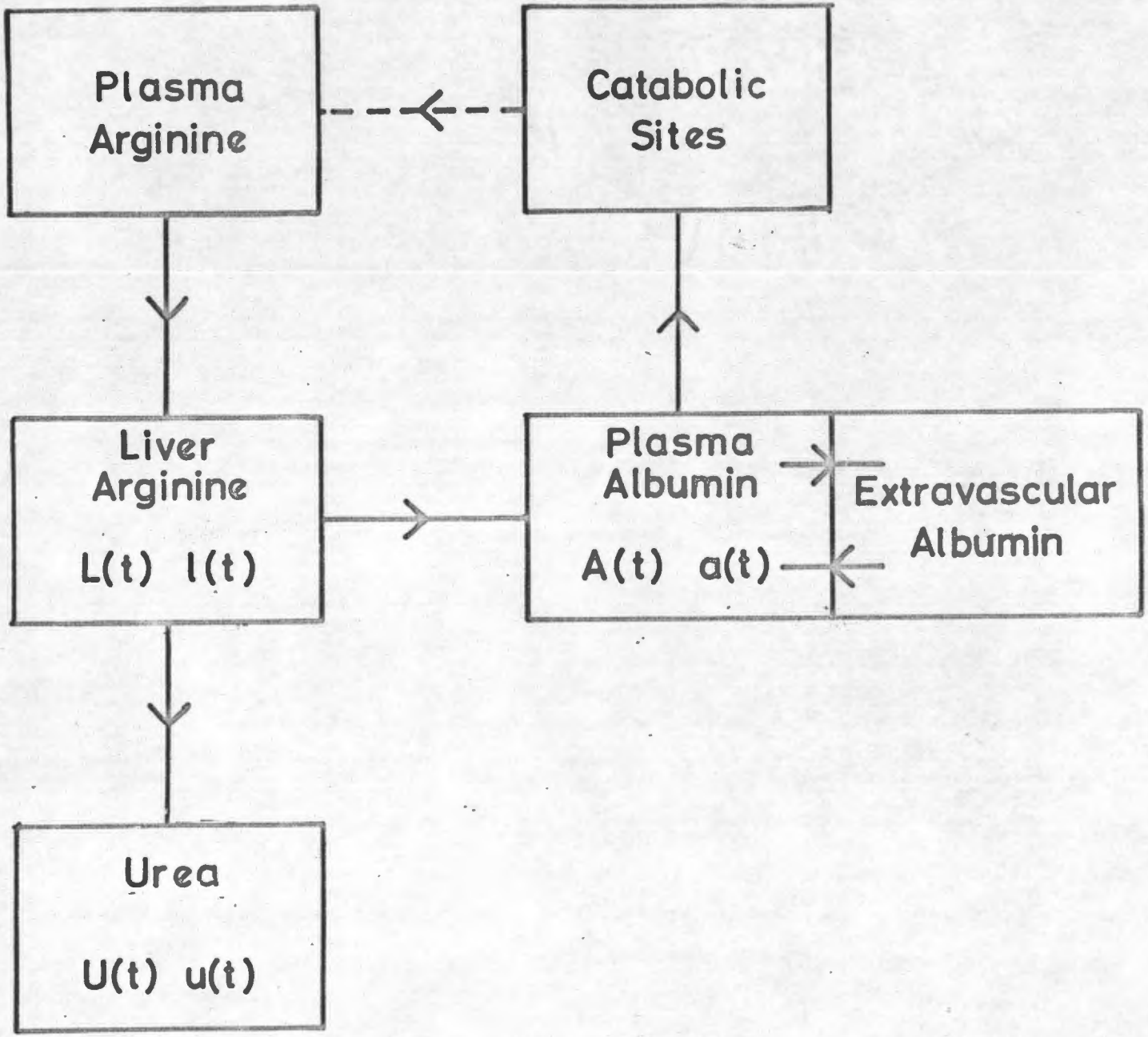

Figure 6: Scheme for incorporation of liver arginine into urea and plasma albumin. L(t), l(t), A(t), a(t), U(t), u(t) - see

following discussion is based largely on their expositions (see Figure 6):

If during time, t_o, t_1, a fraction k_a, of liver arginine, L, enters albumin,

$$A(t_o,t_1) = k_a \cdot \int_{t_o}^{t_1} L(t)dt$$

where $A(t_o,t_1)$ = amount of liver arginine entering albumin and
$L(t)$ = mg. liver arginine at time, t, available for synthesis of urea and albumin.

If n is the constant ratio of mg. albumin to mg. arginine in the protein, then $nA(t_o,t_1)$ is the quantity of albumin formed during this interval. (For albumin/arginine in man n = 16.3, in rabbits n = 19)[95].

If a similar fraction, k_u, of liver arginine enters urea during the same interval, the amount of arginine incorporated is

$$2.9\ U(t_o,t_1) = k_u \cdot \int_{t_o}^{t_1} L(t)dt$$

where $U(t_o,t_1)$ = amount of urea synthesized
2.9 = ratio of molecular weight of arginine to that of urea (174/60).

Since arginine-G-^{14}C (arginine labelled with ^{14}C in the guanidine-carbon position) behaves in a manner identical to that of unlabelled arginine, it may be substituted for the stable amino-acid. In this case, the total radioactivity entering albumin-arginine during t_o, t_1 is

$$a(t_o,t_1) = k_a \cdot \int_{t_o}^{t_1} L(t)l(t)dt$$

and entering urea

$$u(t_o,t_1) = k_u \cdot \int_{t_o}^{t_1} L(t)d(t)dt$$

where $l(t)$ = radioactivity of arginine guanidine-^{14}C per mg. liver arginine, L, at time t

and $L(t)l(t)$ = total radioactivity of liver arginine, L, at time t.

Provided k_u and k_a are constant during the experimental interval, t_o, t_1, then

$$\frac{k_a \cdot \int_{t_o}^{t_1} L(t)dt}{k_u \cdot \int_{t_o}^{t_1} L(t)dt} = \frac{k_a \cdot \int_{t_o}^{t_1} L(t)1(t)dt}{k_u \cdot \int_{t_o}^{t_1} L(t)1(t)dt} = \frac{k_a}{k_u}$$

This equation states that during time, t_o, t_1, the quantity of arginine entering albumin, divided by the quantity required for urea synthesis, equals the total guanidine-^{14}C entering albumin divided by that entering urea. The synthesis rate of albumin may now be derived:-

$$\frac{\text{Synthesis rate of protein guanidine-C}}{\text{Synthesis rate of urea}} = \frac{\text{Total activity in protein guanidine-C at t}}{\text{Total activity in urea C at t}}$$

By appropriate corrections for the sizes of urea and protein guanidine-carbon pools, one obtains the working formula,

$$\frac{\text{Fractional synthesis rate of albumin}}{\text{Fractional synthesis rate of urea}} = \frac{\text{Specific activity of protein guanidine-C at t}}{\text{Specific activity in urea C at t}}$$

In practice, maximum specific activities are used rather than those at t. The necessary adjustments to obtain these values are in the Appendix under "Methods".

Application of this equation requires the determination of 3 variables for the final calculation of albumin synthesis rate, viz. fractional synthesis rate of urea, specific activity of protein guanidine-C and of urea C. In outline, these are obtained after intravenous injection of ^{14}C-carbonate*, with serial sampling of plasma for urea specific activity measurement over time, t_o, t_1 (from the slope of which curve, urea synthesis rate can be derived), and determination of albumin guanidine-carbon specific activity at time, t_1. The principle of the method is further discussed in Chapter IV and details of procedure are given in the Appendix.

In a series of outstanding papers, McFarlane and his co-workers have discussed the theoretical basis for this technique and the various assumptions that need to be made in applying it[73,96,97,98]. He, Reeve et al.[74] and Rosenoer[99] have justified the method by comparing albumin

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* While the preceding theoretical consideration concerns incorporation of ^{14}C-arginine, in practice ^{14}C-carbonate is injected. This has an advantage over ^{14}C-arginine in that globulin specific activities are less than those of other proteins (reducing purification difficulties) and the problem of re-cycling of ^{14}C is negligible[73]. The same incorporation steps apply to $^{14}CO_2$ (see Figure 5).

synthesis rates so derived with catabolic rates obtained from ^{131}I-albumin studies in animals in whom a steady state can be assumed. Good correlation was obtained by all groups, allowing for the fact that measurement is made over a few hours for synthesis rate and over many days for catabolic rate.

The general validity of measurements of catabolic rate made with ^{131}I-albumin and of short-term assays of synthesis rate with $^{14}CO_2$ provides a means of studying alterations in albumin metabolism induced by various experimental manoeuvres. In this thesis the effects in rabbits of feeding diets of low protein content have been studied, as well as changes consequent on infusions of albumin or removal of protein by plasmapheresis. The results will be considered in relation to the findings of other workers in Chapter VI.

CHAPTER IV.

METHODS AND EXPERIMENTAL DESIGN.

METHODS.

Adult male rabbits weighing 2 - 3 kg. were housed in separate cages in conditions of controlled temperature and humidity. Lateral ear-veins were used for bleeding and intravenous injection, the animal not being anaesthetised for this simple, painless procedure.

Dietary regimes consisted of a normal protein intake (NPD) of 15% mixed protein and a low protein intake (LPD) of 5%. Diets were supplied by the Vereeniging Milling Company and the protein content was regularly checked by the Kjeldahl method. Ad libitum feeding and free access to water was allowed.

Plasma albumin concentration was measured by the method of Fernandez et al.[100] which is based on biuret determination after acid-alcohol extraction of albumin from plasma. Stable urea was measured on an auto-analyser according to Marsh et al.[101].

Plasma volume was determined by isotope dilution after injection of albumin or plasma labelled with ^{131}I, ^{125}I or ^{132}I. These isotopes were obtained from The Radiochemical

Centre, Amersham, England, ^{131}I and ^{125}I in carrier-free form without reducing agent. ^{132}I was eluted from a ^{132}tellurium column with dilute ammonium hydroxide; ^{131}I contamination was reduced to negligible amounts by prior "milking".

Plasma albumin pool was derived from the product of plasma volume and albumin concentration.

Plasmapheresis was performed by bleeding from a lateral ear-vein into sterile, heparinized containers, with immediate centrifugation, followed by re-suspension of red blood cells in sterile normal saline prior to re-injection. A standard amount of 15 ml. plasma was removed, except in Experiment C in which 25 ml. was removed. Time between bleeding and re-injection of red cells was 10 - 20 minutes.

Albumin for infusion was prepared from rabbit plasma by salt precipitation. Globulins were first separated by addition of 50% saturated ammonium sulphate; the pH of the supernatant fluid was then reduced to 4.5 by adding dilute acetic acid. The resulting precipitate was re-dissolved in distilled water, dialysed to remove all traces of salt, and made up as 3 g. % or 6 g.% solutions for infusion.

Details of methods for albumin catabolic and synthesis rate determinations are given in the Appendix. A brief description of the overall principles is presented here.

ALBUMIN CATABOLIC RATE.

Albumin for this purpose was fractionated from rabbit plasma by the ammonium sulphate method described above; its purity was always checked by electrophoresis.

Iodination with ^{131}I or ^{125}I was achieved by the iodine monochloride method of McFarlane[88], residual free iodine being removed by passage through an anion-exchange resin column or by dialysis against distilled water. At least 70% labelling efficiency was obtained at mean substitution levels of 1 atom iodine per molecule albumin. Trichloro-acetic acid precipitation invariably showed less than 1% free iodine in the final sample. In most cases plasma was added to the iodinated preparation and salt precipitation was repeated to minimize contaminant labelled material. Samples were sterilized by Seitz filtration with the addition of small amounts of carrier albumin or plasma to reduce losses and to protect against radiation damage. Final preparations were checked by cellulose-acetate electrophoresis to ensure absolute chemical purity and homogeneity of the labelled product.

For at least 24 hours prior to injection, and throughout the experiment, rabbits were given drinking water containing 0.005% sodium iodide to block thyroidal uptake of radio-iodine released by breakdown. Labelled albumin was administered intravenously in a dosage of 10 - 15 μCi, total

body radioactivity was promptly assayed and, after 5 - 10 minutes, a blood sample was removed from the uninjected ear for plasma volume determination. Plasma and whole-body radioactivity was measured daily for 10 days*. Aliquots of plasma (0.5 ml.) were counted in a well-type scintillation counter (Ecko model N664 A) with a sodium iodide crystal. Separation of the contributions of ^{125}I and ^{131}I was achieved by appropriate voltage discrimination. When ^{132}I was used with other iodine isotopes, samples were counted twice to allow decay of ^{132}I activity to negligible amounts. For assay of whole-body radioactivity, rabbits were placed in a large, well-ventilated tin which was counted in a ring of 6 matched Geiger-Muller tubes. In all cases appropriate standards were counted.

Catabolic rate was calculated from the ratio of daily fall in whole-body radioactivity and mean plasma specific activity during the corresponding 24 hours. This ratio defined the fraction of plasma albumin catabolized per day; the product of this fraction and the plasma albumin pool provided an absolute catabolic rate (mg. albumin per day).

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* In this study urinary loss of radioactivity was not measured directly, but was derived from whole-body counts. Earlier experiments had shown excellent agreement between this method and direct measurement of urinary ^{131}I loss; faecal loss seems unlikely to contribute materially to short-term studies of this sort[15].

