SUMMARY

1. Purified salmine from *Oncorhynchus keta* has been coupled completely with 1-fluoro-2:4-dinitrobenzene and the spectrum of the resulting dinitrophenyl (DNP)-salmine measured in the range 235–420 mμ.

2. The spectrum is similar to the spectrum of DNP-proline.

3. Four-fifths of the *N*-terminal residues in the salmine are proline and the rest mainly arginine, serine and glycine.

4. From the spectrum an average molecular weight of 3800 ± 10% has been calculated for salmine, assuming all the molecules to be simple open-chain peptides.

5. A secondary slow reaction occurs with fluorodinitrobenzene, which is unexplained.

6. A little N^{δ}-DNP-ornithine was found, which it is thought may be in the native protamine and not a degradation product.

I wish to thank Mr D. A. Maguire for his continued valuable technical assistance and Dr J. A. Broben of the Commonwealth Serum Laboratories, Melbourne, for the gift of a sample of protamine from *Salmo irideus*.

REFERENCES

Ando, T., Ishii, S., Hashimoto, C., Yamasaki, M. & Iwai, K. (1952). *Bull. chem. Soc. Japan*, **25**, 132.
Berenblum, I. & Chain, E. (1938). *Biochem. J.* **32**, 295.
Blackburn, S. & Lowther, A. G. (1951). *Biochem. J.* **48**, 126.
Corfield, M. C. & Robson, A. (1953). *Biochem. J.* **55**, 517.
Felix, K. (1953). *The Chemical Structure of Proteins.* (Ed. G. E. W. Wolstenholme and M. P. Cameron), p. 151. London: Churchill.
Felix, K., Fischer, H., Krekels, A. & Rauen, H. M. (1950). *Hoppe-Seyl. Z.* **286**, 67.
Felix, K. & Krekels, A. (1953). *Hoppe-Seyl. Z.* **295**, 107.
Fletcher, C. M., Lowther, A. G. & Reith, W. S. (1954). *Biochem. J.* **56**, 106.
Kossel, A. (1928). *The Protamines and Histones* (translated by W. V. Thorpe). London: Longmans, Green and Co.
Levy, A. L. (1954). *Nature, Lond.*, **174**, 126.
Ma, T. S. & Zuazaga, G. (1942). *Industr. Engng Chem. (Anal.)*, **14**, 280.
Middlebrook, W. R. (1951). *Biochim. biophys. Acta*, **7**, 547.
Mills, G. L. (1952). *Biochem. J.* **50**, 707.
Plaskéev, V., Yarovenko, N. & Pasynskiĭ, A. (1945). *C.R. Acad. Sci., U.R.S.S.*, **49**, 580.
Porter, R. R. & Sanger, F. (1948). *Biochem. J.* **42**, 287.
Rao, K. R. & Sober, H. A. (1954). *J. Amer. chem. Soc.* **76**, 1328.
Rauen, H. M., Stamm, W. & Felix, K. (1952). *Hoppe-Seyl. Z.* **291**, 275.
Sanger, F. (1945). *Biochem. J.* **39**, 507.
Sanger, F. (1949*a*). *Biochem. J.* **45**, 563.
Sanger, F. (1949*b*). *Symp. biochem. Soc.* no. 3, p. 21.
Schroeder, W. A., Honnen, L. R. & Green, F. C. (1953). *Proc. nat. Acad. Sci. Wash.* **39**, 23.
Šorm, F. & Šormova, Z. (1951). *Coll. Trav. chim. Tchécosl.* **16**, 207.
Thompson, A. R. (1951). *Nature, Lond.*, **168**, 390.
Tristram, G. R. (1947). *Nature, Lond.*, **160**, 637.
Tristram, G. R. (1949). *Advanc. Protein Chem.* **5**, 83.
Udenfriend, S. & Velick, S. F. (1951). *J. biol. Chem.* **190**, 733.
Velick, S. F. & Udenfriend, S. (1951). *J. biol. Chem.* **191**, 233.
Waldschmidt-Leitz, E., Kühn, K. & Zinnert, F. (1951). *Experientia*, **7**, 183.

Quantitative Aspects of Glycine Metabolism in the Rabbit

By OLGA B. HENRIQUES,*† S. B. HENRIQUES* and A. NEUBERGER
National Institute for Medical Research, London, N.W. 7

(*Received* 10 *December* 1954)

Schoenheimer, Ratner & Rittenberg (1939) first observed that the amount of labelled amino acid incorporated into proteins, within a short time after administration of the tagged compound, varied greatly with different tissues. These pioneer experiments were done with rats and the amino acid used was L-leucine which had been labelled with both ^{15}N and deuterium. Later work (Tarver & Schmidt, 1942; Friedberg, Tarver & Greenberg, 1948; Greenberg & Winnick, 1948; Winnick, Friedberg & Greenberg, 1948), in which carbon- and sulphur-labelled amino acids were used, has amply confirmed the findings of Schoenheimer *et al.* (1939). The mixed proteins of some tissues such as intestinal tract, liver and kidney were found to contain large amounts of isotopically labelled amino acid within a few hours after administration, while other tissues such as muscle, skin and brain gave only low values, if the comparison was made on a unit weight basis. It was concluded (Schoenheimer & Rittenberg, 1940) that the concentration of isotope found in a particular tissue under the conditions mentioned could be taken as a measure of 'relative chemical activity' of the organ proteins. It has been pointed out (Neuberger, 1952) that this interpretation is based on the assumption that the rate of penetration of the labelled amino acid into the cells of a given tissue is very much greater than the

* Present address: Instituto Butantan, C.P. 65, Sao Paulo, Brazil.
† Fellow of the Brazilian Research Council.

rate of protein synthesis in that tissue. This assumption has not been tested directly in the rat or rabbit, but indirect evidence suggests that it is not correct in the case of the rat brain. Thus Friedberg & Greenberg (1947) observed marked increases of amino-N in all tissues except the brain after intravenous administration of various amino acids. The conclusion that the transfer of the amino acids from the blood to the brain is slow was supported by experiments of Friedberg & Greenberg (quoted by Friedberg *et al.* 1948) showing that intracisternal injection of labelled methionine produced higher radioactivity in brain proteins than in the proteins of plasma, liver or kidney.

With tissues such as liver and kidney, containing proteins which are being replaced at rapid rates, maximum radioactivity is reached within about 1 hr. after administration of the labelled compound (Borsook, Deasy, Haagen-Smit, Keighly & Lowy, 1950); the specific radioactivity then remains fairly constant for some hours and decreases later to reach half the maximum value in periods varying between 3 and 10 days, depending on the species and tissue. It should be possible to calculate turnover rates of proteins from the ascending part of the activity/time curve of the protein, provided that the values of the specific activity of the free amino acid during the relevant period is known. Such data are, however, not available. Most quantitative statements about rates of protein synthesis have been deduced from the slope of the descending part of the activity/time curve, but an accurate quantitative interpretation has been impossible again owing to lack of information on the activity of the precursor amino acid. Thus, Shemin & Rittenberg (1944) based their estimate of the half-life value of the average protein molecule in the rat liver on the assumption that the ^{15}N content of the free glycine available for protein synthesis some time after administration was equal to that of the glycine obtained on hydrolysis of the 'carcass' protein. In the early work on the turnover of plasma proteins, labelled amino acids were used and it was tacitly assumed that the label content of the amino acid 'pool' had dropped to negligible values within 12–24 hr. after administration. In more recent work (Miller *et al.* 1949; Abdou & Tarver, 1951; Humphrey & McFarlane, 1954), labelled protein was transfused and the error arising from reutilization of the labelled amino acid liberated on breakdown of the injected protein is likely to be much smaller under these conditions than when a labelled amino acid is used. However, even in these experiments it would be desirable to know the specific activity of the free amino acid compared with that of the protein-bound amino acid measured at the same time.

The only quantitative data published on changes with time of the radioactivity of a free amino acid in different tissues are those of Barton (1951), who injected ^{14}C-labelled glycine into mice and measured the radioactivity of the formaldehyde liberated by ninhydrin from a protein-free extract of blood or tissue. The assumption was made that the formaldehyde was exclusively derived from glycine. Barton found that 5 min. after injection the radioactivity of the free glycine of the liver was about 5 times that of the free glycine of muscle, but that after 15–20 min. the radioactivities of the free glycine of liver, blood and muscle had become about equal. Experiments on hippuric acid formation from labelled glycine (Arnstein & Neuberger, 1951) suggested that the equilibration of free glycine between liver and other tissues of the rat is a relatively slow process. It was therefore considered desirable to isolate glycine from blood, liver and muscle at various times after administration of labelled amino acid and to estimate its radioactivity. The experiments described in this paper were done with rabbits, and the results differ markedly from those obtained by Barton (1951) with mice. Glutathione from liver and muscle was also isolated and the radioactivity of the glycine obtained by its hydrolysis was measured. In addition, glycine was obtained from the hydrolysates of the mixed proteins of liver and muscle.

