The author wishes to acknowledge his indebtedness to Dr K. O. Pedersen, of the Institute of Physical Chemistry, Uppsala, for the gift of the crystalline insulin referred to, and for communicating some of his unpublished results; to Messrs Burroughs Wellcome Ltd., for the gift of another

sample of insulin; to Dr H. Gutfreund for communicating an abstract of some of the American work prior to publication; to Dr P. A. Charlwood of this Institute for his criticism of the manuscript; and to Prof. E. C. Dodds, F.R.S., for his interest and support.

REFERENCES

- Cecil, R. & Ogston, A. G. (1948). *Biochem. J.* **43**, 592.
 Cecil, R. & Ogston, A. G. (1949). *Biochem. J.* **44**, 33.
 Charlwood, P. A. (1952*a*). *Biochem. J.* **51**, 113.
 Charlwood, P. A. (1952*b*). *Biochem. J.* **52**, 279.
 Coulson, C. A., Cox, J. T., Ogston, A. G. & Philpot, J. St L. (1948). *Proc. Roy. Soc. A* **192**, 382.
 Creeth, J. M. (1952). *Biochem. J.* **51**, 10.
 Crowfoot, D. (1938). *Proc. Roy. Soc. A* **164**, 580.
 Doty, P., Gellert, M. & Rabinovitch, B. (1952). *J. Amer. chem. Soc.* **74**, 2065.
 Fiess, H. A. & Klotz, I. M. (1952). *J. Amer. chem. Soc.* **74**, 887.
 Fredericq, E. & Neurath, H. (1950). *J. Amer. chem. Soc.* **72**, 2684.
 Gralén, N. (1944). Inaugural Dissertation, Uppsala.
 Gutfreund, H. (1948*a*). *Biochem. J.* **42**, 156.
 Gutfreund, H. (1948*b*). *Biochem. J.* **42**, 544.
 Gutfreund, H. (1952). *Biochem. J.* **50**, 564.
 Gutfreund, H. & Ogston, A. G. (1946). *Biochem. J.* **40**, 432.
 Jullander, I. (1945). *Ark. Kemi Min. Geol.* **21 A**, no. 8.
 Kegeles, G. & Gutter, F. J. (1951). *J. Amer. chem. Soc.* **73**, 3770.
 Klotz, I. M. & Curme, H. G. (1948). *J. Amer. chem. Soc.* **70**, 939.
 Lamm, O. (1937). *Nova Acta Soc. Sci. upsal.* **10**, no. 6.
 Miller, G. L. & Andersson, K. J. I. (1942). *J. biol. Chem.* **144**, 459.
 Ogston, A. G. (1949*a*). *Proc. Roy. Soc. A*, **196**, 272.
 Ogston, A. G. (1949*b*). *Biochem. J.* **45**, 189.
 Ogston, A. G. (1952). Private communication.
 Oncley, J. L., Ellenbogen, E., Gitlin, D. & Gurd, F. R. N. (1952). *J. phys. Chem.* **56**, 85.
 Pedersen, K. O. (1950). *Cold Spr. Harb. Symp. quant. Biol.* **14**, 140.
 Pedersen, K. O. (1952). Private communication.
 Perlmann, G. E. & Longworth, L. G. (1948). *J. Amer. chem. Soc.* **70**, 2719.
 Polson, A. (1939). *Kolloidzshr.* **87**, 149.
 Quensel, O. (1942). Dissertation, Uppsala.
 Sjögren, B. & Svedberg, T. (1931). *J. Amer. chem. Soc.* **53**, 2657.
 Svedberg, T. & Pedersen, K. O. (1940). *The Ultracentrifuge*. Oxford University Press.
 Tietze, F. & Neurath, H. (1952). *J. biol. Chem.* **194**, 1.
 Waugh, D. F. (1948). *J. Amer. chem. Soc.* **70**, 1850.

The Metabolism of Collagen from Liver, Bone, Skin and Tendon in the Normal Rat

BY A. NEUBERGER AND H. G. B. SLACK

*The National Institute for Medical Research, Mill Hill, London, N.W. 7 and
 The Rheumatism Research Centre, The University of Manchester*

(Received 18 June 1952)

In a previous communication (Neuberger, Perrone & Slack, 1951) the metabolism of collagen from normal rat tendon was studied. These experiments have now been extended to include collagen obtained from tendon, skin, bone and liver. Some of the results have been the subject of a preliminary communication (Perrone & Slack, 1951).