Synthesis and transfer rate (S + T) was derived according to Matthews[38], the method being based on relative slopes of intravascular and extravascular radioactivity curves.

ALBUMIN SYNTHESIS RATE.

The general methods of McFarlane et al.[98] and Regoeczi et al.[97] were followed. ^{14}C was received in the form of sterile sodium ^{14}C-carbonate (specific activity 20 - 40 mCi/mMol.) from The Radiochemical Centre, Amersham, England, or from Phillips-Duphar, Holland. Each animal received approximately 200 μCi ^{14}C intravenously. Radio-iodinated albumin was simultaneously administered for plasma volume determination and to permit measurement of loss from the plasma during the period of synthesis rate assay. This provided a correction factor for albumin specific activity at zero time. Small samples (2 ml.) of blood were removed for urea specific activity measurement at half-hourly intervals, starting one hour after injection, for a total of 4 - 6 hours. A final large sample (10 ml.) was taken for albumin specific activity determination. During the assay rabbits were allowed access to drinking water but not to food.

Urea specific activity was measured on deproteinized plasma samples, which were then incubated with urease to produce $^{14}CO_2$, which was released by the addition of acid. The volume of gas produced was measured manometrically on

a high vacuum gas-train*, after which it was collected in phenylethylamine-methanol for radioactivity measurement. This was added to a PPO - POPOP mixture in toluene for counting in a Beckman Automatic Liquid Scintillation Counter (PPO = 2,5-diphenyloxazole; POPOP = 1,4-bis-(5-phenyloxazol-2-yl) benzene).

Albumin was extracted from plasma by the acid-alcohol technique[85]. After acid hydrolysis at 108°C, the sample was passed through a carbonate resin column to separate arginine, which was incubated with arginase to produce urea. The sample was then treated as above for $^{14}CO_2$ specific activity measurement.

The plasma urea $^{14}CO_2$ specific activity slope was used to derive synthesis rate of urea. Within 20 minutes of injection, labelled urea is believed to be uniformly distributed throughout the body water[73], so that a falling specific activity results from progressive dilution of the radioactive material by newly-formed stable urea. The slope of this fall reflects the rate of formation of urea, and interpolation to zero time gives theoretical maximum specific activity values. Albumin specific activity

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* The design and method of operation of a locally-modified McFarlane high-vacuum gas-train is described in the Appendix.

measurement at the end of the experiment was similarly corrected to zero time by application of the factor obtained from the fall in radio-iodinated albumin activity.

Albumin synthesis rate was derived from the formula

$$\frac{\text{Urea S.R. x Alb. S.A. at } t_o}{\text{Urea S.A. at } t_o}$$

and was expressed as percentage of intravascular pool synthesized per day, or in absolute terms (mg. albumin synthesized per day).

A worked example of the albumin synthesis rate determination is included in the Appendix.

EXPERIMENTAL DESIGN.

In the first instance it seemed appropriate to apply this new direct method of measuring albumin synthesis to confirm previous conclusions about the body's response to low-protein feeding[15]. Experiment A was designed with this in view. Four rabbits (F1 - 4) were kept on a normal protein diet for almost two months. Synthesis rate was measured twice during this control period, sufficient time being allowed between studies to minimize residual radioactivity in the plasma. Towards the end of this period, catabolic rate was determined from the turnover of ^{131}I-

albumin. Rabbits were placed on a low-protein diet; synthesis rate was measured after 3 weeks and, again, after 6 weeks; catabolism was assessed at the end of this phase. Rabbits were then fed a normal protein diet for purposes of repletion. Synthesis rate was measured twice after similar periods on this diet, and a final catabolic rate study was performed. Each animal was thus subjected to 6 synthesis and 3 catabolic rate determinations over a period of about 5 months.

A further group of 3 rabbits (E2 - 4) was studied twice after similar periods of low protein diet, and twice after repletion on normal diets, i.e. without an initial control study*.

In this experiment, therefore, the response of albumin catabolism and synthesis to dietary protein deprivation was studied, each rabbit acting as its own control for serial comparative purposes.

Experiments B and C were concerned with the effects of plasmapheresis on synthesis and catabolism of albumin. If the body is to restore its total albumin pool after sudden

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* As indicated later (see Appendix), pooled samples were used. The death of E1 after the control period invalidated the use of the original NPD findings for group comparison purposes; the remaining rabbits of the group (E2, 3 and 4) were used to complete the study.

removal of an appreciable portion, it can do so by increasing its synthesis rate or by reducing catabolism. In Experiment B, 15 ml. plasma was acutely removed from each rabbit and synthesis rate was measured immediately after return of red cells (0 hours) and at 2, 4, 8, 16 and 24 hours thereafter. Rabbits were studied in groups of three, plasma samples from each group being pooled; similar control groups were subjected to "mock plasmapheresis", blood being withdrawn and returned after a short time without removal of plasma. Plasma volume and albumin concentration were determined before and at the end of each 4-hour synthesis measurement period.

In Experiment C catabolic rate was measured in a further group of 8 rabbits following removal of 25 ml. plasma. ^{131}I-albumin was injected, two days were allowed for full equilibration, and daily catabolic rate was measured in individual rabbits before, on the day of, and for two days after plasmapheresis. Plasma volume and concentration were measured daily, ^{132}I-albumin being used for the former.

In Experiment D an attempt was made to suppress albumin synthesis by infusing solutions of the protein. Three groups of 3 rabbits were used, 15 ml. 3% albumin solution being infused into two, and 7.5 ml. 6% into the third. Synthesis rate was measured two hours later. In each case an equal volume of normal saline was injected into a control group.

Normal Values in Rabbits

		No. of Rabbits	Mean		S.D.	
Weight	kg.	97	2·7		0·5	
P.A.C.	g/100 ml.	97	3·72		0·34	
P.V.	ml.	97	122·0	(45·0)	21·3	(6·0)
I.V.P.	mg.	97	4530	(1670)	920	(240)
S.R.	mg/day	59	941	(394)	217	(132)
	% IVP/day	59	24·5		5·4	
C.R.	mg/day	38	1720	(591)	550	(180)
	% IVP/day	38	34·4		9·8	

Table 1: Synthesis and catabolism in normal rabbits. PAC = Plasma albumin concentration; PV = Plasma volume; IVP = Intravascular albumin pool; SR = Synthesis rate; CR = Catabolic rate; Values in brackets are expressed per kilogram body-weight.

CHAPTER V.

RESULTS.

In the course of this study, and arising from work previously reported[15], data has accrued on a large number of normal rabbits, mostly used as controls. Table 1 compares their synthesis and catabolic rates as measured by the two different methods. A discrepancy exists which is wholly attributable to inordinately high figures for catabolism, since the synthesis rate data compare well with those of McFarlane[98] and with catabolic rates for rabbit albumin reported by others[38,102-104]. The reason for these high catabolic rates is not apparent; partial denaturation of the albumin fraction is possible, but there was no increased iodide excretion in the first 24 - 48 hours such as might be expected if denatured products were used. While this discrepancy makes it difficult to draw conclusions from the relationship of synthesis to catabolism in this study, changes in catabolism per se may be accepted as valid, since each rabbit acted as its own control before and after an experimental period.

Table 2 and Figure 7 set out the results of Experiment A. Reasonably reproducible results for synthesis rate

		Days after start of diet	Weight	Plasma Albumin Concentration	Plasma Volume	Plasma Albumin Pool	Synthesis Rate		Catabolic Rate	
			kg.	g/100 ml.	ml.	g.	mg/day	%/day	mg/day	%/day
E 2-4	L.P.D.	21		4·05	98·2	3·977	680	17·1		
		42		3·88	92·4	3·585	617	17·2		
	N.P.D.	19		3·55	108·9	3·866	924	23·9		
		44		4·73	116·5	5·510	1328	24·1		
F 1-4	N.P.D.	1	2·5	4·17	134·4 (53·8)	5·604 (2·242)	1020 (408)	18·2		
		35	2·7	3·54	116·9 (43·3)	4·138 (1·533)	1022 (379)	24·7		
		40	2·6	3·88	103·6 (39·8)	4·020 (1·546)			1550 (596)	38·6
	L.P.D.	21	2·2	4·09	83·0 (37·7)	3·395 (1·543)	614 (279)	18·1		
		35	2·3	3·47	105·0 (45·7)	3·644 (1·584)			743 (323)	20·4
		42	2·5	3·31	95·4 (38·2)	3·158 (1·263)	442 (169)	14·0		
	N.P.D.	20	2·8	4·16	101·9 (36·4)	4·239 (1·514)	1149 (410)	27·1		
		48	3·2	4·10	112·0 (35·0)	4·592 (1·435)	1075 (336)	23·4		
		68	3·2	3·71	113·0 (35·3)	4·192 (1·310)			1086 (339)	25·9

Table 2: Experiment A: Effects of low protein diet (LPD). NPD = Normal protein diet; % day = % IVP per day. E2 - 4, F1 - 4 represent groups of 3 and 4 rabbits respectively. Values in brackets are expressed per kilogram body-weight.

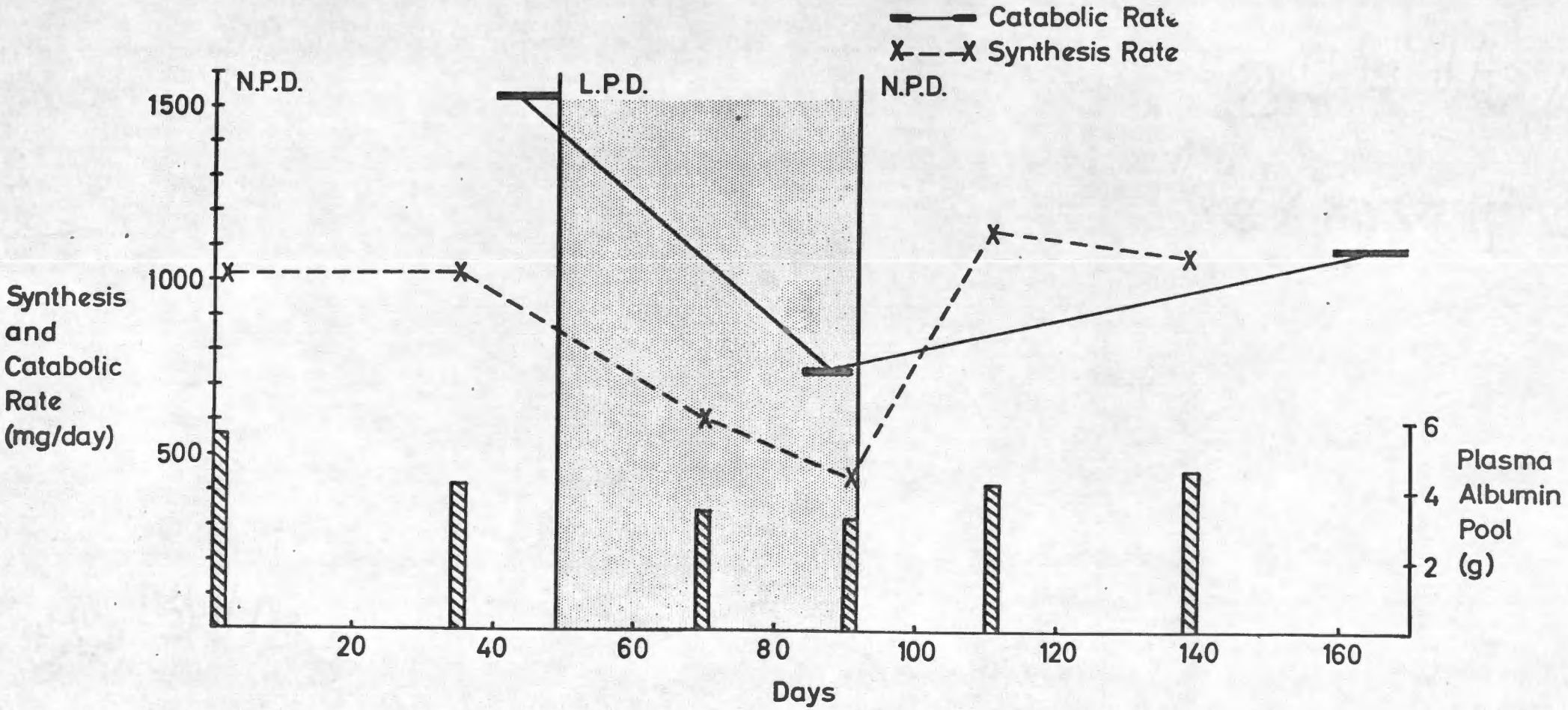


Figure 7: Changes in synthesis, catabolism and plasma albumin pool on LPD.

Hours after Plasmapheresis		Control				Experimental			
		Plasma Albumin Pool		Synthesis Rate		Plasma Albumin Pool		Synthesis Rate	
		g.		mg/day	%/day	g.		mg/day	%/day
0	(a)	3·31	(1·50)	834 (377)	25·2	2·23	(1·02)	597 (274)	26·8
	(b)	3·50	(1·39)	980 (389)	28·0	4·29	(1·64)	1527 (584)	35·6
2	(a)	2·88	(1·43)	625 (309)	21·7	2·03	(1·01)	574 (286)	28·3
	(b)	5·31	(1·90)	1307 (467)	24·6	4·56	(1·09)	894 (213)	19·6
4		4·59	(1·43)	1075 (336)	23·4	3·34	(1·04)	695 (217)	20·8
8	(a)	4·21	(2·41)	1478 (845)	35·1	4·00	(2·13)	1344 (716)	33·6
	(b)	3·77	(1·30)	739 (255)	19·6	2·60	(1·00)	621 (239)	23·9
16		3·62	(1·91)	1177 (621)	32·5	3·76	(2·04)	1087 (590)	28·9
24	(a)	3·41	(1·80)	1023 (540)	30·0	2·64	(1·40)	792 (420)	30·0
	(b)	3·94	(1·62)	1068 (439)	27·1	3·22	(1·39)	1050 (452)	32·6

Table 3: Experiment B: Synthesis rates at various times after plasmapheresis. Control groups were subjected to "mock plasmapheresis". Values in brackets are expressed per kilogram body-weight.