EXPERIMENTAL

Animals. Twenty-two female albino rabbits each weighing about 2 kg. were used. After being fasted for 3 hr. they received intravenously 5 μc (0·2 mg.) of [α-^{14}C]glycine, dissolved in 0·25 ml. of 0·85% NaCl/kg. of body weight. Blood from the aorta (20 ml.), the whole liver and the muscles of both hind legs were rapidly removed from the treated animals, under anaesthesia (pentobarbitone sodium, 40 mg./kg. body wt.) 5, 10, 15, 30 min. and 1, 2, 3, 4, 6, 12 and 18 hr. respectively after the injection of glycine. The animals killed at 12 and 18 hr. received 25 μc of [α-^{14}C]-glycine/kg. body weight. Two of the rabbits killed 15 min. after the injection were treated as follows: the animal was anaesthetized, injected with isotopic glycine, left for 10 min. and prepared so that exactly at 15 min. it could be bled and quickly perfused with warm saline through the aorta before the removal of the muscles. Immediately after removal, the muscles and liver were sliced, pressed against blotting paper and dropped into liquid air. The blood was allowed to clot and was centrifuged. The frozen muscle and liver slices were finely pulverized in a mortar, while kept frozen by dry ice, and immediately extracted as described below.

Extraction of free amino acids and glutathione. Blood serum, muscle powder, or liver powder, were treated with equal weights of 10% (w/v) trichloroacetic acid (TCA). After being thoroughly mixed and left standing for at least 10 min. the different mixtures were centrifuged. The precipitated proteins were resuspended in half the initial volume of 5% (w/v) TCA and the extraction was repeated twice more with half the initial volume of 5% (w/v) TCA. The TCA supernatants were then mixed and measured. An

amount equivalent to 10 g. of organ and the whole serum extract were used for glycine isolation, while the remaining material was used for the isolation of glutathione.

Isolation of glutathione. The glutathione was precipitated as its cuprous mercaptide (GSCu) by the method of Hopkins (1929), with preliminary separation as the cadmium mercaptide, as recommended by Waelsch & Rittenberg (1941).

Separation of protein. The washed proteins were resuspended in 5% TCA containing inactive glycine (1 mg./10 g. solid), left at room temperature for 24 hr. with occasional stirring. The proteins were then transferred to a sintered-glass funnel and thoroughly washed with 5% TCA, by resuspending in the washing medium several times. Using a similar technique the proteins were further washed 3 times with acetone, and extracted once with boiling ethanol–ether mixture (3:1, v/v). After drying over P_2O_5 the proteins were ground to a fine powder in a mortar and sieved through a standard sieve no. 40.

Isolation of glycine. The TCA extracts were shaken 3 times with 3 vol. of ether, to remove the TCA. Glutathione glycine was isolated from 2 to 4 mg. of GSCu after hydrolysis with 6 N-HCl at 105° for 24 hr., in sealed ampoules. Protein glycine was isolated after hydrolysis with 6 N-HCl (10 ml./g. protein) at 105° for 24–30 hr., using 200–500 mg. of protein. TCA extracts, protein or GSCu hydrolysates were treated with 1-fluoro-2:4-dinitrobenzene, and 2:4-dinitrophenylglycine (DNP-glycine) was isolated after chromatography on buffered Celite (Campbell & Work, 1952). After passing through a second column of Celite–buffer mixture with peroxide-free ether as solvent, the solution from the glycine band was collected in a volumetric flask, the volume made up to 50 or 100 ml. and the DNP-glycine was estimated colorimetrically (Krol, 1952) with a Unicam photoelectric colorimeter (wavelength, 460 mμ.). The standard curve was linear over the range 50–300 μg. (in 10 ml. of 0·5% $NaHCO_3$), and all the estimations of glycine were made within this range. For some assays of radioactivity the remaining DNP-glycine was diluted with a known amount of pure inactive DNP-glycine, so as to obtain about 20 mg. of substance after recrystallization. The ether was removed *in vacuo*, and the residue crystallized from ethyl acetate–*cyclo*hexane. In a few experiments 2:4-dinitrophenylserine (DNP-serine) was also isolated; this was done by the method of Campbell & Work (1952).

Radioactivity measurements. These were done either with samples of 'infinite thickness' mounted on polythene disks of 1 sq.cm. area or with solid samples of 'infinite thinness'. The procedure in the second method, which is similar to that described by Fager (1947), was as follows: the dinitrophenyl derivatives of glycine were dissolved in a mixture of acetone and N-NH_4OH (1:1, v/v), and volumes of 0·01–0·02 ml. of the solution, containing 10–200 μg. of the solute, were pipetted on to plastic (polythene) planchets of 2 cm.^{2} area (Popják, 1950) lined with lens paper (Green's lens tissues used for cleaning microscope objectives) which ensured even spreading of the samples. Disks of the lens tissue, 2 cm.^{2} (weight approx. 1·9 mg.), can be punched out conveniently between two layers of hard filter paper. The solvent was evaporated off by gently warming under an infrared lamp or in an oven at 37°. The radioactive counts obtained with a standard sample of dinitrophenyl-[α-^{14}C]glycine were linearly proportional to sample weight in the range of 20–200 μg. and were reproducible within ±5% (S.E.), provided the specific activity of the samples was not too low. (The counting rate of a sample of 200 μg. plated as above is about one-fiftieth of that obtained on an 'infinitely thick' plate of the same material.) As a check on the method, samples of dinitrophenyl-[^{14}C]glycine were diluted with known amounts of unlabelled material and were assayed in 'infinitely thick' samples. The specific activity calculated with the aid of the dilution factor agreed satisfactorily with the specific activity determined on the undiluted material by the thin-plating technique (Table 1). All samples were counted long enough to reduce the counting error to less than 3%. In the case of samples giving a low count, such as some glycine samples obtained by hydrolysis of glutathione or proteins, the specific activities were measured in an automatic counter. A standard consisting of ^{14}C-labelled Perspex supplied by the Radiochemical Centre, Amersham, was used both to check the counting system and to establish correspondence between our two counters.

Table 1. *Comparison of results obtained with the method of 'infinite thickness' and that of 'infinite thinness' in the assay of specific activity of DNP-glycine*

Rabbit no.	Source of DNP-glycine	Specific activity (μc/mg.) 'Infinite thickness'	Specific activity (μc/mg.) 'Infinite thinness'
1	Liver	$1·05 \times 10^{-2}$	$1·04 \times 10^{-2}$
1	Plasma	$1·79 \times 10^{-2}$	$1·74 \times 10^{-2}$
3	Liver	$7·09 \times 10^{-3}$	$7·20 \times 10^{-3}$
3	Plasma	$7·23 \times 10^{-3}$	$7·90 \times 10^{-3}$
3	Muscle	$7·00 \times 10^{-4}$	$8·33 \times 10^{-4}$

RESULTS

The changes with time of specific radioactivity of free glycine in plasma

It will be seen (Table 2) that the specific radioactivity of the free plasma glycine is very high 5 min. after injection of the glycine, decreases sharply within the next 40 min. and then falls more slowly. The differences between animals are fairly large; thus, in the four 15 min. experiments the individual values of the plasma glycine were 126, 71, 90 and 112% of the mean value. However, this is not surprising in view of the rapidity of the changes measured.

The mean values for plasma glycine given in Table 2 cannot be represented by a simple exponential equation. Solomon (1949) discussed the mathematical problems encountered in the case where a labelled substance dissolved in one compartment mixes at different rates with several other, isolated, compartments. It was found by applying the method of least squares to portions of the curve that the results for plasma glycine fit fairly well an equation of the 'three-compartment' curve of the type (Solomon, 1949):

$$f(t) = 37250\,e^{-0·073t} + 15400\,e^{-0·015t} + 542\,e^{-0·0027t} + 292, \quad (1)$$

where $f(t)$ is the radioactivity of plasma glycine expressed as DNP-glycine (in counts/min./2 cm.^{2})

Table 2. *Specific radioactivity of free glycine obtained from serum, liver and muscle of rabbits given* [α-^{14}C]*glycine* (5 μc./*kg. body weight*)

For details of administration of the glycine, isolation and radioassay of the DNP-glycine see text. The time given in column 2 is the interval between injection of [α-^{14}C]glycine and killing of the rabbit.