EXPERIMENTAL

Treatment of animals and administration of labelled glycine

Male albino rats of the Institute stock were used throughout. The animals consisted of three groups of rats: (1) old rats with body weight ranging from 350 to 390 g., all being more than 14 months old, in two series of four rats and three rats

respectively; (2) young adults with body weight ranging from 200 to 250 g. in two series of four and five rats, and a further series of young adults of known age in two groups of three rats each; (3) young rats with body weight ranging from 50 to 70 g. in two series of five and three rats respectively.

All animals were apparently in normal health and had not been used for other experiments. Throughout these experiments they were given the Institute stock diet. Each rat received 10 μ c./100 g. initial body weight of glycine labelled with 14 C in the methylene position. The specific activity of the glycine was 1 mc./m-mole. The required dose was weighed on a microbalance, dissolved in 1 ml. of distilled water and administered by intraperitoneal injection. The animals were killed by stunning at varying times after the injection.

Growth of animals. The old rats showed no marked or consistent changes in body weight (Table 1). The young

adults showed some increases in weight during the experiments, the rate varying somewhat with different animals (Tables 3 and 4). The young rats all increased their body weights very markedly during the experiments (Table 2).

Preparation of samples

Liver and muscle proteins. These were prepared as described previously (Neuberger *et al.* 1951).

Tendon collagen. This was prepared as described (Neuberger *et al.* 1951).

Skin collagen. After shaving the rat to remove hair, the skin was freed from subcutaneous tissue and minced in a meat mincer. It was then immersed for 48 hr. in 5 times its own volume of 20% (w/v) urea. The residue was repeatedly washed in changes of distilled water. The collagen in the residue was converted to gelatin by boiling under reflux for 24 hr. with distilled water. After filtration through Whatman no. 42 paper the solution was reduced in volume by evaporation *in vacuo* on a water bath; acetone was added until no further precipitation occurred and the mixture was then left overnight at 0°. The gelatin was centrifuged off and dried in a desiccator. Samples were hydrolysed by boiling under reflux in 5.8N-HCl for 24 hr.

Liver collagen. The fresh liver was minced with scissors and then ground with sand in a mortar. After extraction for 48 hr. at 4° with 20% (w/v) urea, the residue was repeatedly washed with distilled water; the gelatin hydrolysate was obtained by the same means as that from skin.

Bone collagen. After removal of the head the rat carcass was roughly dissected free from muscle and connective-tissue masses. The carcass was broken up, boiled briefly in water and then rinsed in cold water. It was soaked for 24 hr. in 20% (w/v) urea at 4°. After repeated washing in distilled water the bones were pounded in a mortar and defatted by extraction with 50% (v/v) ether/ethanol. The bony residue was then treated with trypsin (British Drug Houses Ltd.) for 48 hr. at 37°. After repeated washing in distilled water the collagen matrix was converted to gelatin and hydrolysed as described above.

Isolation of 2:4-dinitrophenyl-(DNP-) glycine and radioactivity measurements

The hydrolysates were converted to the 2:4-dinitrophenyl derivatives and the DNP-glycine isolated as described (Neuberger *et al.* 1951). The radioactivities of the samples were also measured as previously described. All results were expressed as counts/min./2 sq.cm. disk of 'infinite thickness'.

RESULTS

Changes in the specific activities of glycine obtained from the mixed proteins of liver and muscle

The specific activities found in glycine obtained from the liver- and muscle-protein samples are very similar to those found previously (see Neuberger *et al.* 1951). Some additional information on the effects of growth and age has been obtained and this will be discussed later.

Changes of specific activity of the glycine obtained from the collagen of liver, bone, skin and tendons

The specific activities of the glycine isolated from hydrolysates of collagens obtained from the four tissues of the old rats at intervals of time ranging from 1 to 7 weeks after administration of the labelled glycine are shown in Table 1. In this series of experiments glycine was not isolated from the mixed proteins of liver or plasma, but in other experiments in which similar amounts (in terms of radioactivity) of [α - 14 C]glycine were administered into rats of the same strain and age, the DNP-glycine samples obtained from the proteins of the internal organs 1 week after injection had activities of 1100–1700 counts/min./2 sq.cm. By comparison the activities of the DNP-glycine samples obtained from the various collagens were extremely low, 109 counts/min./2 sq.cm. being the highest. The variation in the radioactivities was considerable, but bone collagen appeared to be more active than collagen from skin, tendon or liver. The initial activity appeared to change little, at least up to 7 weeks.