Hours after Plasmapheresis	Plasma Albumin Concentration g/100 ml.	
	Tp	To
0	4·08	4·05
2	3·92	3·48
4	3·75	3·52
8	3·24	3·24
16	3·94	3·55
24	3·66	3·46

Tp = Immediately before plasmapheresis.
To = At start of S.R. measurement.

Table 4: The effect of plasmapheresis on plasma albumin concentration.

Day		-1	0	+1	+2
P.A.C.	g/100 ml.	3·87	3·43	3·30	3·44
I.V.P.	mg.	3780	3041	3220	3491
C.R.	%IVP/day	36·9	35·6	36·7	38·0
	mg/day	1401	1123	1120	1277

Table 5: Response of catabolic rate to plasmapheresis. PAC = Plasma albumin concentration; IVP = Intravascular pool; CR = Catabolic rate. Plasmapheresis was performed at the beginning of Day 0.

assay were obtained during the control period on NPD, and these compare roughly with catabolic rate measurements made at the end of the period. On LPD both synthesis rate and catabolic rate are seen to fall; both return to approximately normal values after re-establishment of NPD. The plasma albumin concentration and pool-size are considerably reduced on LPD. Both absolute and fractional figures for synthesis rate and catabolic rate are decreased during low-protein feeding.

The outcome of Experiment B is presented in Table 3. In only one case (one of the two determinations made immediately after plasmapheresis) is there an increase in synthesis rate. In all other instances, including a duplicate study of the above, synthesis rate failed to rise. This applied to both absolute and fractional synthesis rate. Table 4 shows that a fall in plasma albumin concentration and pool was observed after plasmapheresis at the start of synthesis rate measurement.

The response of catabolic rate to removal of plasma is demonstrated in Table 5. From this it will be observed that a fall occurred in plasma albumin concentration and pool-size. Fractional catabolic rate remained unchanged, but because of the smaller plasma pool, the absolute rate of albumin catabolism dropped in the two days following plasmapheresis from a pre-experimental value of about

	Control				Experimental			
	Plasma Albumin Pool		Synthesis Rate		Plasma Albumin Pool		Synthesis Rate	
	g.		mg/day	%/day	g.		mg/day	%/day
I	3·56	(1·41)	812 (321)	22·8	3·89	(1·66)	817 (349)	21·0
II	5·25	(1·73)	793 (261)	15·1	5·22	(1·95)	757 (283)	14·5
III	2·85	(1·49)	915 (478)	32·1	3·23	(1·42)	1063 (467)	32·9

Synthesis Rate after Albumin Infusion

Table 6: Effect of infusion of 450 mg. albumin on synthesis rate. Control groups received equal volume of saline. I and II = Infusion of 3% albumin solution. III = Infusion of 6% albumin solution. Values in brackets are expressed per kilogram body-weight.

1400 mg. to 1120 mg. per day. This reflects a "saving" of approximately 550 mg. in this 48-hour period, which accounts for the greater part of the albumin removed (roughly, 800 mg.). Owing to the small number of rabbits studied, and the wide range of individual variation, it is not possible to show statistical significance, but one may infer that reduced catabolism plays an important role in compensating for the removal of albumin by acute plasmapheresis.

From Table 6 it can be seen that infusion of albumin (isosmolar or hyperosmolar) failed to modify synthesis rate. In each of the three groups, control and experimental animals show similar absolute and fractional rates. The rise in plasma albumin pool and concentration was not significant, and measurement was made only 2 hours after infusion.

CHAPTER VI.

DISCUSSION.

When the protein status of the body is modified, for example by loss or deprivation, certain adaptive responses occur. The distribution of albumin between intra- and extra-vascular pools is thought to alter, as well as rates of synthesis and catabolism[15,37,38,105,106]. This thesis represents an attempt to define the nature of these adaptive responses, in the hope of shedding further light on the central question of albumin homeostasis.

Ample evidence exists to show that synthesis and catabolism may be separately and independently regulated[39,78,103,107,108]. Where acute changes are brought about and steady-state conditions can no longer be assumed to exist, it is necessary to measure rates of change in these two parameters by distinct and unconnected methods. In this thesis deprivation of dietary protein has been shown to result in lowering of both synthesis and catabolic rates for albumin. Since the primary modification is restriction of amino-acid "building-bricks", it can be deduced that the fall in synthesis precedes the fall in catabolism. When protein loss was effected through acute plasmapheresis, compensation appeared to result not

from increased synthesis of albumin, but from reduction in the amount catabolized, which seemed adequate to allow restoration of the albumin pool to normal levels. This failure to produce a change in synthesis by plasmapheresis led to the adoption of infusion of albumin as a possible means of suppressing production. Again, in acute experiments, no change could be detected. Sudden alteration of the plasma albumin pool by removal or addition thus failed to produce a compensatory change in synthesis rate, suggesting that restoration was achieved in these circumstances by variations in catabolism. Some aspects of control of albumin synthesis and catabolism will now be reviewed.

Lowering of Albumin Catabolic Rate in Dietary Depletion - Response to a Smaller Pool or Lower Intake?

Reduction in the catabolic rate of albumin has consistently been found in patients with kwashiorkor[107,109-112], and can readily be shown in human adults and experimental animals fed a low-protein diet[15,71,113-115]. Absolute, as well as fractional, catabolic rates may be affected.

Waterlow[107] has properly questioned whether the fall in catabolic rate reflects the state of depletion or the amount of protein in the diet, at the time of measurement. He concluded that both factors - level of protein intake and degree of depletion - could affect the catabolic rate. The weight of evidence, however, suggests that dietary intake does not play a direct or primary role in the regulation of catabolism; the state of depletion seems to be entirely responsible.

In analbuminaemic subjects, for instance, albumin catabolism is very slow - of the order of 2.5% of the IVP per day[116] - and increases to normal when the IVP is replenished by infusion of albumin, suggesting that it is the extent of the pool that dictates the rate of catabolism. Similarly, a previous study from our laboratory showed that a group of subjects with hypoalbuminaemia of diverse etiology had lower catabolic rates than normal controls, both groups

being on the same protein intake[15]. Friedberg[117] and Rothschild et al.[102] infused albumin into mice and rabbits, respectively, and produced an expanded albumin pool with increased plasma concentration; the greater rate of degradation which followed was regarded as evidence that catabolism was dependent on the size of the albumin pool. In cirrhotic patients, Dykes[118] also observed increased breakdown, after albumin infusions which led to considerable increases in the plasma concentration and pool. The reciprocal of these experiments was reported by Matthews[38] and Hoffenberg et al.[15], who found reduced catabolism after removal of plasma protein by plasmapheresis in rabbits. In these last two sets of experiments, primary modification of the albumin pool, by addition or removal, led to alteration in catabolic rate without change in dietary protein. In a series of papers based on experimental reduction and expansion of plasma albumin pool-size and concentration, Rothschild and his colleagues concluded that degradation was a function of concentration - as it rose, catabolism increased; as it fell, catabolism decreased. Control was probably effected by a mechanism sensitive to concentration, located at the site of proteolytic enzyme activity[102-104,119-121].

Yet, it is clear from previous reports[15,71] as well as the results of Experiment A of this thesis, that dietary deprivation of protein may lead to a fall in catabolic rate. That this fall is secondary to changes in plasma

albumin pool and concentration is well-demonstrated by the recently-published work of Kirsch et al.[39], who showed, in rats on a protein-free diet, that there was a lag period of 6 - 7 days, during which time the pool and concentration became diminished, before catabolism began to fall. This dependence of catabolic rate on the level of albumin in the plasma was further substantiated by the findings of James et al.[115] in infants with kwashiorkor, studied on diets of differing protein composition.

Is Catabolism of Albumin a First- or Zero-Order Process?

A proportionate fall of albumin catabolic rate in response to reduction of plasma albumin concentration or mass would provide the body with a simple and effective means of conserving this protein in the face of threatening depletion or deprivation. Yet a first-order relationship between albumin pool-size or concentration and catabolic rate has not been easy to demonstrate and its existence has actually been denied by some workers[122]. McFarlane[59] has considered the implications of a correlation of this sort, which he regards as a theoretically ideal homeostatic mechanism for plasma proteins. The failure consistently to demonstrate this type of relationship has been alluded to briefly by Hoffenberg et al.[15] and the argument is recapitulated.

If a first-order relationship obtains, the fractional catabolic rate remains roughly constant in the face of a changing intravascular pool. This means that reduction of this pool - by plasmapheresis, urinary or faecal loss, or dietary deprivation - will result in the degradation of a fixed fraction of a smaller pool, i.e. the absolute catabolic rate will be lower. As McFarlane[59] points out, this reduction in catabolic rate may suffice to restore the pool to normal, provided albumin synthesis is allowed to continue at a normal rate and the loss of plasma albumin

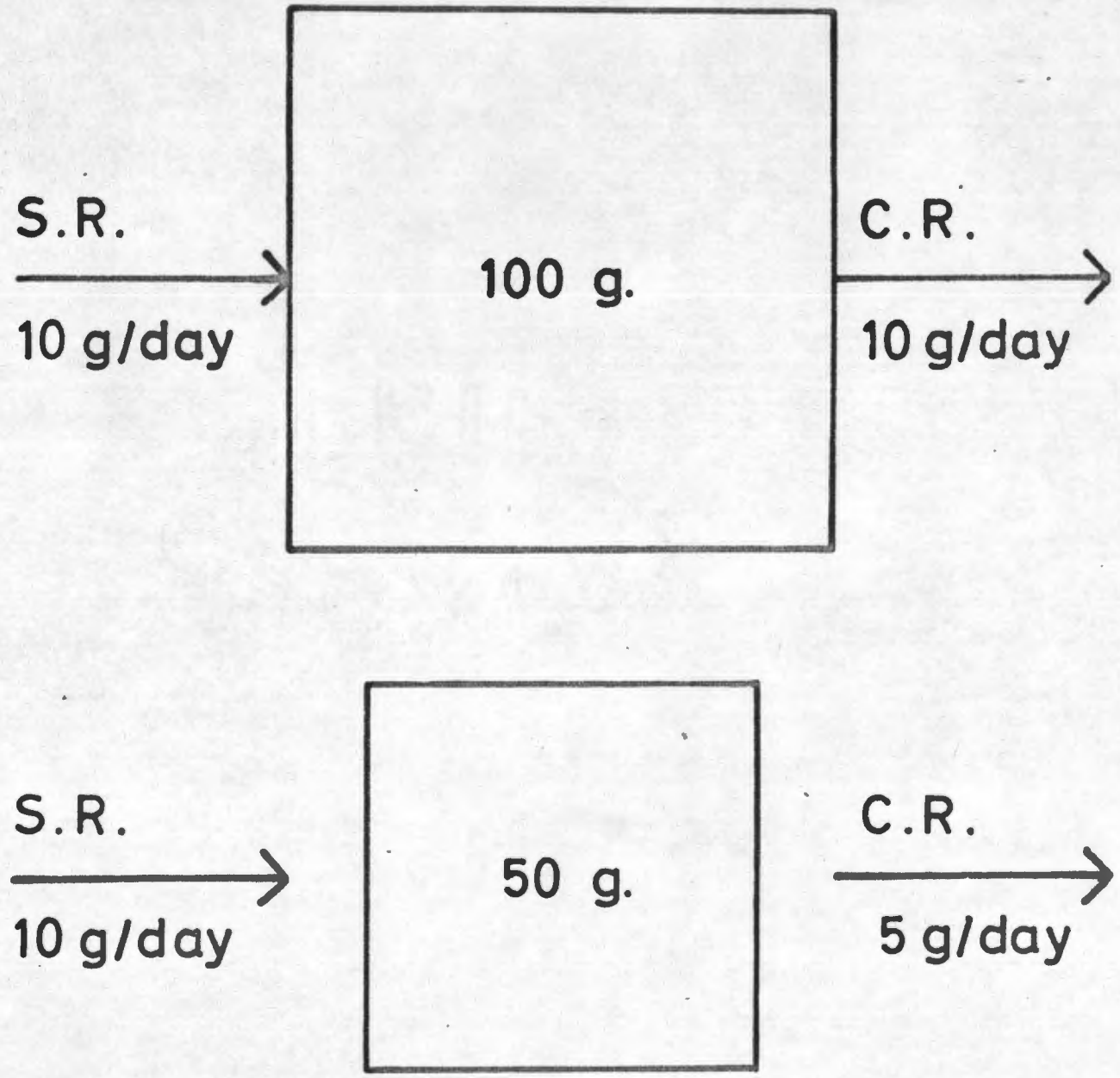


Figure 8: Restoration of pool-size by reduction in albumin catabolic rate.
S.R. = Synthesis rate; C.R. = Catabolic rate.

