Zeitschrift: Bulletin der Schweizerischen Akademie der Medizinischen Wissenschaften = Bulletin de l'Académie suisse des sciences médicales = Bollettino dell' Accademia svizzera delle scienze mediche

Herausgeber: Schweizerische Akademie der Medizinischen Wissenschaften

Band: 21 (1965)

Artikel: Turnover of labelled plasma proteins

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DOI: https://doi.org/10.5169/seals-307604

C.D.: 612.124:621.039.85

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Turnover of labelled plasma proteins

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The use of the term turnover implies the existence of an equilibrium in which absolute synthetic and catabolic rates affecting a protein are equal, a situation which can be assumed to hold in an animal in dietary nitrogen equilibrium. Since of the two the catabolic rate is technically much easier to measure turnover values (or half-lives) in the literature have generally been based on measurements of catabolism carried out in one of two ways. Either a labelled amino acid or a labelled protein was injected and after a suitable interval the rate of loss of protein bound activity from the plasma was measured. These alternative injections have vied with each other almost continuously in popularity since isotopes were first used for this purpose (*Foster*, *Schoenheimer*, and *Rittenberg* 1939) but only in recent years have their relative merits come to be fully appreciated.

Amino acid reutilization

After injecting a labelled amino acid some transfer of the isotope to other amino acids occurs even before the first labelled protein molecules appear. This happens particularly with glycine and with sulphur containing amino acids and requires that catabolic measurements should be based on specific activities of the injected amino acid after it is isolated from the protein and not of the protein as a whole. In these circumstances protein catabolic rates are not affected by 'spread' of the isotope and it would even be permissible, but hardly economic, to inject one amino acid and to base the measurement of catabolic rate on the slope of the specific activity curve of a metabolically related one.

The overriding consideration in planning measurements of this kind is the extent to which the labelled amino acid will be reused for protein synthesis. To this extent will newly labelled molecules continue to be

injected into the plasma after the initial massive arrival and as a result the apparent half-life of the protein will be proportionately increased. This reutilization is a highly individual matter amongst amino acids, apparent half-lives depending greatly on the one used whereas, of course, the true value should not. This was clearly demonstrated in rabbits by *Penn*, *Mandeles*, and *Anker* (1957) using a series of ^{14}C labelled amino acids (cf. Fig. 1) of which glycine was least reincorporated. This amino

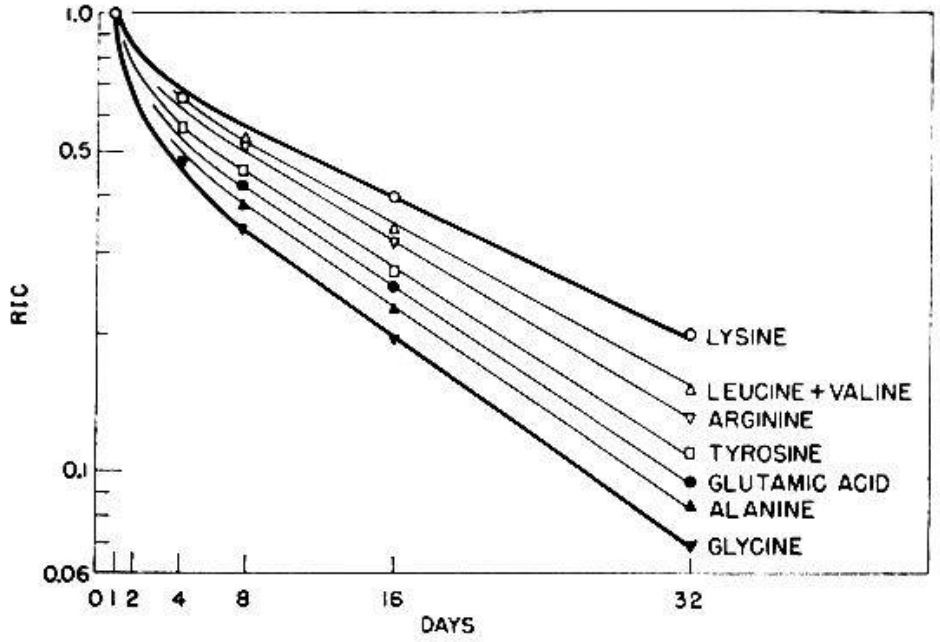

Fig. 1. Amino acid specific radioactivities in plasma albumin after injecting the same ^{14}C-amino acid (cf. *Penn*, *Mandeles*, and *Anker* 1957).