Rabbit no.	Time (min.)	Specific radioactivity of DNP-glycine (counts/min./2 cm.^{2}) Serum	Liver	Muscle
4*	5	35 200	—	—
9	5	50 400	12 500	1 750
1	10	33 900	20 900	1 710
10	10	24 300	10 500	960
2	15	32 400	18 850	1 800
5*	15	18 300	—	—
20†	15	22 900	17 850	1 960
21†	15	28 600	15 800	1 884
3	30	14 800	13 250	1 390
4*	45	7 630	8 200‡	700‡
5*	45	9 590	8 500‡	850‡
11	60	6 540	7 040	581
4*	120	2 810	2 450	1 114
12	120	1 650	1 300	820
5*	180	2 780	1 750	1 820
18	180	1 850	1 320	1 180
19	180	1 440	1 000	1 470
23	240	990	—	1 170
13	240	900	780	760
27	240	1 120	—	1 450
22	360	566	—	750
14	360	500	500	624
24	360	566	—	447
16§	720	372	376	508
17§	1080	292	284	534

— Not determined.

* Bled twice before killing; 10 ml. of blood were taken each time.

† Perfused for 5–7 min. with 0·9% NaCl before removal of leg muscles.

‡ Separate experiments (serum values not being determined).

§ Received 25 μc/kg. body wt.; observed radioactivity was divided by 5.

(Fig. 1). No exact quantitative significance can be attached to these calculations, but it was found that the curve can be fitted only on the assumption that the three experimental coefficients have widely different values. It appears, therefore, that the decrease of the radioactivity in the plasma glycine occurs almost certainly by three or more reactions proceeding with greatly different rates.

The changes with time of specific radioactivity of the free glycine of liver and muscle

The radioactivity of the free glycine of liver increases rapidly and has a rather flat maximum at about 15 min. The value then decreases to about 5% of the maximum figure in 4 hr. and to about 1·5% in 18 hr. The values for muscle change much less with time than those for plasma or liver (Table 2), but there appears to be an indication of the existence of two maxima, one during the first 30 min., and the other at about 3 hr.

However, the ratios of the specific activities of the glycine in the three tissues were less variable and are probably more informative than the actual, observed values. It can be seen (Fig. 2, curve *A*) that after 5 min. the ratio of liver glycine activity to plasma glycine activity was about 0·25 and reached 1·0 between 30 and 60 min. The ratio then appeared to decrease at about 3 hr. to 0·7, rose again and remained at about 1·0 for the period between 6 and 18 hr. We cannot be certain that the maximum of 1·05 at 1 hr. and the minimum at about 3 hr. of the liver/plasma ratio are real phenomena; they might be caused by the variations in animals discussed above. However, the ratios are less variable than the measured activities and a model system can be constructed (see p. 416) behaving in a manner represented by curve *A* (Fig. 2).

The ratio of the activity of muscle glycine to that of plasma glycine (curve *D*) rose from values of 0·03 to 0·09 obtained during the first hour to 1·0 at about 4 hr. and increased steadily up to 18 hr. It can be concluded that penetration from the blood into the liver is a relatively fast process, whilst penetration into muscle is very much slower.

Comparison of the specific radioactivity of free serine with that of free glycine in liver and muscle

In order to analyse in a quantitative manner the results presented above, it was necessary to obtain information about the rate of conversion of glycine into serine in the rabbit. The experiments of Shemin (1946) showed that the conversion of serine into glycine is very fast in the rat and guinea pig, and other, more recent work, summarized by Arnstein (1954) suggests that the reverse reaction, i.e. the transformation of glycine into serine, is also rapid. However, it was uncertain how far these findings apply to the rabbit. Free serine was therefore isolated in a few experiments from liver and muscle of rabbits which had been given [α-^{14}C]glycine; the radioactivity of the serine was compared with that of the glycine isolated from the same source. The results (Table 3) show that in both liver and muscle the radioactivity of serine was of the same order as that of the glycine, already 15 min. after the injection of the latter. If only the α-carbon atom of serine had become labelled, the ratio of the 'molar' radioactivity of DNP-serine to that of DNP-glycine should not be higher than 1. In fact, in two out of the three liver experiments and in all three muscle experiments the ratio found was between 1·23 and 1·86. However, the α-carbon atom of glycine gives rise to radioactivity in both the α- and β-carbon atom of serine (Sakami, 1948) and it may thus be assumed that equilibration between the α-carbon

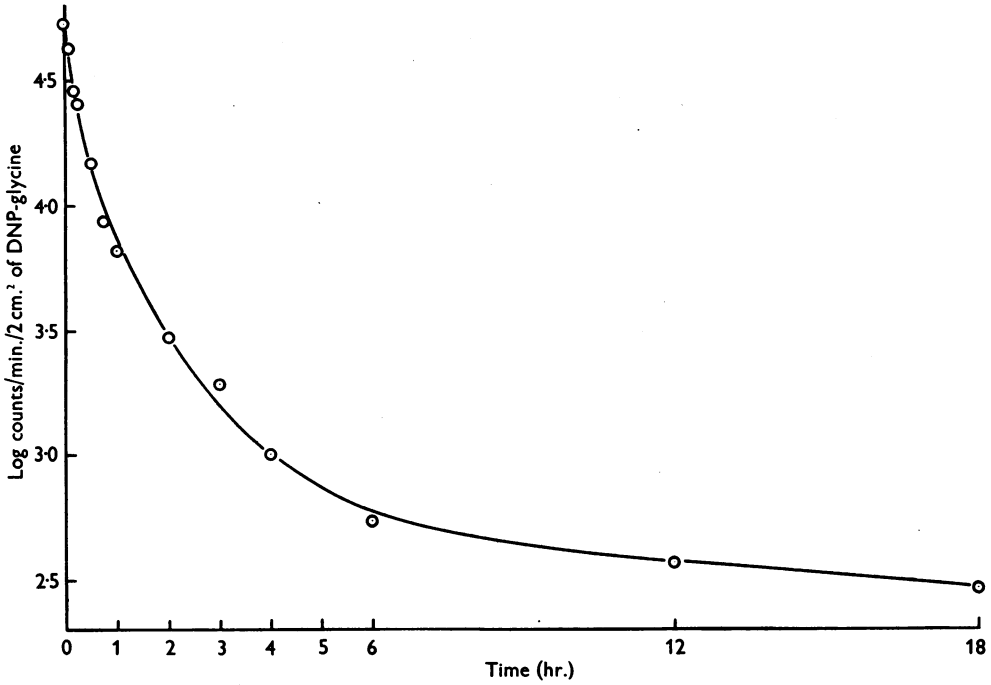

Fig. 1. The change with time of the specific radioactivity of plasma glycine. The experimental points are mean values calculated from the results given in Table 2. The curve is drawn according to eqn. 1.

atom of glycine and the α-carbon atom of serine is complete or almost complete in the rabbit liver within 15 min. and that about 20–30 % of the total radioactivity of the liver serine is due to labelling of the β-carbon atom. Arnstein & Neuberger (1953) found in the rat that with small doses of [α-^{14}C]-glycine, the labelling in the β-position was only 10–20 % of the total radioactivity of the serine. The slightly higher values indicated in the present work for liver may be due to species differences. The even higher ratios found for muscle are most readily explained by the assumption that the muscle cell membrane is more permeable to serine than to glycine and that the labelled serine is transferred from liver to muscle at a faster rate than the labelled glycine.

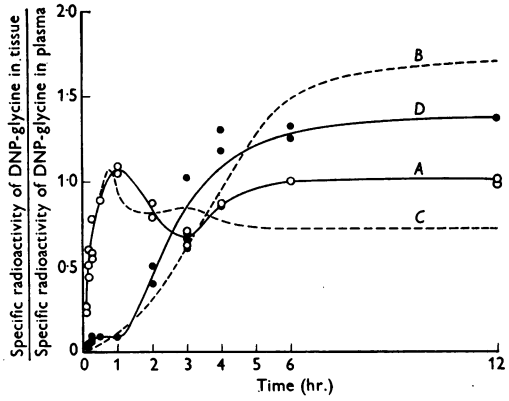

Fig. 2. The change with time of the ratio of the specific radioactivity of the free glycine of liver to that of plasma (○) and of the ratio of the radioactivity of free glycine of muscle to that of plasma (●). The experimental points (curves *A* and *D*) are calculated from the individual values given in Table 2. The theoretical curves (broken lines *B* and *C*), referring to muscle and liver respectively, are obtained from eqns. 1, 10 and 11.

Mathematical treatment of the equilibration of free glycine between plasma, liver and muscle

Using a modification of a differential equation first applied to a related problem by Zilversmit, Entenman & Fishler (1943), the change in the amount (x) of radioactive glycine (expressed as the product of the amount of total glycine (r) and its specific activity) in a tissue is given by the following:

$$dx/dt = p_1 f(t) - p_{1'}(x/r) - (x/r)\,\Sigma p_{2i} + \Sigma p_{2'i} g_i(t), \quad (2)$$

where p_1 is the amount of glycine transferred from plasma to tissue per unit time, $p_{1'}$ the corresponding value for the transfer from tissue to plasma, p_{2i} the rate of transformation of glycine to substance i, $p_{2'i}$ the rate of the reverse reaction, $f(t)$ the specific activity of the glycine in plasma and $g_i(t)$ the specific activity of substance i at time t.