is not maintained. A hypothetical example will illustrate this argument (Figure 8). Let us assume a 100 g. pool of albumin, of which 10% (10 g.) is catabolised per day. In a steady state, synthesis equals catabolism to maintain the pool at 100 g. If half of this pool (50 g.) is suddenly removed, the deficit can be made up by maintaining the fractional catabolic rate at 10% of the pool - now 5 g. per day - provided synthesis continues as before, i.e. 10 g. per day. The net gain to the pool is 5 g./day at first, decreasing as the pool re-expands and the absolute catabolic rate returns to its original value of 10 g./day. Two factors, however, may obscure this simple relationship. First, it has been shown[15,37,38,105,106] that compensatory shifts of albumin take place from the extravascular pool when the intravascular pool is compromised. This, itself, may help to maintain the plasma albumin pool at a higher level, while catabolic rates are reduced. The second obscuring factor would operate if protein-loss or deficiency were severe and long-continued, and if a minimal "essential" daily degradation of albumin were to exist, which would continue, even when the plasma-pool was profoundly depleted. This "essential" catabolism (shown to exist by Rubner in 1908[17]) might disturb a first-order relationship, since, in theory, the fractional catabolic rate of albumin could actually rise to normal or above in these circumstances, although this sequence has not been demonstrated experimentally. Successive stages of depletion might be illustrated (Figure 9). In case (d),

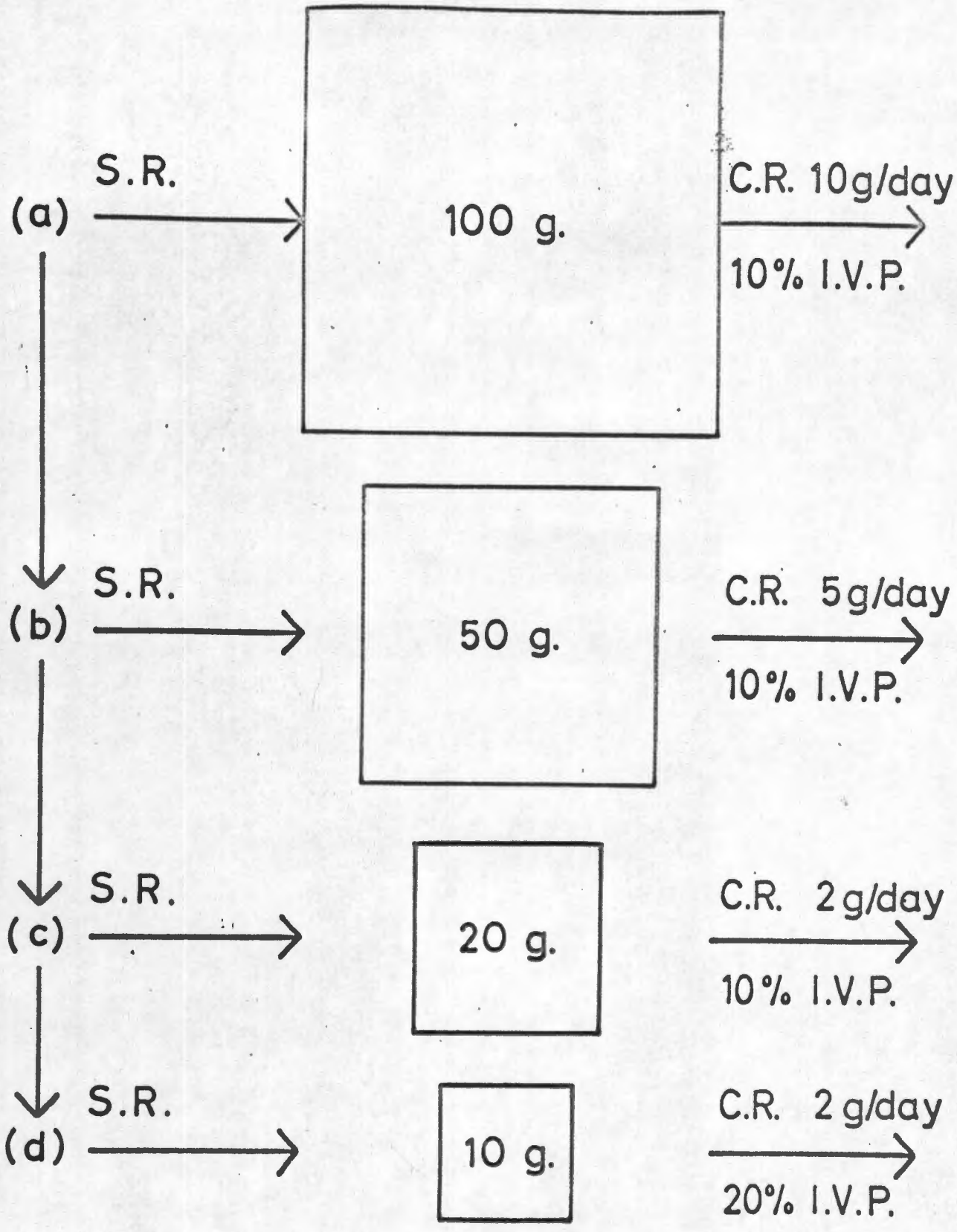


Figure 9: Progressive reduction of albumin pool. Fractional catabolic rate remains constant at 10% IVP/day until (d). SR = Synthesis rate; CR = Catabolic rate.

the minimum "essential" degradation of 2 g./day would constitute 20% of the IVP, i.e. the fractional rate would have increased and a first-order relationship would no longer be apparent.

Apart from these theoretical considerations, a lack of conformance to first-order kinetics is found in some reports of hypercatabolism in albuminuric syndromes and protein-losing enteropathy, despite the presence of low albumin pools[60,123-129]. From their findings in amino-nucleoside nephrotic rats, Katz et al.[127] proposed that albumin breakdown was reduced in the nephrotic syndrome with small intravascular pools; but, beyond a certain degree of albuminuria, the rate of breakdown correlated directly with the extent of albuminuria. They suggested that the kidney becomes a major site of catabolism when albuminuria is severe. This factor of increased local catabolism in conditions of renal or enteric leakage could explain the anomalous high rate of breakdown in the presence of small albumin pools.

Albumin Synthesis in Dietary Depletion.

Until recently, as discussed in Chapter II, measurement of synthesis rate was inferred from the metabolic behaviour of isotopically-labelled albumin. Despite possible fallacies in this inference, some useful and valid information was gained. In children with kwashiorkor, diminished synthesis of albumin was demonstrated[109-111], but the child's capacity to produce albumin was shown not to be impaired, provided adequate dietary protein was available. A rapid return to normal synthesis in the recovery phase was adduced from the immediate increase in plasma albumin levels on restitution of appropriate diets[107,109,110,130].

Using the "synthesis + transfer rate" index described by Matthews[38], Freeman and Gordon[71] and Hoffenberg et al.[15] confirmed that albumin synthesis was reduced in rats, rabbits and human subjects placed on a low-protein diet. The rapid return to normal figures on protein re-feeding strongly suggested that the rate of production depended on the availability of amino-acids, derived from dietary protein, at the site of protein synthesis. This assumption has received basic support from the work of Munro, who has shown that the intact ribosomes of the liver cell, which are responsible for albumin production begin to break down when the amino-acid supply to the cell ceases to be adequate. Fewer polysomes and more oligosomes or

free ribosomes were found in states of deprivation, indicating impaired protein synthetic capacity[131].

The results of Experiment A, in which albumin synthesis was more directly measured by the McFarlane technique, provided complete confirmation of the above assumptions, albumin synthesis falling when dietary protein was reduced. Kirsch et al.[39] have shown that this fall occurs gradually in rats on a protein-free diet (presumably as the amino-acid supply diminishes), and increases abruptly when a normal protein intake is re-established. James et al.[115] in their study of kwashiorkor, add further strength to the postulate that availability of amino-acids from dietary protein regulates the rate at which albumin is produced.

While this conclusion seems ineluctable, other regulating factors must also be considered.

Infusions of Albumin and Globulin and the Role of Plasma Osmotic Pressure in Regulating Albumin Synthesis.

It is tempting to explain the regulation of albumin synthesis in terms of commonly-accepted feedback mechanisms. In such a concept, production of albumin would be controlled by its concentration or total mass in either the intra- or extravascular pool, being stimulated by a fall and inhibited by a rise. From studies of albumin synthesis in hypergammaglobulinaemic human subjects, and after experimental infusion of globulin and dextran in rabbits, Rothschild and his coworkers felt that such a simple regulatory mechanism did exist, but that control was mediated through the osmotic pressure of the plasma, rather than the level of albumin[103, 120,121]. While altered plasma osmotic pressure most commonly results from a change in albumin level, other factors may also play a part. In a recent publication[104], the same group proposed that albumin synthesis varied inversely with the concentration of hepatic interstitial albumin. Clearly, this can only be true if amino-acid supplies and liver function are adequate to permit augmented production when extra demands are made. It should be indicated that the impressive studies of this group are based on a method of measuring synthesis which assumes steady-state conditions during the time of measurement - an assumption which is not necessarily valid.

There seems to be only one reported study which measures synthesis by direct means after attempts to expand the plasma pool. Rosenoer[99] found no demonstrable effect in 5 human subjects infused with 50 g. albumin daily for 6 days; dextran infusion similarly produced no change. Since an increase in plasma concentration and pool took place in all 5 subjects to whom albumin was administered, the author concluded that there was no evidence for a controlling feedback mechanism dependent on albumin concentration. The study of normal rabbits reported in Table 6 confirms this lack of effect of albumin infusions on synthesis rate, even when hyperosmolar solutions were used. Measurement, however, was confined to a period two hours after infusion at which time the plasma albumin concentration was not elevated.

The Value of Plasmapheresis as a Means of Inducing Change.

The technique of plasmapheresis permits the rapid removal of a variable portion of the intravascular albumin mass. Theoretically, this may be compensated for in three ways:-

(a) Increased synthesis, provided dietary protein is adequate and liver function good.

(b) Decreased catabolism (see earlier).

(c) Increased net transfer from the extravascular pool (A temporary device leaving a deficit in the EVP which, itself, will require compensation).

Because of the drastic change induced by this technique, steady-state conditions are unlikely to exist, so that synthesis cannot be equated with catabolism and must be measured by other means. Matthews[38] found a greatly increased value for "S + T" after plasmapheresis in rabbits and argued that a major part of this increase was due to higher rates of synthesis (S), although there was also good evidence that net transfer (T) of albumin from the EVP to the IVP had taken place. Hoffenberg et al.[15] used similar techniques and came to the same overall conclusions. In an attempt to separate S and T more reliably, Matthews[70,132] matched her findings to a series of equations generated by an analogue computer, from the results of which she considered the predominant compensatory reaction to be a striking enhancement of albumin synthesis, with a relatively minor contribution from the EVP. Earlier she had reported a

slight decrease in catabolic rate which she did not regard as a significant compensatory factor[38]. Hoffenberg et al.[15] felt that increased synthesis and decreased catabolism shared equally in the attempt at restoration of the albumin pool. A recent paper by Andersen and Rossing[133] attributes the major compensatory contribution to an increased transfer from the EVP, although they also found increased albumin synthesis and reduced catabolism, i.e. all three mechanisms participated in the recovery process. The methods used to provide data for their deductions cannot be regarded as entirely reliable in circumstances of such profound and dramatic change.

Experiments B and C represent the first direct attempt to measure synthesis and catabolism separately after plasmapheresis. Because of the assumed increase in synthesis discussed above, numerous assays were made at various times after the procedure. As can be seen from Table 3, there is no evidence at all of increased synthesis rate measurable by the McFarlane technique at any time up to 24 hours after plasmapheresis, despite the fact that plasma albumin concentration and, presumably, osmotic pressure had been reduced by the procedure. In view of this finding, the possible regulatory role of catabolism was again assessed. Table 5 shows that catabolism fell significantly after removal of 20 - 25% of the plasma protein pool. Furthermore, the reduction in catabolism alone could compensate for the major portion of the albumin removed and could account for the

restoration of the pool to normal values. There is thus direct and indirect evidence to suggest that plasma albumin, removed acutely by the process of plasmapheresis, is restored by reduction of catabolism, not by an increase in synthesis. A temporary transfer of albumin from the extravascular pool probably takes place.

The finding conflicts with earlier reports of Matthews[38,70,132] and Hoffenberg et al.[15], who deduced an increase in synthesis rate from the enhanced S + T values found after plasmapheresis. The most likely explanation of this conflict is that the present study is concerned with the acute response to a single plasmapheresis, whereas both earlier studies reflected the consequences of daily bleeds over a period of 7 - 10 days. If removal or loss of albumin is sustained in this way, reduced catabolism is unlikely to achieve satisfactory compensation, and other mechanisms probably come into play. Analysis of the response to proteinuria suggests an analogy: after renin-induced albuminuria of short duration, the loss is made up predominantly by decreased catabolism[128]; increased synthesis may be associated with more sustained or more profound loss (see below).

Albumin Synthesis in States of Loss.

It seems that acute expansion of the albumin pool by infusion, or reduction by plasmapheresis, cannot clearly be shown to modify the rate at which albumin is produced. Yet, it is reasonable to assume that some form of control must exist to permit the liver to produce more or less albumin as the demand arises. The continued synthesis in all circumstances of a fixed amount of albumin per day seems most unlikely.