acid labelled with ^{15}N, was indeed among the first to be used for plasma protein labelling purposes by *Schoenheimer*, *Ratner*, *Rittenberg*, and *Heidelberger* (1942) but reutilization in their experiments was on a considerably greater scale because of two additional complicating factors, viz. transamination and a generally greater animal conservation of nitrogen than of carbon. This conservation is strikingly illustrated by a high degree of reutilization of urea nitrogen but sensibly none of its carbon after bacterial digestion of urea in the intestine, a matter which has only recently been studied in detail (*Regoeczi*, *Irons*, *Koj*, and *McFarlane* 1965).

The efficiency with which an injected labelled amino acid is incorporated *de novo* into plasma protein should also reflect the extent of its reincorporation unless the release takes place at a site of synthesis, for example inside parenchymal cells of liver, which would also need to be relatively isolated in a metabolic sense. Although *Maurer* and *Müller* (1955) claimed that this kind of predominantly direct intracellular

transfer, in their case of ^{35}S methionine from albumin to α, β, and γ globulins, took place in rats others were unable to confirm it with ^{14}C proteins (*McFarlane* 1957). Sites of synthesis of plasma proteins are well recognised and such a transfer, if it occurred, would be an important help in deciding whether such sites are also concerned with catabolism about which remarkably little is known.

Unique behaviour of guanidine carbon of arginine

Whereas about 2% of the methylene carbon of injected labelled glycine is incorporated into proteins in the plasma the corresponding proportion in the case of the guanidine carbon of arginine is only $^1/_{10}$ of this because of an exceptionally high rate of exchange of this particular carbon with urea via the Krebs-Henseleit cycle (cf. *Swick* 1958). Reutilization of the guanidine carbon released from proteins should therefore be extremely low. That this is indeed the case was shown by the demonstration that the half-life of the guanidine carbon of albumin in rabbits labelled by injecting $NaH^{14}CO_3$ was the same as of simultaneously injected ^{131}I albumin (cf. Fig. 2). Of the available methods of so called biosynthetic labelling this use of bicarbonate appears to be least associated with