Table 3. *Comparison of the specific radioactivity of free serine with that of free glycine in the liver and muscle of rabbits injected* [α-^{14}C]*glycine* (5 μc/*kg. body weight*)

For details of the experiments see Table 2 and text. The time given in column 2 is the interval between injection of [α-^{14}C]glycine and killing of the animal. The molar radioactivity was calculated by multiplying the counts obtained for DNP-glycine and DNP-serine by 241 and 271 respectively.

Rabbit no.	Time (min.)	Specific radioactivity (counts/min./2 cm.²) DNP-glycine	DNP-serine	Molar radioactivity of DNP-serine/ molar radioactivity of DNP-glycine
		Liver		
101	15	20 890	22 720	1·23
104	120	2 710	3 150	1·31
105	180	1 950	1 500	0·87
		Muscle		
101	15	2 000	2 880	1·64
104	120	1 390	2 290	1·86
105	180	2 020	2 730	1·52

Any attempt to apply this general equation to the results given in Table 2 and Fig. 2 must involve assumptions which are at best gross over-simplifications. First of all we must treat both muscle and liver as homogeneous compartments with respect to glycine. Secondly, we must assume that there is a steady state, i.e. that the quantities of glycine in plasma and in the two tissues and the rates of transfer and the metabolic reactions are constant during the experiment and identical in the various animals. It thus follows that

$$p_1 - p_{1'} + \Sigma p_{2'i} - \Sigma p_{2i} = 0. \quad (3)$$

In addition we postulate

$$p_1 - p_{1'} = 0;\ \Sigma p_{2'i} - \Sigma p_{2i} = 0. \quad (4)$$

This assumption, which is introduced in order to facilitate mathematical treatment, may not be correct. In the beginning of the experiment the value of $\Sigma p_{2'i} g(t)$ is small for all reactions involving the formation of labelled glycine from unlabelled precursors with the exception of the serine $\rightleftarrows$ glycine transformation. Evidence was given above that one of these reactions is extremely fast in rabbit liver and as an approximation we may assume that glycine and serine form a common pool; the term 'glycine' as used in this discussion implies glycine + serine. If we neglect $\Sigma p_{2'i} g(t)$ and put $p_2 = Kp_1$ we obtain

$$dx/dt = p_1 f(t) - (p_1 + Kp_1)(x/r), \quad (5)$$

which can be transformed into

$$\frac{1}{r}\frac{dx}{dt} = \frac{p_1}{r} f(t) - \frac{x}{r}(1+K)\left(\frac{p_1}{r}\right). \quad (6)$$

Equation 1 may be written in the more general form

$$f(t) = a_1 e^{-b_1 t} + a_2 e^{-b_2 t} + a_3 e^{-b_3 t} + c. \quad (7)$$

Integrating equation 6 and substituting $f(t)$ from equation 7 we obtain

$$(x/r) = p_1/r[a_1'(e^{-b_1 t} - e^{-Bt}) + a_2'(e^{-b_2 t} - e^{-Bt}) + a_3'(e^{-b_3 t} - e^{-Bt})] + c'(1 - e^{-Bt}), \quad (8)$$

where x/r is the specific activity of the liver or muscle glycine, p_1/r the fraction of the total liver or muscle glycine transferred to the blood in unit time and

$$B = (p_1/r)(1+K);\quad a_1' = a_1/(B - b_1);$$
$$a_2' = a_2/(B - b_2);\quad a_3' = a_3/(B - b_3);$$
$$c' = c/(1+K).$$

In order to get a value for $K = p_2/p_1$, we put dx/dt in equation 6 equal to zero and obtain

$$1 + K = f(t)/(x/r). \quad (9)$$

In other words, $1+K$ is the ratio of the specific activity of plasma glycine to that of the tissue glycine when the latter has its maximum value.

Application of the equations to the results for the free glycine of liver

With liver the maximum activity of the free glycine appears to be at 15 min. and the value for $1+K$ was therefore calculated from the results (Table 2), using equation 9, to be 1·46. Using equations 8 and 4, smooth curves for several values of r/p_1 were obtained; this ratio may be called a 'renewal time' and designates in this particular case the time (in min.) required to replace the whole of the free glycine of liver by free glycine of plasma. As can be seen from Fig. 3 the best agreement between expected and calculated values for the first 6 hr. is obtained if a 'renewal time' of 17 min. is used. Thus, the equation which is obtained from equation 9 and which gives the best fit is the following:

$$x/r = 180\,000(e^{-0.073t} - e^{-0.085t}) + 12910(e^{-0.015t} - e^{-0.085t}) + 386(e^{-0.00027t} - e^{-0.085t}) + 201(1 - e^{-0.085t}). \quad (10)$$

The later experimental points lie above the calculated curve; this is probably due to the fact that the

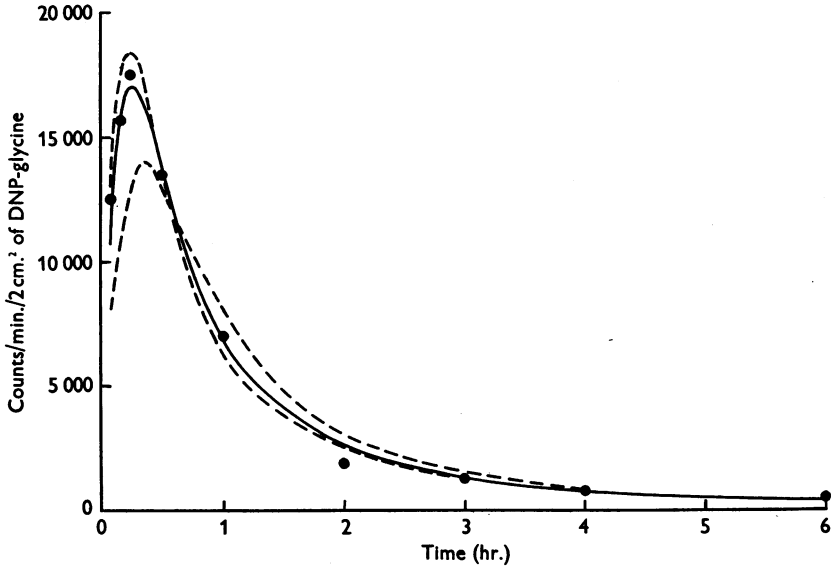

Fig. 3. Changes of specific radioactivity of free liver glycine with time. The experimental points are mean values calculated from the results given in Table 3. The full line is calculated according to eqn. 10, assuming a renewal time of 17 min.; the upper and lower broken lines are calculated in a similar manner, assuming renewal times of 13·5 and 29 min. respectively.

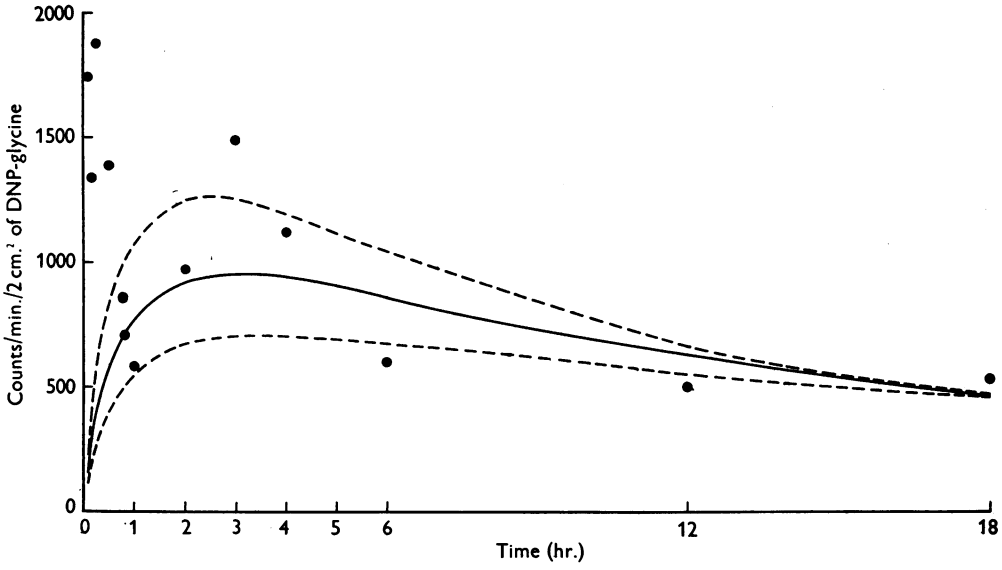

Fig. 4. Changes of specific radioactivity of free muscle glycine with time. The experimental points are mean values calculated from the results given in Table 3. The full line is calculated according to eqn. 11, assuming a 'renewal time' of 1400 min. The upper and lower broken lines are calculated in a similar manner assuming 'renewal times' of 1000 and 2000 min. respectively.

contribution of the term $\Sigma p_{2'i} g(t)$, which has been neglected in our calculations, becomes significant after about 6 hr.