In the very young rats (ages at time of injection 4–6 weeks) the initial activities of the glycine samples from all four types of collagen specimens were very high (Table 2) and not very much lower than corresponding values for muscle or even mixed liver proteins (see Neuberger *et al.* 1951, Table 4). It appears that in young rats differences between tissues with respect to rate of incorporation of labelled amino-acids have largely disappeared within 1 week and what is in fact mainly observed,

Table 1. *Specific radioactivities of 2:4-dinitrophenylglycine samples obtained from collagen isolated from liver, bone, skin and tendon of old rats injected with 10 μ c./100 g. body weight of $\text{NH}_2^{14}\text{CH}_2\text{COOH}$*

Rat no.	Body weight		Duration of experiment (weeks)	Specific activities (counts/min./2 sq.cm. disk, infinite thickness) of DNP-glycine samples of collagen of			
	Initial (g.)	Final (g.)		Liver	Bone	Skin	Tendon
121	362	370	1	38	80	34	—
122	386	378	2	29	—	15	—
123	394	390	3	34	62	40	20
105	358	371	3	42	101	14	24
106	390	370	3	—	79	22	12
101	373	355	7	—	109	28	15
102	385	380	7	—	88	—	18

Table 2. *Specific radioactivities of 2:4-dinitrophenylglycine samples obtained from collagen isolated from liver, bone, skin and tendon of young rats injected with 10 μ c./100 g. body weight of $\text{NH}_2^{14}\text{CH}_2\text{COOH}$*

(The figures in brackets are corrected specific radioactivities. These corrected figures were obtained by multiplying the observed radioactivity by ratio of final body weight to initial body weight.)

Rat no.	Body weight		Duration of experiment (weeks)	Specific activities (counts/min./2 sq.cm. disk, infinite thickness) of DNP-glycine samples of collagen from			
	Initial (g.)	Final (g.)		Liver	Bone	Skin	Tendon
115	79	115	1	681* (908)	670* (893)	{ —	621 (905)
116	80	97	1				663 (804)
139	65	94	1	686 (992)	622 (900)	591 (855)	—
150	69	96	1	645 (898)	665 (925)	562 (782)	—
62	62	102	2	418* (726)	{ 480 (789)	361 (594)	324 (533)
140	60	110	2				
141	65	127	3	385 (752)	401 (784)	321 (627)	—
100	50	160	8.5	235 (752)	220 (704)	—	193 (617)

* Pooled samples.

Table 3. *Specific radioactivities of 2:4-dinitrophenylglycine samples of collagen obtained from liver, bone, skin and tendon of young adult rats injected with 10 μ c./100 g. initial body weight of $\text{NH}_2^{14}\text{CH}_2\text{COOH}$*

Rat no.	Body weight		Duration of experiment (days)	Specific activities (counts/min./2 sq.cm. disk, infinite thickness) of DNP-glycine samples of collagen of			
	Initial (g.)	Final (g.)		Liver	Bone	Skin	Tendon
124	239	239	0.5	474	—	22	—
1	237	237	1	560*	{ —	41	46
125	141	143	1				
126	209	212	3	345	362	34	—
2	233	242	7	290	351	28	68*
127	216	221	7	—	—	—	
3	203	229	14	271	312	25	58*
128	240	251	14	—	—	—	
4	206	252	21	252	294	—	159*
130	212	249	21	—	—	—	
5	205	236	35	260	290	—	174

* Pooled samples.

at this time and later, is growth or net increase of protein and not the turnover of body substance already laid down.