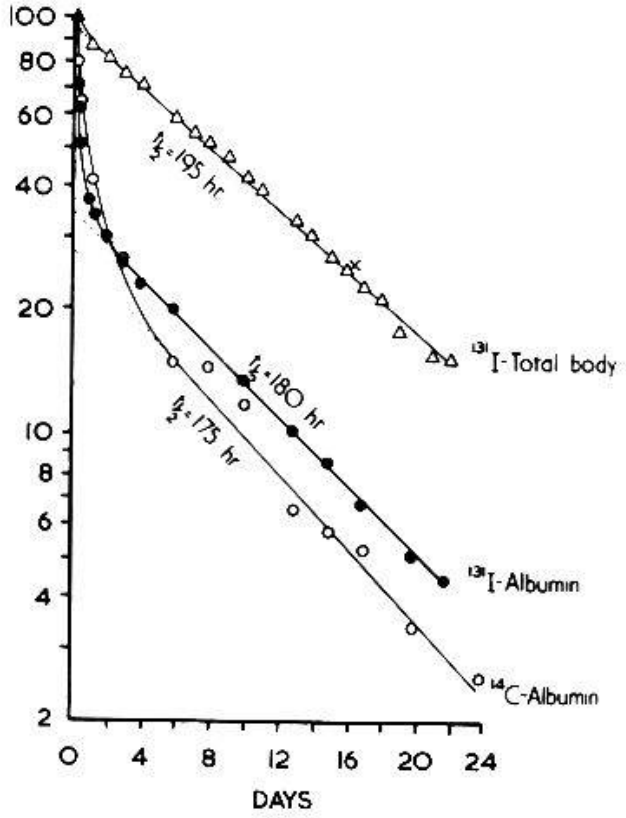

Fig. 2. Comparison of albumin ^{14}C-guanidine carbon radioactivities with ^{131}I-albumin and total body radioactivities in the rabbit after injecting $NaH^{14}CO_3$ and ^{131}I-albumin (cf. *McFarlane* 1963c).

reutilization defects so far as the measurement of plasma protein half-lives is concerned. In addition, although the efficiency with which bicarbonate carbon is incorporated into guanidine carbon of proteins is very low the procedure as a whole is relatively economical since radioactive carbon in this form is at least ten times cheaper than in the form of optically active amino acids.

Biosynthetically labelled proteins

Economic considerations also militate against the alternative procedure of injecting biosynthetically prepared ^{14}C proteins. Using the cheapest available source of ^{14}C amino acids (whole protein of ^{14}C-Chlorella) *Humphrey* and *McFarlane* (1954) were obliged to spend about £ 300 per rabbit to obtain plasma proteins of adequate specific activity for use in other experimental animals. In addition, it is hardly likely that ^{14}C human proteins will be prepared by this method for clinical purposes because of the high level of radiation to which donors would need to be subjected. It has, however, been possible to prepare extremely useful human proteins labelled biosynthetically with ^{35}S-methionine and cystine (*Margen* and *Tarver* 1955; *Volwiler*, *Goldsworthy*, *McMartin*, *Wood*, *McKay*, and *Fremont-Smith* 1955). While the protein transfer procedure has everything in principle to recommend it, even in strictly animal work it is likely to enjoy only a limited application of which there are already several excellent examples (cf. for example, *Miller*, *Bale*, *Yuile*, *Masters*, *Tishkoff*, and *Whipple* 1949).

Reutilization cannot be entirely abolished by passive transfer of labelled proteins. Six hours after injecting most amino acids 3–5% of the dose is present in the form of labelled plasma protein and up to 50% as labelled carcass protein. Muscle proteins which predominate amongst carcass proteins turn over more slowly than do plasma proteins (*Velick* 1956; *Dreyfus*, *Kruh*, and *Schapira* 1960) and the fractional rate of delivery of labelled amino acids into the body water from this source, at least after the first day, will be lower than from plasma proteins. Even when the fractional contributions are equal the effect of transfusing the labelled protein to an unlabelled animal will be to reduce the degree of reutilization to $^1/_{10}$, and this order of reduction can be obtained by substituting bicarbonate for amino acid labelling without the need to inject a donor. Provided, as seems likely, the degree of reutilization of the guanidine carbon of arginine is indeed negligible this procedure should supplant the cross-transfusion one for most purposes. It must be kept in mind, however, that the mandatory low degree of

reutilization is only obtained with bicarbonate in relation to liver-produced plasma proteins, the reutilization of arginine guanidine carbon in all cells other than liver parenchyma being extremely high. This means, unfortunately, that half-lives of γ-globulins and hormone proteins cannot be measured in this way.

In vitro labelling of proteins

All *in vitro* procedures for protein labelling must be regarded with suspicion on two main grounds, viz. that the protein may have been denatured either before labelling or as a result of the labelling procedure. A situation has also arisen in the writer's experience in which an apparently undenatured purified protein—as judged by the usual physicochemical criteria—was found to be denatured after iodination by a procedure which had been shown to be harmless when used with native protein. In this case all signs pointed to a combination effect.