Application of the equations to the results for free glycine of muscle

Fig. 4 shows that the initial values for free glycine in muscle, while much lower than the corresponding values for liver, are high compared with the later muscle values. It was thought that these high initial values might be due to very small amounts of highly radioactive glycine present in blood or extracellular fluid associated with muscular tissue. It was found, however, that perfusion of the muscles with saline (Table 2, rabbits nos. 20 and 21) gave essentially the same results as in the non-perfused animal (Table 2; rabbit no. 2) killed at the same interval after administration of the radioactive glycine. But apart from the difficulty arising from these high values, it has been found impossible to fit a theoretical curve satisfactorily to the experimental points. It was necessary in the first place to fix a maximum; inspection of Table 1 suggests a maximum at 3 hr. which would give a value of $1+K=1{\cdot}36$, but it was found impossible to find a value for renewal time which would give a reasonable fit. A great number of values for K and p_1/r were then tried and the best fit was obtained with a value of $1+K=1{\cdot}8$ and a value for $r/p_1=1400$ min. But, as can be seen (Fig. 4) even this curve does not represent the experimental results at all well. By inserting these values into equation 8 we get the following:

$$x/r = -370(e^{-0{\cdot}073t} - e^{-0{\cdot}0013t}) - 795(e^{-0{\cdot}015t} - e^{-0{\cdot}0013t}) - 282(e^{-0{\cdot}0027t} - e^{-0{\cdot}0013t}) + 162(1 - e^{-0{\cdot}0013t}). \quad (11)$$

Using equations 1, 10 and 11 we have calculated ratios for liver glycine/plasma glycine and for muscle glycine/plasma glycine for various times. The agreement for the liver/plasma ratio between theoretical and experimental values is of the right form up to 6 hr.; in particular the theoretical treatment correctly predicts that the ratio should reach a value of about 1·0 at approx. 1 hr. to decrease again later (Fig. 2). For the interval between 6 and 18 hr. the theoretical value is lower than that obtained from the experimental results. This is due largely to formation of labelled glycine from glutathione which has a negligible isotope content compared with that of free glycine in the first 2 hr. but later this relationship is reversed (Table 4). The theoretical treatment used neglects this factor and thus underestimates the radioactivity of liver glycine after 4 hr. For the muscle/plasma ratio the agreement between calculated and observed values is also quite good (Fig. 2). In particular, as predicted by the theory, the ratio is below 0·1 for the first hour, reaches unity at about 4 hr. and then remains above 1·0 for the following 14 hr. However, during the first 30 min., the ratio as obtained from the experimental results is significantly higher than the predicted value.

The incorporation of labelled glycine into the glutathione and protein of liver and muscle and into serum proteins

The labelled glycine was rapidly incorporated into the glutathione of the liver (Table 4) but more slowly into the glutathione of muscle (Table 5). The specific activity of the glycine obtained from the liver GSH reached a maximum in the interval between 1 and 4 hr. after injection and surpassed that of the free glycine of liver towards the end of that period. The specific activity of the glycine isolated from the muscle GSH remained lower than that of the free glycine of muscle throughout the period of the experiment (Table 5). The conclusions can be drawn that the rate of turnover of muscle

Table 4. *Comparison of the specific radioactivities of DNP-glycine samples obtained by hydrolysis of glutathione (GSH) of liver, mixed proteins of liver and mixed proteins of serum with those obtained from free glycine of livers of rabbits injected* [α-^{14}C]*glycine* (5 μc/*kg. body weight*)

The time in column 2 is the interval between injection of the labelled amino acid and killing of the animal.

Rabbit no.	Time (min.)	Specific radioactivity of DNP-glycine (counts/min./2 cm.^{2}) from: Free glycine of liver	Liver GSH	Liver proteins	Serum proteins
9	5	12 350	118	8	0
10	10	10 500	217	29	0
11	60	7 040	1 670	171	101
12	120	1 300	962	126	128
13	240	780	1 400	158	228
14	360	500	1 100	162	243
16*	720	376	764	150	164
17*	1080	284	440	150	254

* Received 25 μc/kg. body wt.; observed specific activities were divided by 5.

glutathione is appreciably lower than that of the liver glutathione.

The specific activity of the glycine from the mixed liver proteins rose sharply during the first hour and then remained more or less constant for the following 17 hr. It is of interest that the specific radioactivity of the liver protein glycine was still only about half that of the free glycine of liver at 18 hr. (Table 4). The radioactivity of the glycine obtained from the mixed serum proteins was not detectable during the first 10 min.; but then it rose sharply and, after a few hours, it was considerably higher than that of the glycine isolated from the mixed liver proteins (Table 4). The specific radioactivity of the glycine obtained from the mixed muscle proteins rose very slowly and the maximum value found was only about 5% of that observed for the glycine from the mixed liver proteins (Table 5).

Table 5. *Comparison of the specific radioactivities of samples of DNP-glycine obtained by hydrolysis of muscle glutathione (GSH) and mixed proteins of muscle with those obtained from the glycine of muscle of rabbits injected* [α-^{14}C]*glycine* (5 μc/*kg. body weight*)

The time in column 2 is the interval between injection of the labelled amino acid and killing of the animal.

Rabbit no.	Time (min.)	Specific radioactivity of DNP-glycine (counts/min./2 cm.²) from: Free glycine of muscle	Muscle GSH	Muscle proteins
9	5	1750	16·6	0
10	10	964	5·6	0
11	60	581	40	0
12	120	820	83	9·9
13	240	760	200	7·0
14	360	624	206	11·3
16*	720	508	238	9·9
17*	1080	534	346	23·0

* Received 25 μc/kg. body wt.; observed specific activities were divided by 5.

Mathematical treatment of the values of the radioactivity of glycine obtained from the glutathione and of mixed proteins of liver and muscle

In the following calculations it is assumed that the glutathione of liver and muscle represent two homogeneous 'compartments' and that the only reaction by which glycine is lost from the glutathione 'compartment' is the hydrolysis of GSH to give free glycine. It is also assumed that the concentrations of free glycine and of GSH and the rates of interconversion are constant during the experiment. It then follows that

$$p_{3,1}=p_{3,2}=p_3, \qquad (12)$$

where $p_{3,1}$ is the amount of free glycine converted into GSH in unit time and $p_{3,2}$ the amount of GSH-glycine converted into free glycine in unit time. We can then write, following Zilversmit *et al.* (1943),

$$\mathrm{d}(u/r_3)/\mathrm{d}t=p_3/r_3(x(t)/r-u/r_3), \qquad (13)$$

where u/r_3 is the specific activity of the GSH-glycine, r_3 the amount of GSH-glycine present in the tissue and $x(t)/r$ the specific activity of the free glycine of the tissue at time t. By substituting equation 8 for $x(t)/r$ and integrating we get

$$\frac{u}{r_3}=\frac{p_3}{r_3}\left\{\frac{a_1'p_1}{r}\frac{(\mathrm{e}^{-b_1t}-\mathrm{e}^{-p_3t/r_3})}{(p_3/r_3-b_1)}+\frac{a_2'p_1}{r}\frac{(\mathrm{e}^{-b_2t}-\mathrm{e}^{-p_3t/r_3})}{(p_3/r_3-b_2)}+\frac{a_3'p_1}{r}\frac{(\mathrm{e}^{-b_3t}-\mathrm{e}^{-p_3t/r_3})}{(p_3/r_3-b_3)}+\frac{[(a_1'+a_2'+a_3')\,p_1/r+c']\,(\mathrm{e}^{-p_3t/r_3}-\mathrm{e}^{-Bt})}{(p_3/r_3-B)}\right\}+c_1'(1-\mathrm{e}^{-p_3t/r_3}). \qquad (14)$$

Theoretical curves were constructed substituting the numerical data given in equations 10 and 11 into equation 14, and using various values for p_3/r_3. Figs. 5 and 6 show that reasonably good agreement between calculated and observed values for the radioactivities of the glycine from liver-GSH and for that for muscle-GSH is obtained, if the following figures for p_3/r_3 are used: liver-GSH, 1/650; muscle-GSH, 1/1200. Similar calculations, which were made for the two protein fractions, gave values for p_3/r_3 1/7200 for mixed liver proteins and 1/30 000 for mixed muscle proteins (Figs. 5, 6). For reasons to be discussed later, this value for muscle protein is not believed to have any quantitative significance. Values for p_3/r_3 can also be obtained by a graphical method (Zilversmit *et al.* 1943) consisting of measurement of areas of the products $u/r_3(t_2-t_1)$ and $x/r(t_2-t_1)$. This method which does not involve the arbitrary selection of values for K, p_1/r or p_3/r_3 gave the following renewal times (r_3/p_3): 890 min. for liver-GSH, 1660 min. for muscle-GSH, 8950 min. for liver proteins and 34 500 min. for muscle protein.