With young rats the specific activities of the collagen-glycine samples decreased markedly with lengthening of the interval between injection of glycine and killing of the animal. This decrease can be due to the dynamic state of collagen in young animals and/or to the deposition of collagen of decreasing isotope content in the later stages of the experiment ('growth dilution'). This point has been fully discussed already (Neuberger *et al.* 1951); the rough corrections which have been made assume that practically all the isotope is introduced into the collagen at the beginning of the experiment and that the total amounts of the various collagens in the rat are linearly proportional to body weight. The first assumption would certainly be incorrect for adults, but may be justified as a very crude approximation in very young rats in which the initial isotope content of the collagen glycine is relatively high. We have not found any data on

changes of collagen content with age for such tissues of the rat as have been investigated in the present work, but the results of Lowry, Hastings, Hull & Brown (1942) indicate that there is no increase in the collagen content of muscle until the rat has passed reproductive age. The observed specific activities were therefore multiplied by the ratio of final to initial body weight. All the 'corrected' figures for young rats (Table 2) for the activities of glycine from collagen of bone, liver, skin and tendon show some fall during the first 3 weeks. Data for later periods in these young rats are scanty, but the few figures available suggest that a further decrease in activity in collagen after 3 weeks is slow. As noted previously, the activities of glycine from liver and mixed muscle proteins, especially the former, fall markedly with time in rats of all ages.

The activities found in the young adult rats are more difficult to interpret. Table 3 shows the activities of glycine from the collagen of the four tissues in young adult rats of weight range 200–250 g., but

Table 4. *Specific radioactivities of 2:4-dinitrophenylglycine samples of collagen obtained from liver, bone, skin and tendon of young adult rats of known age injected with 10 μ c./100 g. body weight of $\text{NH}_2^{14}\text{CH}_2\text{COOH}$ and killed 1 week later*

Rat no.	Age at death (days)	Body weight		Specific activities (counts/min./2 sq.cm. disk, infinite thickness) of DNP-glycine samples of collagen of			
		Initial (g.)	Final (g.)	Liver	Bone	Skin	Tendon
5V†	103	273	274	185	208	40*	59
6G	103	264	274	174	221	72	40
7V	103	270	285	231	113	94	25
6R	151	317	318	174	127	81	11
7G	151	321	325	162	170	94	17
8R	151	318	327	155	176	86	34

* Derived from skin from the tail of the rat.

† Letters V, G, R indicate litter mates.

whose exact ages were not known. It will be seen that bone and liver collagen were fairly active and were actually 4–10 times as active as the collagen of skin and tendon. The few figures available for liver collagen suggest some active turnover during the first week. After the first week there appeared to be no appreciable loss of activity in any of the collagen fractions. A further group of young adult rats of known age was studied, glycine from the collagen of liver, bone, skin and tendon being obtained 1 week after injection of the radioactive glycine (Table 4). In addition, liver- and muscle-protein glycine were isolated, the results being similar to those reported previously. It will be seen (Table 4) that the increases in body weight during the week of the experiment are not large in either of the two series of three rats, though the rats aged 151 days were considerably heavier than those aged 103 days. Although there was considerable variation in individual figures, the glycine from bone and liver collagen was found to be considerably more active than that from skin, whilst tendon collagen was least active. Furthermore, the activities of all the collagen samples, with the exception of that from skin, were on the average lower in the rats aged 151 days than those in rats aged 103 days. A similar apparent decrease in activity with increasing age was seen in the glycine from liver and muscle protein.

DISCUSSION

Some general considerations on the interpretation of isotope data relating to protein metabolism

In this work and in an earlier paper (Neuberger *et al.* 1951) it was found that the injection of an amount of [α - ^{14}C]glycine which produces a high isotope content in the mixed proteins of liver and plasma leads to a relatively slight labelling in the glycine residues of various collagens of the adult rat. The deduction made, that collagens as a group are metabolically almost inert or have turnover rates which are small by comparison with those of liver and even those of

the mixed proteins of muscle, is, however, based on certain assumptions which have also been implied by other workers in the field of protein metabolism, but which have not been clearly stated. Isotope methods, as used at present, can only detect such changes in protein molecules as consist of a hydrolysis to amino-acids and complete synthesis of the protein from free amino-acids. It is possible that a protein may be broken down to peptides and these may be preferentially utilized for the synthesis of the original protein or for the synthesis of a different protein. If the hydrolysis or synthesis of such peptides from amino-acids is slow relative to the rate of synthesis of the protein, little incorporation of the labelled amino-acid will be observed. The protein may also be changed in other ways not involving the breaking of peptide linkages, or one protein may be transformed into another without the production of free amino-acids or the severance of covalent bonds. In all these cases degradation or synthesis of protein may not be reflected in changes of isotope content. It is concluded, therefore, that any statement on protein metabolism made on the basis of isotope results can refer only to such changes in which amino-acids are produced or in which amino-acids are the starting material of protein synthesis.