Obviously, if the amino-acid building blocks are not supplied, synthesis must be compromised. This shortage of amino-acids could result from deficiency of dietary protein or defective absorption from the gut. In both circumstances, albumin synthesis has been shown to be reduced (References 15, 39,71,109,110,115 and Experiment A of this thesis for low protein diets; References 129,134,135 for malabsorption). Also, since synthesis of albumin is confined to the liver, severe dysfunction of that organ will be reflected by diminished production[99].

The question arises whether the liver ever responds to situations of need by increasing its output of albumin to above-normal levels. In the nephrotic syndrome and in certain protein-losing gastro-intestinal conditions, direct loss of albumin from the body, leading to reduced plasma albumin concentration and osmotic pressure, might constitute a

stimulus to increased hepatic production. Blahd et al.[123] found high rates of synthesis in nephrotic patients fed on high-protein diets; Baumann et al.[136] reported normal rates in 6 of 7 proteinuric patients; in both investigations the analytical methods may not have been entirely reliable, as judged by modern criteria. In vitro studies of albumin production by the perfused liver of nephrotic rats reflect a greater-than-normal synthetic activity[137-139] which may be related to increased liver size[140,141]. It is interesting to speculate whether augmented capacity for synthesis depends on structural adaptive changes to increase the number of functioning hepatocytes.

Assessment of the response of hepatic albumin production to the demands imposed by protein-losing gastro-intestinal disorders is complicated by the possible inter-relationships of diminished availability of amino-acids (through anorexia or malabsorption), hypercatabolism and protein-loss. The recent studies of Jeejeebhoy and his colleagues[134] support the view[142] that the liver is capable of increased synthesis in this situation, provided amino-acid supply is sufficient.

Available information is not adequate to define a complete regulating mechanism for albumin synthesis. Acute manipulation of the plasma albumin pool appears to have little or no effect on the liver's productive capacity; in more

chronic circumstances (sustained plasmapheresis, nephrotic syndrome or protein-losing enteropathy) the liver seems capable of supplying the extra demands.

CHAPTER VII.

CONCLUSIONS.

Evidence, obtained from studying the body's response to low protein diet and to acute alteration of the plasma albumin pool by plasmapheresis and infusion, has contributed towards an understanding of the mechanisms by which the pool is normally maintained at a more-or-less constant level. Synthesis of albumin adds to this pool; catabolism subtracts from it; and a two-way exchange with an extra-vascular system constitutes a third modifying influence. It is the interaction of these three factors that requires elucidation.

Synthesis clearly depends on the availability of amino-acids to hepatic cell ribosomes which are responsible for albumin production; when supplies are deficient, polysomes decrease in number, oligosomes and free ribosomes increase, and the synthetic capacity of the liver is diminished. In this thesis, albumin synthesis is measured directly to provide the first in vivo demonstration of its dependence on a diet of adequate protein content. If dietary protein is not adequate, decreasing amounts of albumin are released from the liver into the plasma, and the intravascular pool-size and concentration diminish. This diminution appears to bring about a compensatory reduction of catabolism and, possibly,

increased transfer of albumin from the extravascular pool(s). These reactions might help to sustain plasma albumin levels for a while, but continued deficiency of protein must inevitably result in reduction of albumin concentration and its consequences, as seen in the clinical syndromes of kwashiorkor or adult hypoproteinaemia. On protein re-feeding, the amino-acid supply is drastically increased, synthesis reverts to normal (possibly, supernormal) levels, plasma albumin and any extravascular deficit is restored, and the need for continued compensatory reduction in catabolism falls away.

The observation that a fall in the plasma albumin pool could be partially or wholly compensated by reduction in the amount of albumin catabolized per day, led to a further examination of the role of catabolism and synthesis in the homeostasis of body albumin. Acute manipulation of the pool by plasmapheresis or infusion of albumin was employed in the hope of providing a more rational explanation of the regulatory processes.

After a sharp loss of a moderate amount of the plasma albumin pool (through, for example, plasmapheresis or renin-induced proteinuria), the deficit can theoretically be made up by reduction of catabolism, although transfer from the extravascular pool appears to provide temporary support. The work reported here shows that this reduction can compensate for a major part of the albumin removed at a single plasmapheresis

bleed; there is no direct evidence of increased synthesis during the following 24 hours. If the loss is severe or prolonged (continued plasmapheresis, the nephrotic syndrome or protein-losing enteropathy), these compensations may be inadequate, and increased synthesis may take place, provided sufficient dietary protein is available. The albumin-sparing effect of lowered catabolism may not be found in severe proteinuria or enteric protein-loss, in both of which hypercatabolism may result from enhanced local breakdown.

Infusions of albumin, which augment the plasma pool, are likewise countered by increased catabolism, rather than decreased synthesis, in short-term experiments. Additional transfer probably takes place to the extravascular pool(s). If the extra albumin load is continued or massive, a renal threshold is exceeded and albuminuria results. Synthesis has not been shown to fall under acute conditions, but on hypothetical grounds it may do so when the load becomes inordinate.

In general, catabolism seems to be governed by the plasma albumin concentration, decreasing as it falls, increasing as it rises. In this way a simple, but effective, method of regulating the albumin pool comes into effect. This direct, or first-order, relationship may be obscured by shifts of albumin between intra- and extra-vascular compartments, or by essential degradative losses that continue,

even when the albumin pool is greatly diminished. Dietary factors do not exert a primary influence on catabolism, but affect it indirectly by altering the plasma albumin level. Synthesis of albumin can only continue at normal rates, if dietary protein is adequately supplied and absorbed, and if liver function is good. Changes in synthesis rate do not seem to follow acute modification of the plasma pool, but the amount of albumin produced probably varies inversely with the plasma albumin concentration or osmotic pressure, if these show a severe or prolonged deviation from normal values.

Plasma albumin concentration thus appears to be the prime mediating factor with respect to both synthesis and catabolism. If it falls, catabolism is reduced and synthesis is increased; if it rises, the reverse changes occur. This regulation probably depends on sensitivity to albumin concentration at catabolic sites within cells containing proteolytic enzymes, and at synthetic sites in the polysome-containing hepatocytes.

While this thesis, by providing some direct measurements of albumin synthesis, has shed a little light on the problem of albumin homeostasis, much remains to be done. In particular, sites of catabolism must be identified, and control of these sites, at a cellular level, must be elucidated; more direct knowledge is needed of albumin synthesis in disease and under experimental conditions of prolonged change; and the partition of albumin between intra- and extra-vascular compartments needs precise measurement.

SYNTHESIS

Depends on available amino-acids (dietary, absorbed) and liver function.

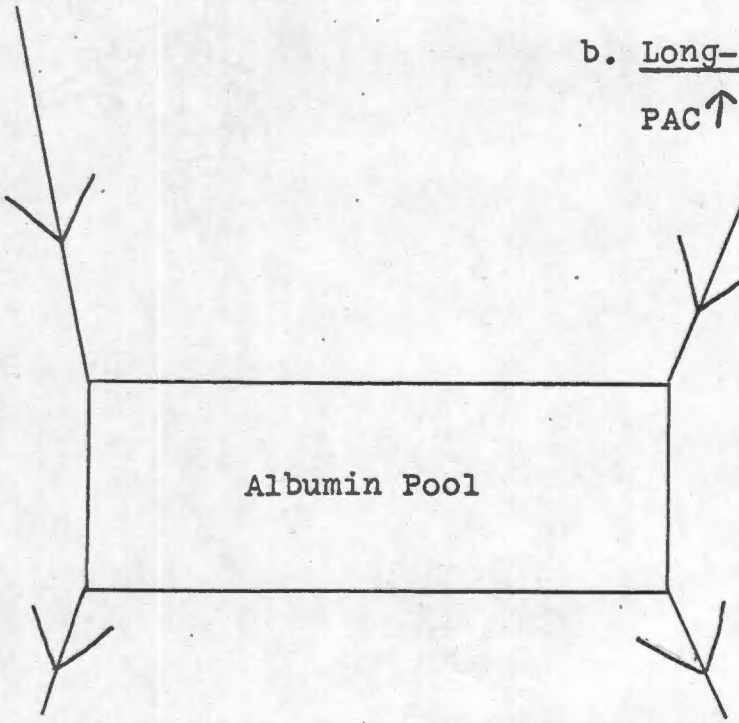
ALBUMIN INFUSION

a. Short-term or small

PAC↑ → CR ↑
IV → EV (?)
SR: no change

b. Long-term or large

PAC↑ → CR ↑
IV → EV
SR ↓
(+ albuminuria)



PLASMAPHERESIS

a. Short-term or small

PAC↓ → CR↓
EV → IV (?)
SR: no change

b. Long-term or large

PAC↓ → CR↓
EV → IV
SR↑

LEAKAGE (renal or GIT)

As for plasmapheresis, but CR may be increased by local breakdown if leakage prolonged or severe.

A concept of the homeostatic regulation of albumin metabolism.

PAC = Plasma albumin concentration; CR = Catabolic rate; IV = Intravascular; EV = Extravascular; SR = Synthesis rate; GIT = Gastro-intestinal tract.

And, finally, to put this all in proper perspective, I quote the words of another:

> "The old scientific ideal of epistēmē - of absolutely certain, demonstrable knowledge - has proved to be an idol. The demand for scientific objectivity makes it inevitable that every scientific statement must remain tentative for ever. It may indeed be corroborated, but every corroboration is relative to other statements which, again, are tentative ..... "

Sir Karl Popper.

The Logic of Scientific Discovery.
London, 1959.

CHAPTER VIII.

SUMMARY.

This thesis is concerned with the regulation of plasma albumin metabolism.

In Chapter I a short historical survey covers some of the concepts held before it was realised that the body's protein stores participate actively in its metabolic processes. It is pointed out that some tissue proteins are more labile than others - that is to say, they are more readily depleted or repleted in times of deficiency or abundance. Plasma albumin appears to reflect the protein status of the body, and may easily be altered by simple measures, to provide information about adaptive processes concerned with its homeostasis.

Chapter II provides a background to the use of radioactive iodine-labelled albumin in the study of endogenous albumin metabolism. Some terms are introduced which form part of the "jargon" of the new field of "compartment analysis" which seeks information from the behaviour of labelled tracer substances in the body. Present-day concepts of albumin distribution, synthesis and catabolism are presented with discussion of some of the problems inherent in the investigative methods and their limitations. In particular,

it is pointed out that albumin catabolism can be measured reliably, but synthesis rate determination is less valid when standard labelled tracer studies are performed.

To provide more direct and significant information about albumin synthesis, a newly-described technique was adopted. Chapter III describes the principle underlying this technique, which measures the incorporation of radioactivity into urea and into the guanidine-carbon of albumin-arginine after administration of ^{14}C-carbonate. The chemical and mathematical basis for the method is presented and elucidated. As part of this technique it was necessary to measure accurately the specific activity of very small amounts of $^{14}CO_2$. This was done on a high-vacuum gas-train using differential freezing in liquid nitrogen and ethanol-CO_2. Important modifications were introduced to the original gas-train, described by McFarlane, which have simplified the technique and made it more accurate. This modification is described in detail in the Appendix.

Chapter IV sets out the methods and experimental design of the project. Again, only principles underlying the methods are discussed, details being presented separately in the Appendix. The work was initiated to study changes in albumin synthesis and catabolism in rabbits in response to various manoeuvres - feeding of a low-protein diet, removal of plasma by plasmapheresis, and addition of albumin to the

plasma by infusion. Rabbits were studied before, during and after a period of low-protein feeding, using ^{131}I-albumin to measure catabolism and ^{14}C-carbonate to measure synthesis. Plasmapheresis was performed as a contrasting method of producing partial depletion of body albumin: catabolic rate was measured daily in some rabbits; synthesis rate was determined in others at various times up to 24 hours after plasmapheresis, groups of 3 rabbits being used for each assay. In all cases, matched "mock plasmapheresis" controls were studied. Infusions of isosmolar and hyperosmolar albumin were also given to see whether synthesis rate could be modified.

The results of these experiments are presented in Chapter V. On low protein feeding both catabolic and synthesis rates were shown to fall. When the plasma albumin mass was reduced by plasmapheresis, catabolism again fell, but no definite change could be detected in synthesis rate up to 24 hours after the procedure, by which time the plasma pool of albumin had partially returned to normal. Infusions similarly failed to alter the rate of synthesis of albumin.

Chapter VI collates the results of these and other reports of experimental modification of the plasma albumin pool. The first part of the chapter deals with albumin catabolism and shows how it alters in response to changes in the plasma albumin concentration. While this might represent

a first-order relationship, discussion follows of factors which tend to obscure it. Synthesis is next reviewed, and it is accepted that reduction occurs if dietary protein is inadequate or if liver function is poor. Less certainty exists about responses of synthesis rate to changes in albumin pool-size or concentration. The evidence favouring control of synthesis by plasma albumin levels is inconsistent, and is not supported by the acute in vivo experiments reported in this thesis.

Chapter VII presents an overall consideration of plasma albumin homeostasis, and puts forward an hypothesis to explain regulation of catabolism and synthesis in response to change. In particular, it is suggested that adaptive responses vary according to the magnitude and duration of the changes induced. Lack of consideration of these variables might explain some of the discrepancies in present-day views.

Chapter VIII, the present summary, is followed by an Appendix of detailed methods for measuring albumin catabolism and synthesis, with a brief account of the operation of a high-vacuum gas-train and of modifications to the McFarlane model which were introduced locally. A list of references to published work concludes the thesis.