DISCUSSION

Penetration of plasma glycine into liver and muscle

It is believed that several conclusions of a qualitative character can be drawn from the present work with some confidence. The rate of fall of radioactivity of the plasma glycine with time, which is first very rapid and later slows down considerably, indicates that the glycine is lost from plasma through several reactions, some of which are fast, whilst others are slow. The results could be reasonably well expressed by an equation containing three exponential terms (Fig. 1), but it is clear that the numerical values of equation 1 are unlikely to

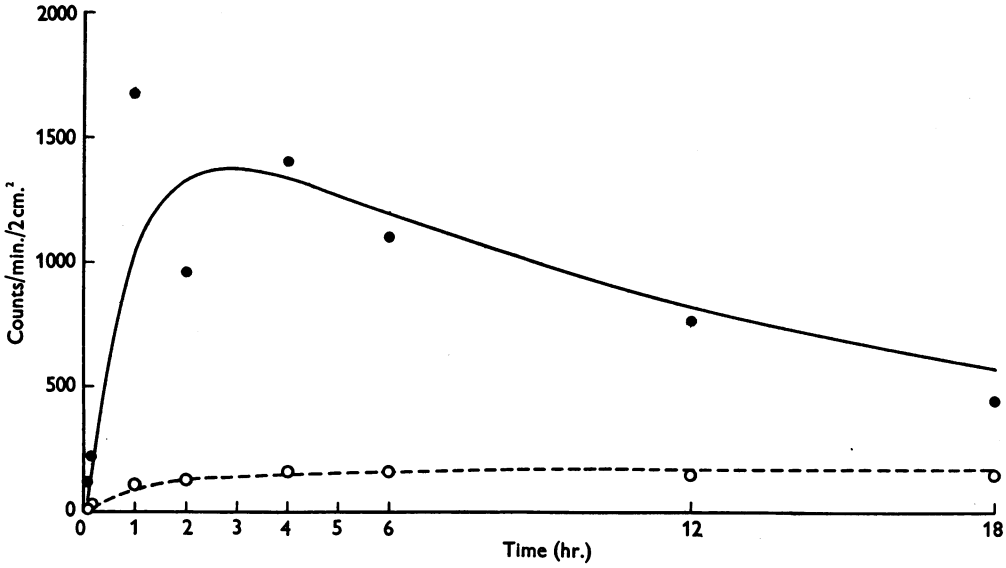

Fig. 5. The changes of the specific radioactivities of glycine obtained by hydrolysis of liver glutathione and liver-protein glycine. The full circles denote experimental points for glutathione (Table 4), while the open circles refer to the protein (Table 4). The theoretical curve for glutathione (full line) was obtained according to eqn. 14, using a renewal time of 650 min., while for the protein curve (broken line) a 'renewal time' of 7200 min. was assumed.

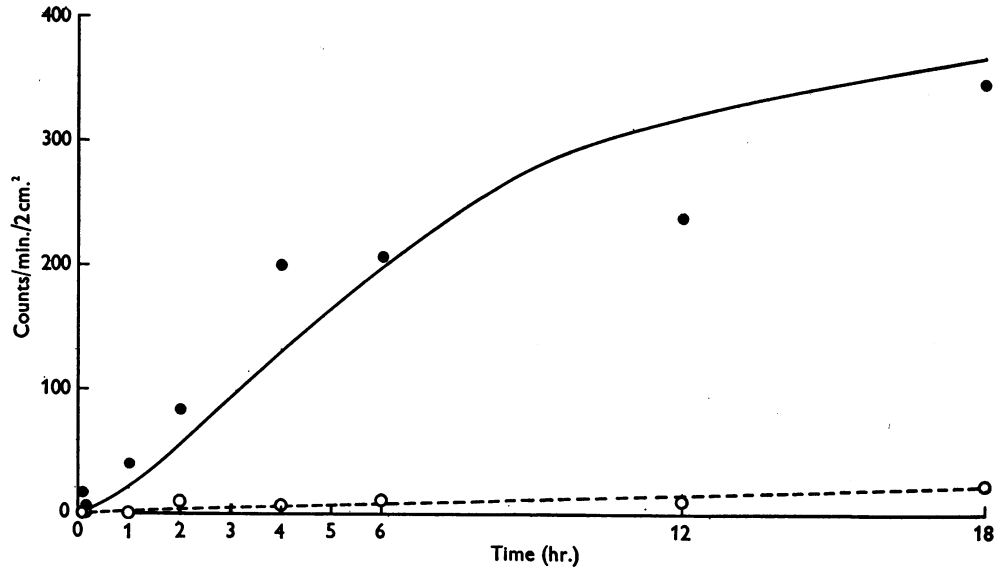

Fig. 6. The changes of the specific radioactivities of glycine obtained by hydrolysis of muscle glutathione and muscle-protein glycine. The full circles denote experimental points for glutathione (Table 5), while the open circles refer to protein (Table 5). The theoretical curves for glutathione (full line) and for protein (broken line) were calculated according to eqn. 14, using 'renewal times' of 1200 and 30000 min. respectively.

correspond with constants of any definite reactions. Similarly, it may be concluded that the exchange of glycine between plasma and liver is fairly rapid, while it is much slower between plasma and muscle. However, great difficulties arise when an attempt is made to make quantitative deductions from results such as those presented here. We have postulated, for example, that the concentrations of glycine in the different tissues and the rates of exchange are identical in all the rabbits used and do not change with time. The results (Table 2), in fact, show that differences between animals are not negligible and the assumption of a 'steady state' in any one animal is probably an approximation. The choice of the constants in equation 1 is also somewhat arbitrary and so is the choice of a value for K (equation 9). As had already been pointed out (p. 414), the assumption that identical quantities of glycine migrate in unit time in the two directions across the membrane of the liver or muscle cell is also questionable. It is clear therefore that no quantitative significance can be attached to the calculations presented here, and their only justification is that they provide an idea of the orders of magnitude of the rates at which the various reactions take place.

The rate of exchange between the glycine in plasma and the glycine in the liver cell will depend both on the rate of blood-flow through the liver and on the rate of diffusion through the cell membrane or across any organized structure inside the liver cell. The maximum rate of exchange, i.e. the rate obtained in the absence of any permeability barriers, can be calculated if the rate of blood-flow through the liver and the concentrations of glycine in the plasma and liver cell are known. The position is somewhat complicated in the case of the liver, as blood is supplied by both the portal vein and the hepatic artery. We may assume, however, that rapid mixing between portal and systemic blood will occur in the heart, lungs and the large vessels and that it will be complete within 1–2 min. after injection of the glycine. The rate of blood-flow in rabbit liver was found by Grayson & Johnson (1953) to be 112 ml./100 g. liver/min. However, the validity of the method used by these workers has been questioned (Linzell, 1953). In man, measurements by different methods indicate that about 1500 ml. of blood pass through the liver/min. (Wakin & Mann, 1953) and assuming a liver weight of 1·5 kg. the average value is about 100 ml./100 g./min. In the dog the figures obtained vary between 40 and 90 ml./min. per 100 g. tissue (Grindley, Herrick & Mann, 1941). We therefore assume that the value for the rabbit lies between 50 and 100 ml./min.

The following concentrations of glycine in rabbit tissues were found by Krueger (1950): whole blood, 8·4 mg./100 ml.; liver, 47·2 mg./100 g.; muscle, 18·2 mg./100 g. Dubreuil & Timiras (1953) reported slightly different figures: plasma 5·2; liver, 45·6; muscle, 33·4 (all per 100 g. or 100 ml.). Using the method of Krueger (1949) we obtained with six rabbits the following mean values (with extreme limits in brackets): plasma, 5·0 (4·2–6·1) mg./100 ml.; liver, 46 (34–58) mg./100 g.; muscle, 26 (18–35) mg./100 g. The glycine content of the erythrocytes is higher than that of the plasma (see Arnstein, 1954), but the exchange between red cell and plasma is slow (Ussing, 1943; Christensen, Cooper, Johnson & Lynch, 1947). It is therefore unlikely that a significant amount of erythrocyte glycine is transferred to the liver cell in one passage of blood through the liver. Thus, assuming a haematocrit value of 45 %, a rate of blood flow of 50–100 ml./100 g./min., a plasma glycine content of 5 mg./100 ml. and a liver glycine content of 45 mg./100 g., we can calculate that the maximum amount of glycine which can be transferred to the liver is between 1·3 and 2·6 mg./min./100 g. of tissue.