A further assumption which is generally made is that the amino-acids present in cells and arising from the reversible breakdown of proteins equilibrate readily with the labelled amino-acid administered. This is unlikely to be completely true. When a labelled amino-acid is fed or injected the isotope content of the labelled compound will be very high, immediately after administration, in the blood and in the intracellular spaces of some tissues, but will decrease rapidly in a few hours owing to mixing with a larger 'pool' and to various metabolic reactions (Arnstein & Neuberger, 1951). The maximum isotope content observed in a particular protein will thus not only be a function of the dose of isotope and the turnover rate of the protein in

question, but will also depend on the rate at which the labelled amino-acid penetrates to the site where the protein under investigation is synthesized. Permeability barriers may prevent complete equilibration between the intracellular amino-acid and the amino-acid present in the blood or extracellular space and thus the amino-acids liberated *in situ* by protein breakdown may be preferentially used for synthesis. Such permeability barriers may account, at least to some extent, for the relatively low maximum isotope contents found in brain and muscle, but it is unlikely that they will explain the extremely low values found with some collagens. For permeability factors to be rate-determining it is necessary that the rate of penetration of an amino-acid to the site of synthesis should be smaller than the rate of incorporation of the amino-acid into the protein. With collagens, the rate of incorporation of labelled glycine is so low that it is almost certain that, at least in the rat, it is the rate of protein synthesis as defined above which determines the overall rate of isotope incorporation.

Purity of the collagen samples

Another, less general, point which has to be considered is the purity of the collagen specimens. Collagens may be defined as a group of proteins, present mainly in fibrous form in the extracellular spaces of most or all tissues, showing a typical pattern under the electron microscope and having, at least in the case of mammalian collagens, an unusual amino-acid composition characterized by high glycine, proline and hydroxyproline content, presence of hydroxylysine, absence of tryptophan, and low content of other aromatic amino-acids, cystine and methionine. Collagen samples prepared by the methods used in the present work gave negative tests for cystine and tryptophan, had a very weak and indistinct absorption in the ultraviolet (265–295 m μ .) and contained between 13.5 and 14.0% hydroxyproline as measured by the method of Neuman & Logan (1950). Paper chromatograms of hydrolysates of these samples revealed the presence of only minute traces of phenylalanine and tyrosine. It would appear, therefore, that these samples were reasonably pure, but it is impossible to assert that the method of preparation has completely eliminated all proteins other than collagens. With skin, for instance, it was found (Harkness & Neuberger, 1952) that the glycine isolated from the soluble proteins had, soon after administration of the labelled compound, a high isotope content similar to that of glycine from plasma proteins, and the presence of even a small amount of such proteins in our early collagen specimens would give misleading results. With samples obtained more than 1 week after administration contamination would be somewhat less

serious since the labelling in the soluble proteins decreases rapidly with time. Moreover, the glycine content of collagen is about 5–10 times greater than that of most globular proteins and it is therefore unlikely that the glycine derivative isolated contained more than 1% of material derived from sources other than collagen. It is probable that contamination was much less than this figure. It is suggested, therefore, that the radioactivities measured represent substantially the activities of the glycine residue in collagens. It may be mentioned, however, that 'procollagen' is probably included since it is not soluble in urea.

Comparison of the extent of isotope incorporation into different collagens of the adult rat

In the earlier paper (Neuberger *et al.* 1951), it was reported that the glycine isolated from the collagen of the tendon of the adult rat was almost completely inactive, and this finding is confirmed by the present work (Tables 1 and 4). The conclusion which has been put forward tentatively, that this may apply to collagens generally, has, however, to be slightly modified. Although the tendon samples were obtained from the limbs, feet and backs of the animals, as well as the tails, dissection is not easy and at least half of the sample consisted of tail tendon. Since the tail has a lower temperature, and therefore presumably a lower rate of metabolism, the observed activities of the mixed tendon-collagen samples may be particularly small. In this connexion it is of interest that one sample of skin collagen which was taken from the tail had a somewhat lower activity than samples taken from other parts of the body (Table 4). Collagens from skin and liver had definitely higher activities in adults than tendon collagen and must be presumed to turn over somewhat faster. The collagen of bone had the highest activity both in the very old rats (Table 1) and in the animals aged 103 and 151 days (Table 4), whilst in the group of young adults, the age of which was not known, bone and liver collagen had similar, moderately high, activities as compared with the very low activities of skin and tendon collagen (Table 3). The consistently high relative activity of bone collagen in all age groups suggested that the matrix of bone is replaced continuously at a slow rate, and this is in accordance with the observation that the epiphyses of the rat remain open during the life of the animal.