APPENDIX OF DETAILED METHODS.

Note: Details of preparation of reagents marked * are presented in Appendix C.

A - ALBUMIN CATABOLIC RATE.

1. Fractionation of Albumin.

(i) To 1 volume (vol.) plasma add 2 vols. normal saline.

(ii) Add 3 vols. ammonium sulphate solution, saturated at 37°C.

(iii) Centrifuge (globulin precipitate may be redissolved in water, if needed).

(iv) Bring supernatant to pH 4.6 with 10% acetic acid.

(v) Centrifuge; redissolve albumin precipitate in distilled water.

(vi) Dialyse overnight against distilled water at 4°C to remove salt.

(vii) Measure protein concentration by biuret method.

2. Iodination.

Note: Approximately 15 mg. protein is used in not more than 0.5 ml. volume.

(i) Adjust pH of protein solution to 8.5 with alkaline glycine buffer* (tube A).

(ii) To radioiodine solution of appropriate activity in another tube add 0.3 ml. iodine monochloride working solution*.

(iii) Adjust pH of this tube to 8.5 with alkaline glycine buffer (tube B).

(iv) Rapidly squirt contents of tube B into tube A.

(v) Remove free iodine by passage through Deacidite FF resin in chloride form or by dialysis against distilled water.

(vi) Check recovery of radioactivity in eluate.

(vii) Check free iodine content of eluate, by counting supernatant after trichloroacetic acid precipitation of an aliquot, to which carrier stable plasma has been added.

(viii) Sterilize by Seitz filtration.

(ix) Check final activity and sterility prior to use.

(x) Check purity of fractionation and efficiency of labelling by cellulose acetate electrophoresis.

3. Calculation of Results.

(a) Standard Catabolic Rates.

(i) Take immediate post-injection whole-body counts and those of the 10-minute plasma sample to represent 100% of the administered dose. After

correction for appropriate standards, plot successive daily whole-body and plasma counts as a percentage of the dose on log-linear paper.

(ii) Derive best-fitting curves, using least mean squares method.

(iii) From the graphs, calculate daily urinary loss (equivalent to fall in whole-body activity) and mid-point of plasma activity for the corresponding 24-hour period.

(iv) Determine daily fractional catabolic rate (FCR) from

$$\frac{\text{Urinary excretion in 24-hours}}{\text{Mean plasma activity}} \times 100\%$$

If values for S + T are desired, further steps are:-

(v) Plot daily extravascular activity values, derived by subtracting plasma from whole-body activity.

(vi) Calculate slopes of IV and EV curves and daily EV/IV ratio.

(vii) $\text{Fractional S + T} = b + c.^{X_3}/_{X_1}$

where b = slope of IV curve

c = slope of EV curve

$^{X_3}/_{X_1}$ = ratio of EV/IV activities

(viii) Calculate absolute values for CR and S + T from fractional rates and plasma albumin pools (plasma volume x albumin concentration).

(b) Catabolic Rates in Plasmapheresis Experiments.

Note: Since day-to-day changes were to be measured, best-fitting curves could not be used for whole-body and plasma activities.

(i) For each rabbit calculate fall in whole-body and plasma radioactivity for a 24-hour period, after correcting counts for standards. Express both as percentage of administered dose.

(ii) Calculate daily FCR from

$$\frac{\text{Daily fall in whole-body activity (actual figures)}}{\text{Mean plasma activity}} \times 100\%$$

(i.e. do not use smoothed best-fitting curves).

(iii) Taking Day-1 as the 24-hour period preceding plasmapheresis, Day 0 as the following 24 hours, and Days 1, 2, etc. as succeeding 24-hour periods, calculate mean figures for the whole group of rabbits subjected to plasmapheresis in this way.

Note: By taking raw data, not those derived from calculated best-fitting curves, and by using daily measurements instead of average figures obtained from a 7 - 10 day study, one introduces a more

irregular distribution of results. This accounts for the failure to provide significant statistical backing for the results of Table 5.

B - ALBUMIN SYNTHESIS RATE.

1. Preparation for Measurement of Urea SA.

   (i) Add known amount of stable urea in 2 ml. distilled water to 1 ml. plasma samples.

   (ii) Add 6 ml. distilled water, 0.5 ml. 10% sodium tungstate and 0.5 ml. 0.66 N sulphuric acid.

   (iii) Centrifuge after standing.

   (iv) Place protein-free supernatant in round-bottom flask and evaporate to dryness.

   (v) Redissolve dry residue in about 0.5 ml. distilled water and 0.5 ml. phosphate buffer*.

   (vi) Adjust pH to 7.0 with CO_2-free 1 N sodium hydroxide*.

   (vii) Sample is now ready for gas-train (Step 3).

2. Preparation for Measurement of Albumin SA.

   (a) Separation of Albumin from Plasma.

   (i) To 5 ml. plasma add equal vol. of 10% cold trichloroacetic acid.

   (ii) Centrifuge, and wash precipitate with 5% cold trichloroacetic acid.

   (iii) Centrifuge, and add 15 ml. absolute alcohol to protein precipitate.

   (iv) Mix very thoroughly and centrifuge.

   (v) Dialyse supernatant albumin solution overnight against distilled water at $4^{\circ}C$.

(vi) Concentrate to about 5 ml. and determine accurate vol. and protein concentration to give total amount of albumin extracted (2a (vi)).

(b) Acid Hydrolysis of Albumin.

(i) Calculate amount of 6 N HCl required for hydrolysis, on basis of 100 ml. for 250 mg. protein.

(ii) Add appropriate amounts of water and concentrated HCl to protein, taking volume of solution into account.

(iii) Transfer to hydrolysis tubes, seal, and incubate at 108°C for 16 hours.

(iv) Transfer to round-bottom flask, evaporate to dryness, wash with distilled water and re-evaporate.

(c) Neutralization with Resin and Separation of Arginine.

(i) Dissolve amino-acid residue in 3 - 4 ml. distilled water.

(ii) Adjust pH to 7 with solid Deacidite FF resin in the carbonate form*.

(iii) Pass through column containing about 5 mms. of the same resin, adding distilled water washings.

(iv) If total protein (2a (vi)) was less than 100 mg., collect eluate in round-bottom flask, add 2 ml. activated arginase*, adjust pH to 9 with 1 N sodium hydroxide and incubate for 16 hours at 37°C.

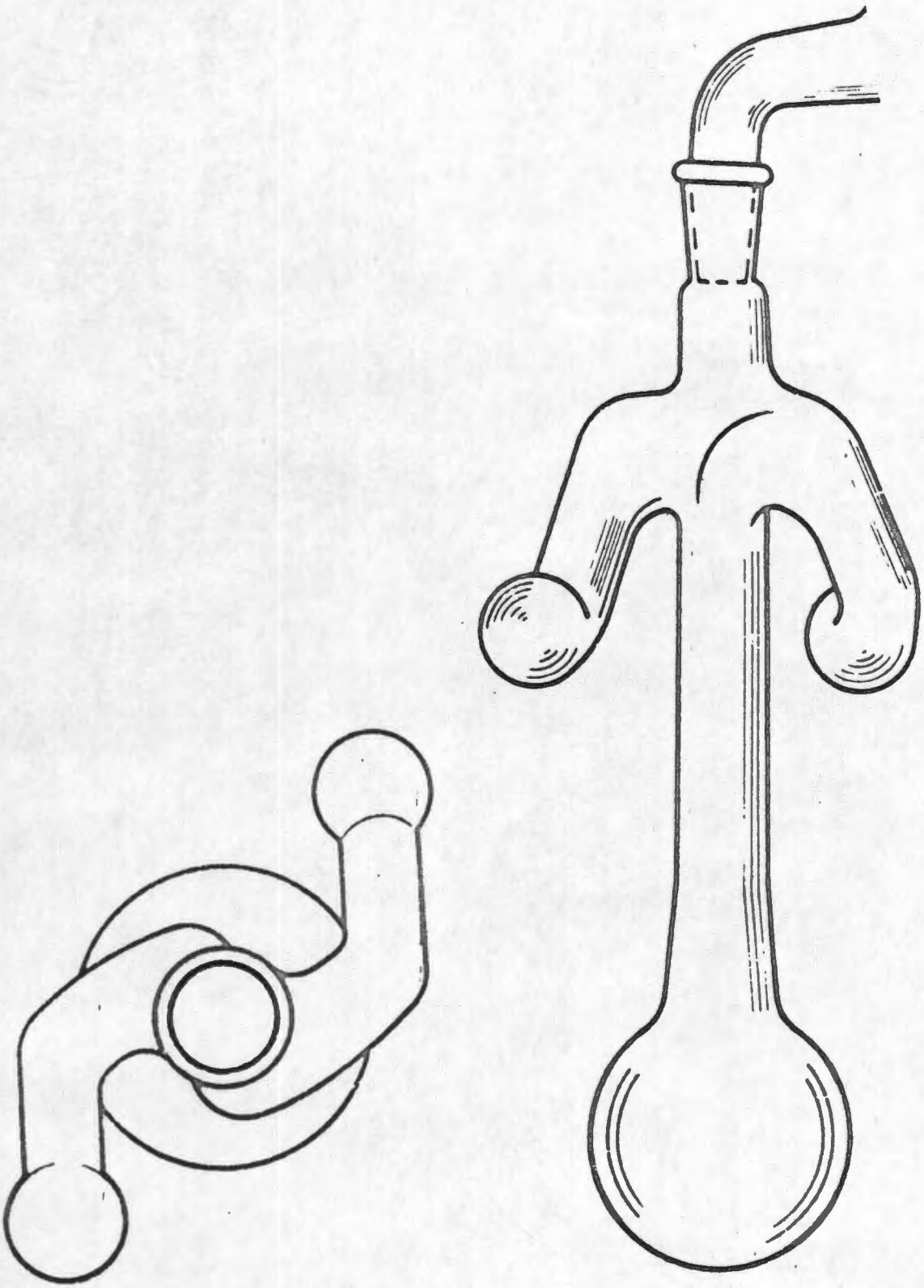


Figure 10: Reaction vessel (lateral and top elevations) showing side-arms for urease and acid, which allow each to be tipped in separately.

If total protein (2a (vi)) was more than 100 mg., evaporate eluate to 1 - 2 ml. volume, pass through column of mixed resins*. When sample is on column, add distilled water and collect 5 - 10 ml. aliquots. Spot one drop from each aliquot on filter paper, dry in oven, spray with ninhydrin and re-dry to develop (arginine stains bluish-purple). Combine aliquots containing arginine in a round-bottom flask, add 3 ml. activated arginase*, adjust pH and incubate, as above.

(v) Reduce pH to 1 - 2 by adding a small amount of 4 M citric acid.

(vi) Evaporate to dryness.

(vii) Proceed as for urea - Steps 1(v) and 1(vi).

(viii) Sample is now ready for gas-train (Step 3).

3. Measurement of $^{14}CO_2$ SA.

(i) Transfer sample to reaction-vessel (Figure 10), one side-arm of which contains 1 ml. urease*, the other 1 ml. citric-tungstic acid*.

(ii) Evacuate reaction-vessel after freezing contents under CO_2-ethanol, and de-gas urease and sample with thawing.

(iii) Tip urease into sample and incubate at 22°C for about one hour.

(iv) Tip acid into sample, agitate gently to release CO_2, and transfer reaction-vessel to gas-train.

(v) After manometric determination of volume, collect gas in 2.0 ml. phenylethylamine-methanol*.

(vi) Add this to 8.0 ml. scintillation fluid* in counting vial.

(vii) Count.

4. Calculation of Albumin Synthesis Rate (see Appendix E).

(i) Determine SA of each sample as ^{14}C counts per minute per milligram carbon.

(ii) Correct urea SA for stable urea added.

(iii) Plot serial urea SA measurements on log-linear paper and derive best-fitting line by least mean squares method.

(iv) Determine slope of line (k_1) and interpolated intercept on Y axis at time 0.

(v) Correct albumin SA to time 0 by factor obtained from fall in ^{131}I-albumin activity over period of measurement.

(vi) Calculate fractional albumin synthesis rate (% IVP per hour) from

$$\frac{k_1 \text{ (urea synthesis rate per hour)} \times \text{albumin SA at } t_o \times 100}{\text{Urea SA at } t_o}$$

C - REAGENTS USED FOR THESE PROCEDURES.

(1) Alkaline Glycine Buffer.

i. Dissolve 7.5 g. glycine in 75 ml. distilled water.

ii. Add 25 ml. 1 N sodium chloride = stock solution.

iii. Add 2 ml. 1 N sodium hydroxide to 18 ml. of this stock solution (pH 9 - 9.5).

(2) Iodine Monochloride Solution.

i. Dissolve 150 mg. sodium iodide in 8 ml. 6 N HCl.

ii. Add 108 mg. sodium iodate monohydrate in 2 ml. water (iodate solution should be forcibly injected into NaI-HCl mixture to avoid precipitation of iodine).

iii. Dilute to 40 ml. with distilled water.

iv. Shake with 5 ml. carbon tetrachloride. If organic solvent has faint red colour, remove and repeat carbon tetrachloride treatment until no trace of pink remains. Then pipette off residual carbon tetrachloride and remove final traces by aerating with moist air for 1 hour.

v. After final removal of solvent, make up to 45 ml. with distilled water. This is stock ICl solution, and is 0.033 M with respect to ICl and normal to HCl.

vi. Make working solution by diluting stock solution with 9 vols. 2 M sodium chloride.