The value of r/p_1 to give the best fit for the experimental results was 17 min. Assuming r to be 45 mg./100 g. liver, we obtain for p_1, i.e. the amount of glycine exchanged between plasma and liver (/100 g./min.), a value of 2·65 mg. which agrees quite well with the upper limit calculated above. In view of the many assumptions made, this possibly fortuitously close agreement should not be overemphasized, but it is safe to conclude that the maximum rate of exchange calculated from blood-flow figures, without taking into account permeability barriers, and the corresponding value calculated from isotope data, are of the same order of magnitude. It thus appears that the rate of exchange of glycine between plasma and the liver cell is mainly determined by the rate of blood-flow and not at all or only slightly by diffusion barriers at the cell membrane.

The position is different with muscle. It has already been pointed out (p. 416) that the early values for free glycine in muscle are surprisingly high compared with the later values. If 20 % of the total muscle space is extracellular and if the glycine concentration in the extracellular space is the same as in plasma, it can be calculated that about 3 % of the total glycine of muscle is extracellular. If this fraction were to exchange rapidly with plasma glycine, the radioactivity in the first 15–30 min. could be mainly due to this interstitial glycine fraction. However, the fact that perfusion did not affect the radioactivity of the free muscle glycine during this early period (Table 2), is not in accordance with such an explanation. It is more likely that muscle is not homogeneous with respect to the rate of exchange with plasma glycine and this might be caused by local differences of blood supply. In resting muscle the majority of the capillaries are collapsed, and probably only a small fraction of

muscle cells are in close proximity to circulating blood. The rate of blood-flow through muscle is probably very variable, but a value of about 6 ml./100 g./min. is not likely to be very far from a true average value (see, for example, Barcroft & Swan, 1953); in the quadriceps muscle of the dog a figure of 6·9 ml./100 g./min. has been reported (Green, Lewis, Nickerson & Heller, 1944). We can calculate, as we have done for liver, maximum rates of exchange, i.e. rates operating in the absence of diffusion barriers, for two likely limits of rates of blood-flow, using the data for glycine concentrations given above. For the improbably low rate of blood-flow of 3 ml./100 g./min., p_1 would be 0·082 mg. of glycine/100 g. muscle/min. and p_1/r would equal 1/315. For a rate of blood-flow of 10 ml./100 g./min. the corresponding values would be 0·275 mg./100 g./min. and 1/94 respectively. From the radioactivity/time curve we have calculated a value of 1/1400 for p_1/r of muscle glycine; assuming r to be 26 mg./100 g., this is equivalent to a rate of exchange of 0·019 mg. of glycine/100 g. muscle/min. This is about 4·5 times smaller than the figure predicted on the basis of a low rate of blood-flow of 3 ml./100 g./min. It thus appears that the diffusion of glycine from the plasma to the muscle is impeded by a permeability barrier, which, together with the low rate of blood-flow, accounts for the relatively slow equilibration between plasma glycine and muscle glycine.

The turnover of glutathione in rabbit liver and muscle

The synthesis of glutathione (GSH) from the three constituent amino acids has been studied extensively by Bloch and his colleagues and evidence was obtained (Snoke & Bloch, 1952) that in the last step of the synthesis glycine combines with L-glutamylcysteine. On the other hand, GSH may also be formed in the cell by reaction of γ-glutamyl peptides with cysteinylglycine (Fodor, Miller, Neidle & Waelsch, 1953). It is thus possible that GSH may be synthesized by several mechanisms and that the changes in radioactivity observed may be the result of several reactions occurring at different rates. The biosynthesis of GSH in the liver and intestine of rats and rabbits has also been studied *in vivo*. In these experiments there were used [^{15}N]glycine (Waelsch & Rittenberg, 1941), [^{15}N]ammonia and [^{15}N]glutamic acid (Waelsch & Rittenberg, 1942) and also [^{35}S]cystine (Anderson & Mosher, 1951); the isotope contents of free glycine or glutamic acid were not determined, and the values of the turnover rates of GSH which were calculated are therefore somewhat uncertain. However, on making certain assumptions, Waelsch & Rittenberg (1942) deduced from their results that the 'half-life' of GSH in the liver of rats and rabbits was less than 4 hr. In the present work the radioactivity of the free glycine in liver and muscle was measured and our results indicate that the 'renewal time' is about 10–13 hr., corresponding to a 'half-life' value of 7–9 hr. This value indicates that the turnover of GSH in rabbit liver is somewhat slower than that deduced by Waelsch & Rittenberg (1942). The uncertainties in our calculations have been already stressed, but the estimate given is believed to be correct within ± 30 %.

The changes in radioactivity of the GSH-glycine of muscle may only reflect the rate of penetration of labelled GSH made elsewhere and transported to muscle. However, as already pointed out by Anderson & Mosher (1951), the absence of GSH from plasma and its presence in erythrocytes suggests that it does not readily penetrate into cells and is not transferred from one organ to another. Thus we may assume that the radioactivity/time curve is a measure of the rate of synthesis of GSH in rabbit muscle. The renewal time calculated is between 22 and 25 hr., i.e. about twice the value found for liver GSH. But the concentration of GSH in rabbit liver is approx. 240 mg./100 g., while in rabbit muscle it is 30 mg./100 g. (Gregory & Goss, 1939). Thus the rates of synthesis and degradation of GSH (/100 g. of tissue/hr.) are about 20 mg. for liver and 1·0–1·5 mg. for muscle.

The turnover of proteins in rabbit liver and muscle

Effect of radioactivity of free amino acid. The doubtful character of the many assumptions which are made, generally implicitly, in calculations of turnover values of tissue proteins on the basis of isotope data, has been emphasized by several workers (see, for example, Neuberger, 1952; Reiner, 1953*a*, *b*; Fisher, 1954). The results obtained in the present work on the relative specific radioactivities of free glycine and protein glycine both in liver and muscle indicate that it is not justifiable to assume that the activity of the free amino acid decreases to negligible values within a few hours after administration. Thus even after 18 hr., the specific radioactivity of the free glycine in liver is almost twice as high as that of the protein glycine (Table 4); the few results which we have obtained for intervals greater than 18 hr. suggest that the specific radioactivities of the two fractions become equal about 50 hr. after injection. It follows that the descending portion of the activity/time curves of liver protein represents the net result of two opposing reactions: (*a*) the loss of labelled amino acid from the protein, and (*b*) incorporation of amino acid of somewhat lower isotope content into the protein. It is almost certain therefore that the 'half-life' values such as that of 5–7 days calculated by Shemin & Rittenberg (1944) for liver protein of the rat and that of 4–5 days obtained by Neuberger, Perrone & Slack (1951) are

too large. The figure of a renewal time of 120 hr. (corresponding to a 'half-life' value of 3·5 days) obtained in the present work, is based on a treatment which takes into account the radioactivity of the free amino acid and is therefore likely to give more reliable information of the rate of protein synthesis in rabbit liver.

With rabbit muscle the specific radioactivity of the free glycine at 18 hr. is more than 20 times greater than that of the protein glycine (Table 5). It is thus impossible to calculate even the order of magnitude of protein synthesis in muscle from the radioactivities of the protein glycine without knowing those of the free glycine. But it can be concluded that the low incorporation of labelled glycine into muscle protein is not entirely due to the slow penetration of the labelled amino acid into the muscle cell. The value of a renewal time of 500 hr. obtained in the present work cannot be considered reliable in view of the low counts of the muscle protein glycine and other reasons discussed above. But it indicates, in agreement with the conclusions of Shemin & Rittenberg, that protein synthesis is much slower in muscle than in the liver.

It appears from the present work that the incorporation of a labelled amino acid into protein may be determined by at least three factors: the rate of synthesis of the particular protein, the rate of blood flow to the organ concerned and the permeability to the labelled amino acid of the cell in which the protein is situated. The present results also show that the mammalian organism cannot be considered as a homogeneous medium with regard to the distribution of labelled amino acid. A more satisfactory model is a system of semi-isolated compartments communicating with a central compartment—the blood and the extracellular fluid—at widely differing rates. Deductions such as those of Sprinson & Rittenberg (1949) and San Pietro & Rittenberg (1953) from the rate of the excretion of ^{15}N in the urine after administration of [^{15}N]glycine and assuming a homogeneous glycine pool, are therefore based on an over-simplification and are probably not quantitatively reliable.