The most prolific cause of denaturation so far as labelled proteins for human use is concerned is the heat treatment of plasma pools which is obligatory in most countries to avoid the risk of disseminating hepatitis virus. Almost without exception commercially available labelled albumins are prepared from plasmas which have been heated for this purpose at 60° for up to ten hours and as much as 30% of the protein may be denatured.

Self-radiation damage

For practical purposes *in vitro* labelling consists almost exclusively of combination with iodine and a second major cause of denaturation is self-radiation damage by the iodine isotopes. This often takes place while hot commercial preparations are stored awaiting results of sterility tests before being dispensed. The specific activity below which this kind of damage occurs at an insignificant rate is widely accepted as 5–10 μc/mg protein (*Bloom*, *Crockett*, and *Stewart* 1958; *Yalow* and *Berson* 1957; *Cohen* 1959), as is also the proper precautionary procedure to be used to avoid damage, namely, prompt dilution with inactive protein. Proteins labelled with ^{125}I are becoming commercially available and since this isotope has no beta radiation these should have a much longer shelf-life than ^{131}I-proteins. With labelled proteins which are to be used for tracer purposes self-radiation damage can generally be avoided but this may not be true of very high specific activity preparations such as are sometimes used for isotope therapy (*Helmkamp*, *Goodland*, *Bale*, *Spar*, and *Mutschler* 1960) or for estimating levels of hormone antibodies

(*Berson*, *Yalow*, *Bauman*, *Rothschild*, and *Newerly* 1956; *Hunter* and *Greenwood* 1962).

Conditions governing protein iodination

Of the numerous factors which affect the quality of iodine labelled proteins the most sensitive one is the proportion of iodine incorporated into the molecule. In general this should not exceed one atom per molecule but in the case of fibrinogen the upper limit is at a mean of 0.6 atoms (*McFarlane* 1963a). The presence of denatured labelled protein gives rise to falsely exaggerated values for the extravascular pool mass of the protein in a recipient animal. This arises from the fact that removal of denatured molecules from the blood by reticulo-endothelial cells for the most part is complete within the first few hours and is indistinguishable from simultaneous loss into the extravascular space. The proportion of total fibrinogen in this compartment is unusually low—only 20% in man and rabbit—and values around 50% which have been observed and which were thought to be normal were later realized to be due to overiodination of the protein.

No question of reutilization of the iodine label is believed to arise if the animal or patient has received enough inactive iodide in the drinking water to block the thyroid gland. That this is so was shown by *Cohen*, *Holloway*, *Matthews*, and *McFarlane* (1956) when they gave high activities in the form of iodine labelled plasma proteins by mouth and failed to find any significantly labelled proteins in the plasma of rabbits. *Margen* and *Tarver* (1957) in an article entitled "The de-iodination of proteins labelled with iodine" claim, however, that iodine may be lost from proteins by a reaction which does not involve rupture of peptide bonds and they are confident that this occurs at least with human ^{131}I-albumin. Their proof depended on finding a shorter half-life (17 days) for albumin labelled biosynthetically in another human by injecting ^{35}S methionine. De-iodination without reincorporation will certainly shorten the half-life but attempts to bring this about *in vitro* have not been successful. Other workers (*Cohen*, *Freeman*, and *McFarlane* 1961; *Beeken*, *Volwiler*, *Goldsworthy*, *Garby*, *Reynolds*, *Stogsdill*, and *Stemler* 1962) find 17 days to be within the normal range of half-lives of iodine labelled albumin in humans, and the value of 27 days obtained by *Margen* and *Tarver* for biosynthetic ^{35}S-albumin would lie outside their normal range. In this connection ^{35}S half-lives are usually amongst the longest to be found in the literature for a protein (*Armstrong*, *Bronsky*, and *Hershman* 1955; *Goldsworthy* and *Volwiler* 1957). A higher efficiency

of utilization of the sulphur containing amino acids for protein building in many humans and animals may be mainly responsible for this. Thus while *Goldsworthy* and *Volwiler* (1956) found only 1% of orally administered ^{14}C-ε-l-lysine to be incorporated into total circulating plasma proteins of dogs in similar circumstances 4–7% incorporation of oral ^{35}S-cystine occurred.