The collagen in certain organs of the rat increases in old age (Lowry *et al.* 1942; Myers & Long, 1946) and the possibility was considered (Neuberger *et al.* 1951) that such incorporation of isotope as was observed in the very old rats was due to a slight net increase of collagen and not to reversible degradation of material already laid down. The fact, however, that similar values for the various collagens

were found in the old animals and in rats aged 103 and 151 days suggests that collagen is not completely inert. The differences now observed between different collagens also indicate probably that the collagen molecules are continuously but very slowly replaced, even in the adult animal. But compared with liver, kidney or even muscle, turnover rates are very low, with the possible exception of bone collagen, and the contribution which is made by this group of extracellular proteins to the 'dynamic state' of the body is small.

*The metabolic activity of collagens
in young animals*

In rapidly growing rats the labelled glycine is used for collagen synthesis, as shown by the high activities of the DNP-glycine obtained soon after administration of the labelled compound. The sharp decrease in specific activity observed with later samples (Table 2) is probably mainly due to the fact that the activity of the free glycine in the body decreases sharply within hours of administration. Since growth continues, collagen of rapidly decreasing activity is deposited thus leading to a loss of specific activity. The 'corrected' results (Table 2) indicate that there is a decrease not only in specific activity, but also in total radioactivities of all four collagen fractions. If collagen were inert, total activity should have increased. It seems likely, therefore, that collagen, including that of tendon and skin, is metabolically more active in young rats than in adults. This may be associated with the relatively large amounts of 'procollagen' found in growing animals (Bresler, Finogenow & Frenkel, 1950).

SUMMARY

1. [α - ^{14}C]Glycine was injected into rats of varying ages. The animals were killed at different intervals of time and samples of mixed proteins of liver and muscle and of collagen from skin, tendon, bone and liver were obtained. Dinitrophenyl-glycine was isolated from the hydrolysate of these proteins and its radioactivity determined.

2. In rats more than 14 months old, the activities of the glycine samples obtained from all the collagen fractions were very low, bone collagen showing the highest values. In young adults which were still growing slowly, low activity was observed with skin and tendon collagen, but appreciably higher values were obtained with collagen from bone and liver. With young, rapidly growing rats initial activities were high with all collagen fractions.

3. The difficulties inherent in the interpretation of isotope data in relation to protein metabolism are discussed, and it is concluded that only such changes can be detected as involve participation of free amino-acids in protein turnover. The criteria of purity of collagen are also examined.

4. Subject to the limitations mentioned the results suggest that, even in the adult rat, collagen fibres are continually, but very slowly, replaced, the turnover rates being highest for collagen from bone. It is likely that in young rats this change is somewhat faster. However, in all age groups all collagens showed turnover rates which are low compared with those of intracellular proteins.

The authors wish to thank Dr J. C. Perrone for help in the preparation of some of the samples and Mrs F. Higginson for technical assistance.

REFERENCES

- Arnstein, H. R. V. & Neuberger, A. (1951). *Biochem. J.* **50**, 154.
Bresler, S. E., Finogenov, P. A. & Frenkel, S. Ya (1950). *C.R. Acad. Sci. U.R.S.S.* **72**, 555.
Harkness, R. D. & Neuberger, A. (1952). *Biochem. J.* **51**, xxii.
Lowry, O. H., Hastings, A. B., Hull, T. Z. & Brown, A. N. (1942). *J. biol. Chem.* **143**, 271.
Myers, V. C. & Long, W. W. (1946). *J. Geront.* **1**, 441.
Neuberger, A., Perrone, J. C. & Slack, H. G. B. (1951). *Biochem. J.* **49**, 199.
Neuman, R. E. & Logan, M. A. (1950). *J. biol. Chem.* **186**, 549.
Perrone, J. C. & Slack, H. G. B. (1951). *Biochem. J.* **49**, lxxii.