(3) Phosphate Buffer.

Make 41.75 g. sodium pyrophosphate and 5.70 ml. orthophosphoric acid up to 500 ml. with distilled water.

(4) CO_2-free 1 N Sodium Hydroxide.

i. Prepare CO_2-free water by boiling distilled water to half original volume. Store in tightly stoppered container.

ii. Mix 10 ml. 10 N sodium hydroxide and 90 ml. CO_2-free water.

Note: Solution must be freshly made for each determination.

(5) Resins. (Deacidite FF is supplied in the chloride form).

a. In Hydroxide Form.

i. Wash chloride form of resin with water until eluate is clear.

ii. Wash with 2 litres 4 N sodium hydroxide.

iii. Wash with 2 litres 2 N sodium hydroxide until eluate is chloride-free on testing with silver nitrate.

iv. Wash with distilled water to pH 7.0.

b. In Carbonate Form.

i. Wash hydroxide form of resin several times with

0.5 N. sodium bicarbonate.

ii. Wash with distilled water to pH 7.0.

c. Mixed Resins.

Mix equal quantities of chloride and hydroxide resins.

(6) Activated Arginase Solution.

i. Dissolve 8.90 g. sodium maleate in about 200 ml. distilled water.

ii. Bring to pH 9.7 - 9.8 with 1 N sodium hydroxide.

iii. Add 11.15 g. manganese sulphate ($MnSO_4.4\ H_2O$) and make up to 500 ml.

iv. Bring to pH 7.0 with 1 N HCl.

v. Add 500 mg. arginase to 500 ml. of this manganese/maleic buffer and incubate for 3 hours at 37°C.

vi. Store at 4°C.

Note: Solution must be freshly made every two months.

(7) Urease Solution.

i. Make up as 1 mg/ml. solution in phosphate buffer.

ii. Store at 4°C.

Note: Solution must be freshly made every month.

(8) Citric/Tungstic Acid Solution.

i. Add 10 ml. 10% sodium tungstate to 3.8 ml. 1 N HCl.

ii. Make up to 100 ml. with distilled water.

iii. Mix equal vols. of this solution and 4 M citric acid.

(9) Phenylethylamine-Methanol*.

i. Re-distil phenylethylamine from commercial sources.

ii. Mix equal vols. of this and methanol.

iii. Store in dark glass bottle in a dark cupboard.

(10) Scintillation Mixture.

i. Make up 0.5% 2,5-diphenyloxazole (PPO) and 0.05% 1,4-bis-(4-methyl-5-phenyloxazole-2-yl) benzene (POPOP) in anhydrous toluene.

ii. Use 8 ml. of this solution per counting vial.

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* The superiority of phenylethylamine over commonly-used hyamine for absorbing CO_2 has been well-substantiated (143,144).

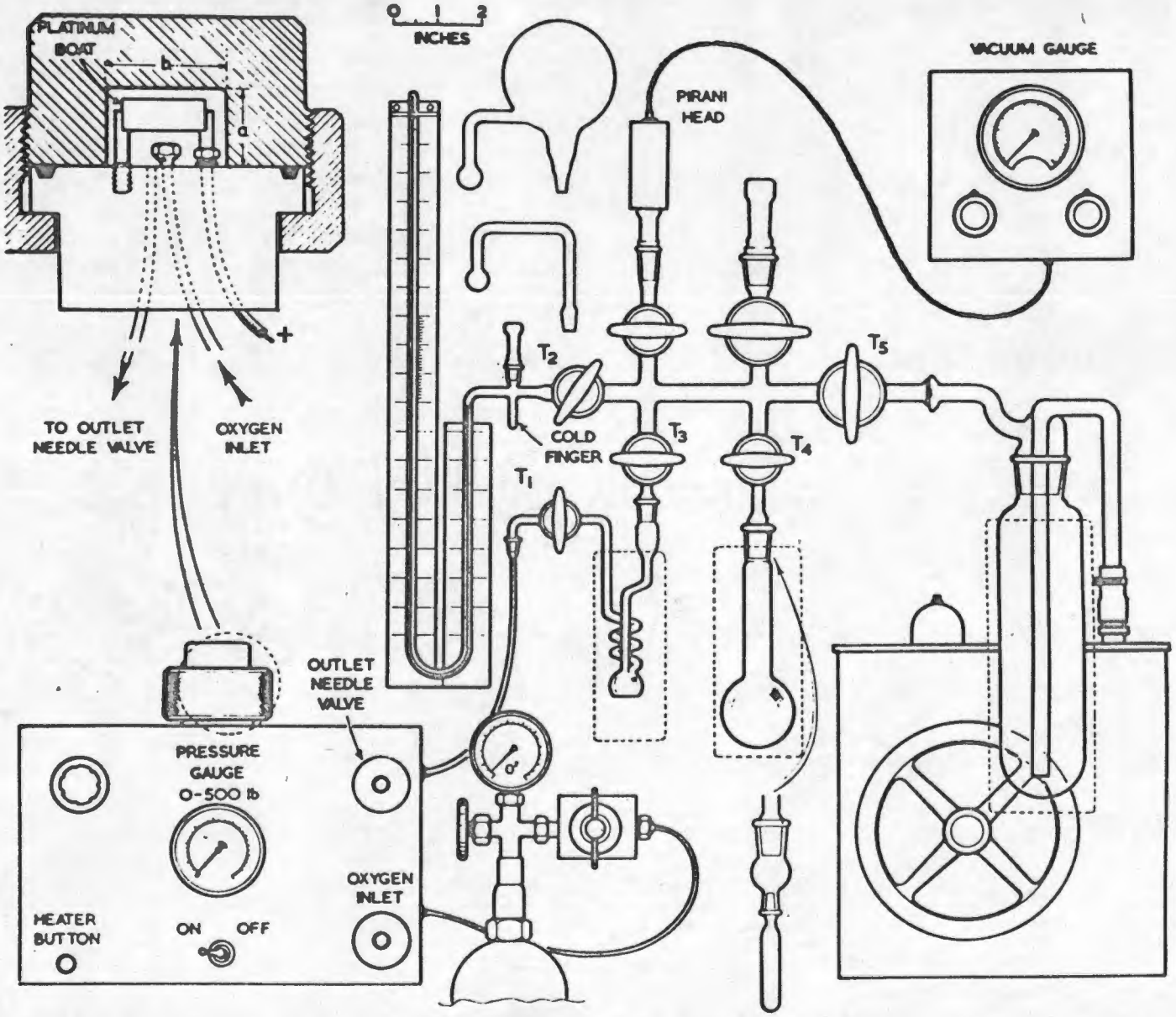

Figure 11: The McFarlane high-vacuum gas-train[145]. The left side of the diagram is occupied by a plan of a tissue combustion-bomb which was not used in the present work. A reaction vessel (Figure 10) was connected below tap, T_1.

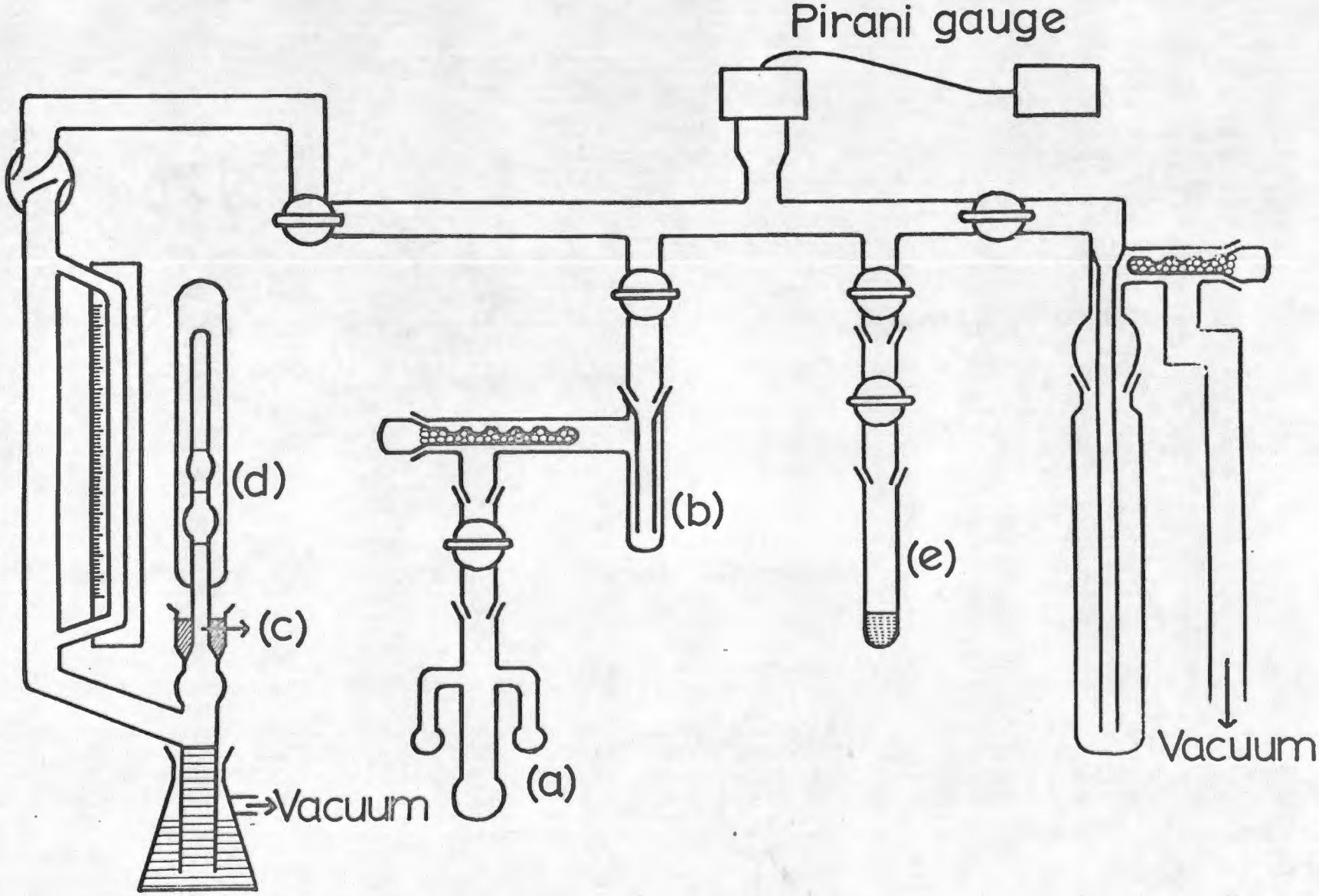

Figure 12: Modification of McFarlane high-vacuum gas-train (see text). (a) Reaction vessel (see Figure 10). (b) Cold finger. (c) Cuff for liquid nitrogen. (d) Modified MacLeod chamber. (e) Tube containing phenylethylamine.

D - THE HIGH-VACUUM GAS-TRAIN.

Modification of McFarlane High-Vacuum Gas-Train Model.

The accurate measurement of $^{14}CO_2$ was greatly facilitated by McFarlane's application of differential freezing of CO_2 in a high-vacuum gas-train[145]. In this technique, CO_2 is trapped under high vacuum by freezing in liquid nitrogen, other contaminating gases being eliminated by trapping in ethanol-dry ice. The CO_2 is released by thawing, measured manometrically and condensed into phenylethylamine for radioactive assay.

Since contamination by molecules of gas other than CO_2 could lead to serious errors, a high-vacuum system was devised with a series of taps, which would allow maintenance of a vacuum, and which could be closed during transfer of CO_2 from one point to another. Leakage into or out of the system could seriously affect readings.