In addition to the rapid elimination already referred to of grossly denatured iodine-labelled proteins some acceleration of the steady exponential rate of disappearance of protein bound activity has been observed (*McFarlane* 1956). On the other hand no treatment of proteins has been known to result in a retardation of the catabolic rate and provided reutilization of the label can be excluded it may be assumed that the longest observed half-life of an iodine-labelled protein will be closest to the true catabolic rate. For this reason the identity of ^{14}C guanidine carbon and ^{131}I-albumin half-lives shown in Fig. 2 is regarded as particularly convincing evidence against significant reutilization of the guanidine carbon.

Biological testing of iodine labelled proteins

The demonstration that half-lives of carefully iodinated proteins truly reflect protein catabolic rates depends on comparisons which have been made of ^{14}C biosynthetically prepared proteins with corresponding ^{131}I ones in the same animal (*Campbell, Cuthbertson, Matthews*, and *McFarlane* 1956; *Cohen* et al. 1956; *McFarlane* 1963a). An additional criterion can be relied upon provided the protein is homogeneous in a metabolic sense. Catabolism is believed to be a first order process occurring in close proximity to the plasma pool. Thus a constant fraction of this pool should be catabolised per day and the total activity excreted daily in the urine should represent a constant fraction of the mean protein bound activity in the plasma on the same day. If this holds throughout including the first day it can be assumed that no significant amount of denatured protein is present in the preparation. The test can be used to estimate the proportion of denatured protein in the preparation but in carrying it out it is important to include with urinary activities any changes occurring in the level of non-protein bound activity in the body water at the same time (*McFarlane* 1962).

In patients or experimental animals not in nitrogen equilibrium the net unbalance between synthesis and catabolism is best obtained from separate estimates of the two. Although, as we have seen, the iodine label is not itself involved in protein synthesis at least in principle it is possible

to deduce synthetic and catabolic rates independently after injecting an iodine labelled protein. This arises from the fact that the rate of decay of the total body activity depends on the rate of protein catabolism while that of plasma protein specific activity depends principally on the rate of synthesis of new unlabelled protein (*Matthews* 1961). However, so much information is required about the distribution of the protein, which may not be constant during the experiment, that the method is unlikely to have wide application.

Absolute measurement of synthesis rates

Direct measurement of synthetic rates depends invariably on obtaining data about precursor specific activities (*Swick* 1958; *Reeve, Pearson* and *Martz* 1963; *McFarlane* 1963b). A recently introduced method of doing this for liver produced plasma proteins depends on measuring specific activities of protein guanidine carbon and of urea carbon within a few hours of injecting $NaH^{14}CO_3$. The following relationship is believed to hold for a hypothetically closed system in which urea and protein guanidine carbon arise from the same precursor, namely the guanidine carbon of intracellular arginine (*McFarlane, Irons, Koj*, and *Regoeczi* 1965).

$$\frac{\text{Fractional synthesis rate of the plasma protein}}{\text{Fractional synthesis rate of body urea}} = \frac{\text{Maximum specific activity of the labelled protein in the plasma}}{\text{Specific activity of plasma urea at } t_0}$$

The maximum protein specific activity referred to is the value observed after 4–6 h in the plasma increased by 10–15% depending on estimates of the proportion of new labelled protein which has been catabolised or has escaped into the extravascular space in this time. The specific activity of urea at t_0 is obtained by extrapolating the curve of plasma urea specific activity to zero time, and the fractional synthesis rate of urea is given by the slope of this curve. Because of endogenous catabolism of urea in the gut this rate is substantially greater in man and rabbits than that based on urinary output.

Estimates of the fractional synthesis rate of albumin and fibrinogen by this method are not very different from catabolic rate estimates in animals in equilibrium. The two need not correspond exactly, however, since the measured synthesis rate is the one effective over a period of a few minutes following the injection of bicarbonate and the catabolic rate is a mean value prevailing over several days. Assuming that rates of protein synthesis by the liver are not sensitive to short-term fluctua-

tions in dietary nitrogen intake the values should presumably be the same, but effects of dietary changes have still to be examined. The above formula is proposed to replace earlier ones (*Reeves* et al. 1963; *McFarlane* 1963c and 1964) mainly in order to avoid complications which arise from loss of labelled urea by endogenous catabolism.

The essential measurements can be made on blood samples taken over a period of 5–6 h from the time of injection and a second measurement could be made almost immediately if allowances were made for the degree of protein labelling persisting from the first one. It is obviously important that the absolute rate of synthesis of urea should be constant during the period of measurement and experiments are required to obtain reassurance on this point. Meantime, clinical investigations are in progress using injections of 100–200 μc $NaH^{14}CO_3$ which are considered to be free from significant radiation hazard, and some preliminary results have been published (*McFarlane* 1964).

Summary

Measurements of plasma protein turnover depend on the use of isotopes and are beset with many technical difficulties which are reviewed. Hitherto, the most reliable measurements have been obtained by transferring to an unlabelled animal protein from the plasma of a donor animal which has been injected with a labelled amino acid. This procedure which is not entirely free of reutilization effects, is prohibitively expensive even for use in animal experiments, and because of the radiation hazard to the donor is unlikely to be much used in clinical research.

An alternative one which dispenses with the need for a donor is to inject ^{14}C-labelled bicarbonate and to follow specific activities of the guanidine carbon of arginine in liver-produced plasma proteins. Reutilization appears to be no greater than in the case of biosynthetically labelled proteins (excepting guanidine labelled ones) and the method can be used to obtain independent values for synthetic and catabolic rates in unbalanced conditions. However, for highest accuracy an iodine-labelled protein should be injected simultaneously. Conditions for iodinating proteins so that they behave in the plasma in the same way as biosynthetically labelled proteins are outlined.

Zusammenfassung

Bestimmungen des Turnover von Plasmaproteinen werden mit Hilfe von Isotopen durchgeführt und sind mit mancherlei Schwierigkeiten be-

haftet. Bisher erhielt man die zuverlässigsten Werte durch Verabreichung von Proteinen aus dem Plasma eines Tieres, dem zuvor eine markierte Aminosäure injiziert worden war. Dieses Vorgehen, das von Wiederbenützungseffekten nicht ganz frei ist, erweist sich selbst im Tierversuch als unverantwortlich teuer. Die klinische Verwendung ist infolge der Gefährdung des Spenders durch Strahlenschädigung nicht zu empfehlen.

Ein alternatives Vorgehen, das kein Spendertier verlangt, besteht darin, ^{14}C-markiertes Bikarbonat zu injizieren und die spezifische Aktivität des Guanidin-Kohlenstoffatoms des Arginylrestes in den von der Leber produzierten Plasmaproteinen zu verfolgen. Der Wiederverwendungseffekt erscheint nicht größer als im Falle von biosynthetisch markierten Proteinen (mit Ausnahme der mit Guanidin markierten Eiweiße). Die Methode kann verwendet werden, um auch unter pathologischen Bedingungen unabhängige Werte für die Synthese- und Abbaugeschwindigkeiten zu erhalten. Die höchste Genauigkeit erhält man durch gleichzeitige Injektion eines jodmarkierten Proteins. Der Autor beschreibt sodann, in welcher Weise bei der Jodination der Proteine vorgegangen werden muß, damit sie sich im Plasma wie biosynthetisch markierte Proteine verhalten.

Résumé

Les difficultés techniques inhérentes à l'utilisation des isotopes, lors de la détermination du turnover des protéines plasmatiques, sont passées en revue. Jusqu'à ce jour, les résultats les plus exacts ont été obtenus par le transfert à un animal non marqué des protéines plasmatiques d'un animal donneur, qui avait reçu un acide aminé radioactif. Cette méthode n'est pas exempte d'effets de réincorporation et est particulièrement onéreuse, même chez l'animal d'expérience. Il est peu probable qu'elle soit employée en clinique à cause des dangers d'irradiation qu'elle fait courir au donneur.