The original gas-train used by McFarlane is shown diagrammatically in Figure 11. Experience with this over the past two years has led to several modifications (a) to reduce leakages through poorly-fitting taps and (b) to provide traps for moisture contamination. The system in use at present is shown diagrammatically in Figure 12, the modifications from McFarlane's

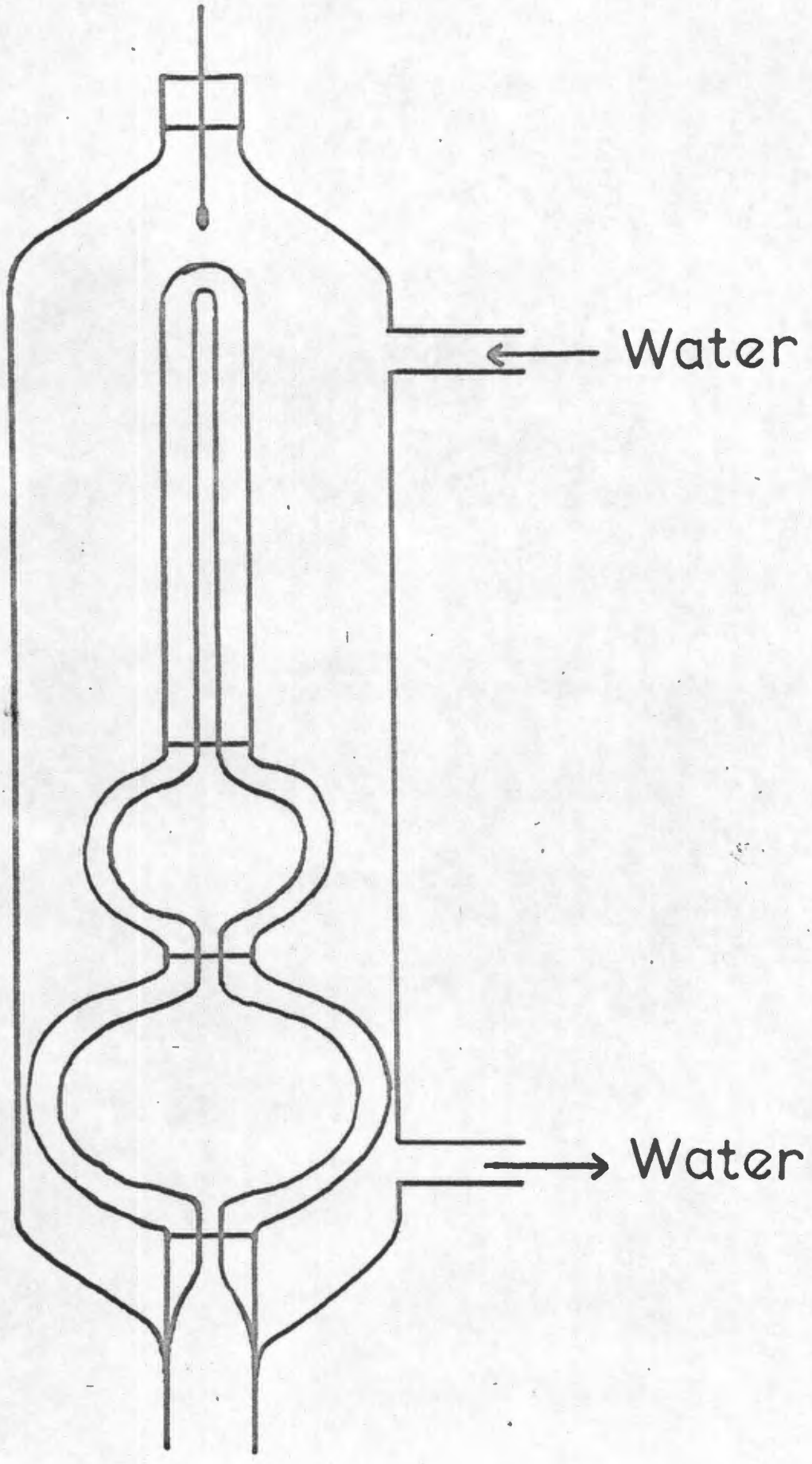

Figure 13: Modified MacLeod chamber showing 3 marks for varying gas volumes.

original model being:

i. Elimination of the two-way tap from the reaction vessel.

ii. Introduction of a moisture-trap (containing phosphorus pentoxide) between the reaction vessel (a) and cold-finger (b).

iii. Elimination of the cold-finger for trapping CO_2 for volumetric analysis, and replacement by a cuff (c) for liquid nitrogen, which freezes CO_2 prior to release into the MacLeod chamber.

iv. Introduction of a modified MacLeod chamber at (d) for measurement of CO_2 volume, thus increasing sensitivity by compressing the gas to obtain larger mercury fluctuation. This chamber has 3 marks (Figure 13) to which mercury can be adjusted according to the volume of CO_2 in the sample.

v. Elimination of the redundant access to the atmosphere.

vii. Introduction of a second moisture trap on the pump side of the system.

This simplified, less troublesome model, was built to specification by Mr. A. Monk of Scientific Glassblowers, Ltd., who is also responsible for providing the reaction vessels.

Principles of Procedure for Use of High-Vacuum Gas-Train.

i. After termination of urease action by addition of citric-tungstic acid, the reaction vessel is placed on the gas-train (a), frozen in CO_2-ethanol and the system is evacuated.

ii. $^{14}CO_2$ is trapped under liquid nitrogen in cold-finger (b), contaminating gases being removed by suction.

iii. $^{14}CO_2$ is released by thawing, but retained in the cold-finger by closure of the taps on either side; CO_2-ethanol is applied to the cold-finger to trap contaminant gas.

iv. $^{14}CO_2$ is transferred to the manometer under a liquid nitrogen cuff (c), the mercury column is raised to prevent leakage back, and thawing is brought about by addition of warm water to the cuff.

v. By raising the mercury column to one of the 3 marks on (d), a manometric reading of $^{14}CO_2$ volume is obtained - scale readings are translated to volume of CO_2 from previously-constructed calibration graphs.

vi. The mercury column is lowered and $^{14}CO_2$ is transferred to phenylethylamine in tube (e) frozen under liquid nitrogen.

vii. Both taps connecting (e) are closed, the tube is removed, stoppered and allowed to thaw.

viii. Solution is transferred to a counting-vial containing 8 ml. PPO-POPOP mixture.

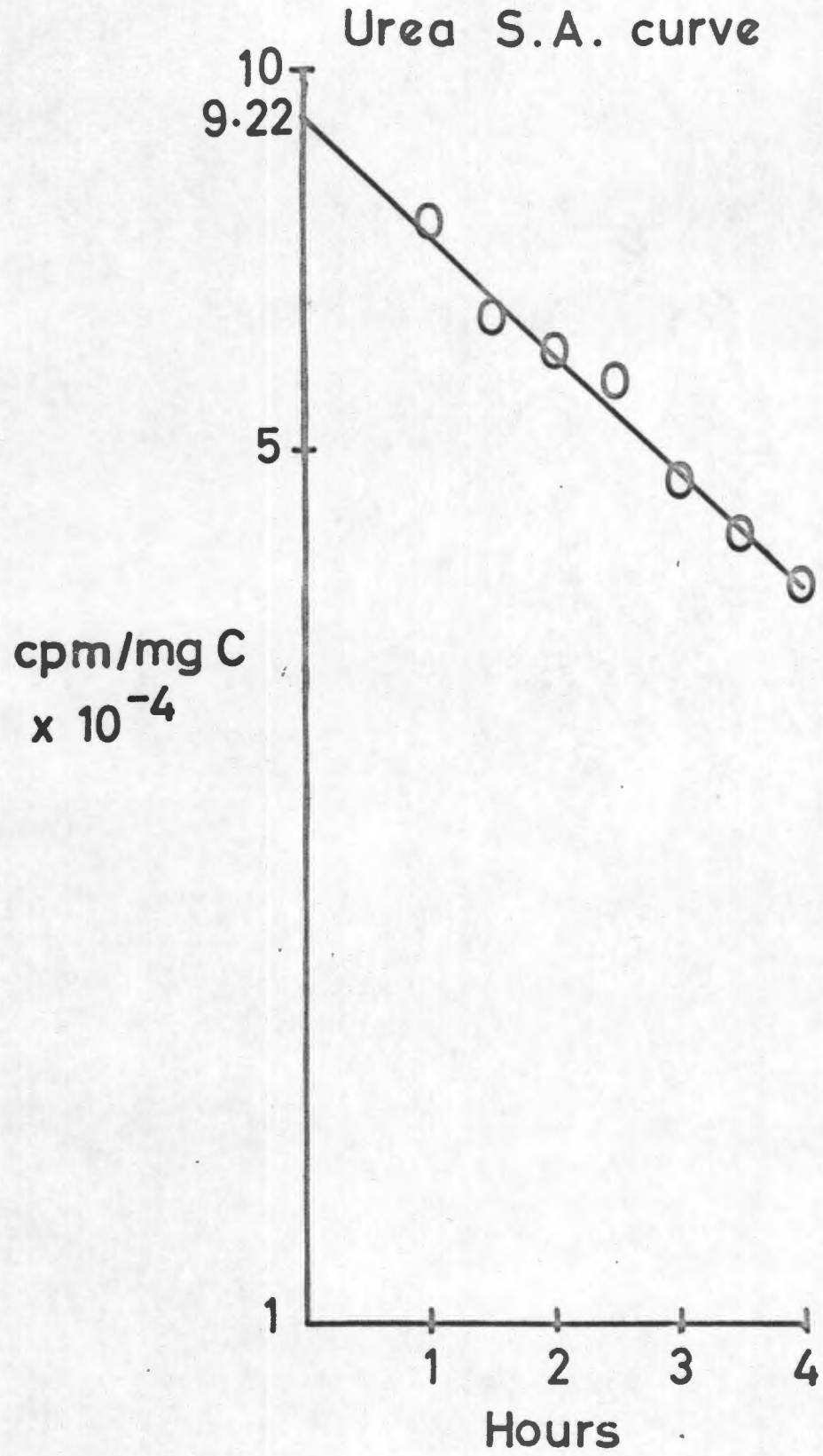

Figure 14: Plasma urea ^{14}C SA curve, interpolated to zero time to give theoretical maximum SA (9.22 cpm/mg. C x 10^4).

Hours	cpm	mg C	S.A. cpm/mg C	Stable* Urea mg/100 ml.	Corrected S.A. cpm/mg C
1·0	2663	0·273	9753	29·5	75875
1·5	2506	0·293	8556	31·0	63756
2·0	2341	0·291	8052	31·0	60000
2·5	2095	0·262	7995	33·0	56450
3·0	1908	0·269	7103	35·5	47120
3·5	1944	0·303	6419	35·5	42582
4·0	1682	0·279	6036	36·5	39110

* = Stable urea on original plasma samples

$k_1 = \frac{0.693}{3.2} = 0.217 = 21.7\%/\text{hour}$

Alb. S.A.(T_4) = 3398 cpm/mg C

$\frac{\text{Sample A}}{\text{Sample B}} = \frac{100}{67}$

Alb. S.A.(T_0) = $3398 \times \frac{100}{67}$ cpm/mg C

Alb. S.R. = $3398 \times \frac{100}{67} \times \frac{21.7 \times 24}{92200}$

= 28·7 % IVP/day

P.V. = 75·3 ml. P.A.C. = 3·14 g/100 ml.

I.V.P. = 2·36 g.

Alb. S.R. = 28·7 x 2·36 = 677 mg/day

Table 7: Worked example of calculations for albumin synthesis rate determination.

E - WORKED EXAMPLE OF ALBUMIN SYNTHESIS RATE MEASUREMENT.

Three rabbits served as a control group for a plasmapheresis experiment. $^{14}CO_2$ (200 µCi of $Na_2{}^{14}CO_3$) and ^{131}I-albumin (1 - 2 µCi) were injected intravenously into each animal. After 10 minutes, 1 ml. blood was removed from the uninjected ear for plasma-volume determination (Sample A) and pooled for measurement of plasma albumin concentration. Starting one hour later, half-hourly blood samples of 2 ml. were removed, and equal aliquots of plasma from each were pooled for stable urea and urea ^{14}C SA measurements. A final sample of 10 ml. was taken at 4 hours, ^{131}I activity was counted (Sample B), and equal aliquots were then pooled for urea and albumin SA determination.

Two mg. stable urea were added to each pooled plasma sample prior to urea ^{14}C SA measurement; in the final assessment of this, correction was made for the added urea. Table 7 shows the observed and corrected figures for this experiment. The latter were plotted on log-linear paper, as shown in Figure 14. A best-fitting curve was drawn through these points and interpolated to zero time to give a theoretical maximum urea SA.

Albumin-arginine ^{14}C SA was determined on the final sample and corrected to zero time by a factor equal to

$$\frac{\text{Counts per ml. Sample A}}{\text{Counts per ml. Sample B}}$$

The fractional synthesis rate of urea (k_1) was derived from the slope of the urea SA curve (0.693/half-time).

Table 7 shows successive steps for the calculation. The following abbreviations were used :

cpm = counts per minute of ^{14}C per sample

mg C were derived from gas-volume

corrected SA = urea ^{14}C SA after correction for added stable urea

k_1 = slope of plasma urea ^{14}C SA curve

Alb. SA (T_4) = SA of guanidine-^{14}C of albumin-arginine 4 hours after injection

Alb. SA (T_o) = SA of guanidine-^{14}C of albumin-arginine corrected to zero time

Alb. SR = Albumin synthesis rate

IVP = Intravascular albumin pool

PV = Plasma volume

PAC = Plasma albumin concentration.

F - STATISTICAL METHODS.

The following standard statistical methods were used:-

1. Calculation of Mean and Standard Deviation.

$$\bar{x} = \frac{\sum x}{n}$$

$$S.D.^2 = \frac{\sum (x - \bar{x})^2}{n - 1}$$

$$= \frac{\sum x^2 - \frac{(\sum x)^2}{n}}{n - 1}$$

2. Comparing the Means of Two Small Samples from Normal Populations.

(a) Unknown variances, σ_1^2 and σ_2^2, assumed to be equal.

$$\text{Student's } t = \frac{\bar{x}_1 - \bar{x}_2}{s\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

$$\text{Where } s^2 = \frac{\sum_1 (x - \bar{x}_1)^2 + \sum_2 (x - \bar{x}_2)^2}{n_1 + n_2 - 2}$$

Degrees of freedom are $n_1 + n_2 - 2$

(b) Unknown variances, σ_1^2 and σ_2^2, not assumed to be equal.

$$d = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}}$$

Treat d as Student's t but degrees of freedom are

$$f = \frac{1}{\frac{u^2}{n_1 - 1} + \frac{(1 - u^2)}{n_2 - 1}}$$

$$\text{where } u = \frac{s_1^2/n_1}{s_1^2/n_1 + s_2^2/n_2}$$

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Mr. Alf Monk, of Scientific Glassblowers, Ltd., has earned my admiration by demonstrating his artistry, not only in

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