Un autre procédé, qui ne nécessite pas le recours à un donneur, consiste à injecter du bicarbonate marqué au ^{14}C et à suivre les activités spécifiques de l'atome de carbone de la guanidine (présent dans le résidu arginyl) des protéines plasmatiques synthétisées dans le foie. La réincorporation de l'atome radioactif ne semble pas supérieure à celle qui intervient avec les protéines marquées par biosynthèse. Cette méthode permet d'évaluer de façon indépendante les vitesses de synthèse et de catabolisme dans des situations non équilibrées. Cependant, un résultat plus précis est obtenu par l'injection simultanée d'une protéine marquée par l'iode. L'auteur décrit ensuite les conditions d'iodation des protéines, qui

entraînent un comportement plasmatique semblable à celui obtenu par les protéines marquées par biosynthèse.

Riassunto

La determinazione del metabolismo proteico del plasma è possibile soltanto mediante isotopi. In questo lavoro vengono esaminate le difficoltà tecniche di tali determinazioni. Le misurazioni più esatte furono ottenute iniettando ad un animale normale le plasmaproteine di un animale trattato con aminoacido marcato. Questo metodo, non sprovvisto di effetti di riutilizzazione, è estremamente costoso, anche negli esperimenti biologici. E inoltre poco probabile che lo si adoperi in clinica, considerati i pericoli per il donatore. Un altro sistema che elimina la presenza di un donatore consiste nell'iniettare bicarbonato marcato al C^{14} e di studiare poi le attività specifiche del C di guanidina, nell'arginina delle proteine plasmatiche elaborate dal fegato.

La riutilizzazione non sembra maggiore che con le proteine biosintetiche marcate (salvo nel caso della guanidina) e questo metodo può essere adoperato per lo studio dei processi anabolici e catabolici negli stati patologici. Per ottenere dei risultati più precisi bisognerebbe pertanto iniettare contemporaneamente una proteina marcata con iodio.

L'autore descrive in seguito come trattare le proteine con lo iodio affinchè si comportino nel plasma alla stessa maniera delle proteine sintetiche marcate.

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Discussion

L. Donato (Pisa): I think that Dr. *McFarlane's* method for biosynthetical labelling of proteins is extremely interesting. Having heard for the first time about it one year ago in Oak Ridge, I have been a little disappointed today since I was hoping to hear more about it. In fact this method was presented as the first available approach to the measurement of synthesis rate in humans. The importance of such an achievement would be obvious: it would become possible to investigate separately synthesis and catabolism, and therefore to study unsteady state conditions. I wonder why you did not tell us about it today.

The second question concerns the comparison between the C^{14} and I^{131} labelled protein. As far as I can see your comparison was essentially based on the slopes of the plasma curves. These were fairly close to each other in the final monoexponential part, while the two curves differed widely in their initial part. I wonder how much one can rely on the simple slopes comparison.

A. S. McFarlane (London): The use of $^{14}CO_2$-labelling to measure absolute synthesis rates is continued in our laboratory and will be referred to in the latter part of my published paper. However, in the short time available for oral presentation and in the light of the subsequent papers in the symposium, I thought it advisable to concentrate today on the catabolic aspects of plasma protein turnover.

In comparing the behaviour of iodine- and carbon-labelled proteins excretory data, such as one obtains from using iodine, is not available in the case of carbon. It is necessary to rely on the ^{14}C half-life in the plasma and in doing so it is important to remember that a small degree of reutilization may be present — presumably not affecting the half-life by more than 2%. The best procedure would, of course, be to transfer guanidine carbon labelled proteins to unlabelled animals. In this case I am confident that no sensible reutilization will be observed.