Heterogeneity of tissue proteins. Another, major, difficulty arises from the fact that almost all isotope data published were obtained with unfractionated tissue proteins, and the deductions made are likely to be wrong if the components of such a mixture are replaced at greatly different rates. Thus skin has been considered to be a metabolically rather inert tissue, but it has been shown (Harkness, Marko, Muir & Neuberger, 1954) that the cellular proteins incorporate isotopically labelled glycine at a high rate, while the extracellular fibrous proteins are indeed fairly inert. The high glycine content and the large concentration of collagen in skin are thus responsible for the low overall incorporation of isotope, particularly if glycine is used as the labelling agent. A similar situation may exist in muscle. But with liver there is reason to believe that treating the organ as metabolically homogeneous may be justified as a very rough approximation. Thus Hultin (1950) fractionated chicken liver after administration of [^{15}N]glycine and found that microsomes showed higher incorporation than fractions containing nuclei, mitochondria or cell fluid. But the differences were relatively small. Peters & Anfinsen (1950*a*) used chemical methods for fractionating, and found that all protein fractions had similar isotope contents excepting material which was identified as newly formed serum albumin. Brunish & Luck (1952) found significant, but not very large, differences between nuclear and cytoplasmic proteins, whilst Åquist (1951) found only very small differences between these two fractions. But recently Keller, Zamecnik & Loftfield (1954) have reported that the rate of incorporation of labelled valine and leucine is much greater with microsomes of rat liver than with any other fraction.

Translocation of proteins. Both the calculations used by Shemin & Rittenberg (1944) and those used by ourselves assume that the tissue under investigation is an open system with respect to amino acids, but is a closed system in regard to protein, i.e. it is assumed that the protein does not leave the tissue before it is degraded. Such a treatment cannot be applied to endocrine or exocrine glands which continuously secrete a large fraction of the newly synthesized protein, and also probably not to tissues such as the intestine. Leblond & Stevens (1948) deduced from the rate of mitoses that the epithelium covering the villi of the duodenum and ileum of the rat is renewed every 1·5–2 days, and similar values were obtained by McMinn (1954) for the cat. Thus it seems likely that the high rate of incorporation of isotope into the proteins of the intestinal tract and the subsequent rapid decrease are not merely the expression of the turnover of proteins in a static cell, but represent largely or even mainly the shedding of the epithelial layer and the continuous formation of new cells. In such cases isotope analysis of the proteins of the tissue itself cannot give complete information about the total rate of protein synthesis in the tissue. Liver, apart from forming its own tissue or 'sessile' proteins, also synthesizes all the plasma albumin, and fibrinogen, and also most of the α- and β-globulin (Miller & Beale, 1954; Miller, Bly & Beale, 1954). The plasma and sessile liver protein synthesized during the first hour will have a very high isotope content. But while the sessile protein remains in the liver, the plasma protein is quickly excreted into the blood. The rapidity of this excretion is shown by two independent observations. Peters & Anfinsen (1950*b*) found by immunological methods that unwashed liver slices, which

probably included traces of blood and tissue fluid, contained only 1·5 mg. serum albumin per 1 g. tissue. This was greatly reduced on washing; moreover, most of the serum albumin formed on incubation, appeared in the medium. In the present work it was found (Table 5) that the serum proteins had already 1 hr. after injection 60 % of the activity of the liver protein. Since this newly synthesized serum protein must have mixed with at least 50 times its own weight of circulating plasma protein, it would follow that the average retention time of plasma protein in the liver is considerably less than 1 hr. Isotope incorporation into liver protein in the first hour may thus be caused to a slight extent by plasma protein, but all the later values can be almost entirely interpreted in terms of the metabolism of the sessile proteins.

Total rate of protein synthesis in rabbit liver. In order to calculate the total rate of protein synthesis of the liver, the rate of the synthesis of the sessile proteins as obtained by the assay of the liver proteins themselves has to be added to the value calculated from the radioactivity changes of the circulating plasma proteins. The average amount of liver proteins of the rabbits used in this work was 6 g./kg. body wt.; assuming a 'renewal time' of 120 hr., the rate of synthesis of sessile liver proteins is 1·2 g./kg./day. The total concentration of albumin, of α- and β-globulin in the rabbit is approx. 5·75 g./100 g. plasma and the plasma volume 4 ml./100 g. body wt. (Dr A. S. McFarlane, private communication). Thus the amount of circulating serum proteins made in the liver is 2·3 g./kg. body wt. The total amount, which includes plasma protein present in the lymph, interstitial fluid and in cells, appears to be 2·5 times the value found in the blood (Dovey, Holloway, Piha, Humphrey & McFarlane, 1954). Thus the total amount of albumin, α- and β-globulin is about 5·75 g./kg. body wt. Taking a renewal time of 8 days (Dovey *et al.* 1954) it follows that the liver produces 0·72 g. of serum proteins/kg./day. Thus, of the 1·9 g. of protein/kg. body wt./day which are formed in the liver, 60 % are 'sessile' or liver proteins proper and 40 % are plasma proteins. The doubtful character of the many assumptions involved has already been emphasized, but the fact that the 'renewal time' for liver proteins as calculated from the descending and ascending parts of the activity/time curves differ not too greatly, is significant. It is therefore believed that the values for protein synthesis in the liver given here are likely to be of the correct order of magnitude.

General. Finally, we wish to draw attention to two additional assumptions made in the present paper and also in earlier interpretations of isotope data on protein metabolism *in vivo*. The isotope methods at present available measure only protein synthesis in which the free amino acids present in the cell are the ultimate, but exclusive, precursors. Formation of a new protein in which the peptides resulting from the breakdown of another, unlabelled, protein molecule are used, cannot be detected by the methods employed in the present work, unless the peptides were to equilibrate at a fast rate with the labelled free amino acid. How far such a re-utilization of peptides occurs in protein synthesis *in vivo* is still uncertain, but the possibility exists that we may have greatly underestimated the rate of protein metabolism. Lastly, it may be questioned whether the assumption of randomness of protein breakdown is justified. Proteins present in a homogeneous cell fluid are likely to be degraded in a random manner, but the life expectation of a protein molecule forming part of an organized structure, such as mitochondria or fibres, is likely to be dependent on the 'age' of the structure concerned. It is thus possible that the behaviour of haemoglobin (Shemin & Rittenberg, 1946) may not be exceptional. If the 'life-span' of microsomes, or mitochondria is short relative to the 'life-expectation' of the protein or if protein metabolism occurs in the intact particle, at a similar rate as in the cell fluid, the assumption of randomness may be justified and this indeed may apply to tissues such as liver. However, it appears likely that the metabolic inertia of collagen and possibly that of actomyosin is not an intrinsic property of the molecules themselves, but an expression of the morphological stability of the fibrous structures of which these proteins form a part.

SUMMARY

1. Tracer doses of [α-^{14}C]glycine were given to rabbits and the radioactivity of the free glycine in plasma, in the liver and in muscle was measured at times ranging from 5 min. to 18 hr. after injection. In some experiments free serine in liver and muscle was also isolated and its radioactivity assayed. Glutathione, the mixed proteins of liver and muscle and the mixed serum proteins were hydrolysed and the radioactivity of the resulting glycine was measured.

2. The radioactivity of the plasma glycine decreased sharply during the first 30 min., more closely in the next few hours and very slowly in the period from 6 to 18 hr. after injection. These results were interpreted as indicating that dilution of the labelled plasma glycine occurred by several reactions with widely different rates.

3. The exchange of plasma glycine with liver glycine was found to be fast, and the time required for the replacement of the whole of the glycine of liver by plasma glycine was calculated to be between 15 and 20 min. It was concluded that

permeability barriers play only an insignificant part in this exchange and that the limiting factor is the rate of blood-flow.

4. The exchange between plasma glycine and muscle glycine was very much lower and the 'renewal time' with this tissue was calculated to be about 20–25 hr. It was concluded that this slow equilibration is caused both by the low rate of blood-flow in muscle and the existence of a permeability barrier.

5. Comparison of the radioactivities of free glycine and free serine indicated a very fast and reversible conversion of glycine into serine. The specific radioactivity of muscle serine was appreciably higher than that of muscle glycine suggesting that the muscle cell is more permeable to serine than to glycine.

6. The 'renewal times' of glutathione were calculated to be about 10–12 hr. in the liver and 20 hr. in muscle.

7. The bearing of the present results on calculations of protein turnover are considered and the various assumptions made are critically discussed. It is pointed out that incorporation of labelled amino acid into tissue protein will depend on the rates of blood-flow, of cell penetration and of protein synthesis.

8. Making certain assumptions, it was calculated that approx. 1·9 g. of protein are synthesized in the liver/day/kg. rabbit. About 40% of this total synthesis is concerned with plasma proteins and 60% with liver proteins proper.

9. The low incorporation of labelled glycine into muscle proteins is caused in part by the low rate of penetration of glycine into muscle; however, it is concluded that the rate of protein synthesis in muscle is much smaller than in liver, although no quantitative deductions can be made.

The authors wish to thank Dr G. Popják for his invaluable help and advice on radioactivity measurements using solid samples of 'infinite thinness', Dr J. E. S. Bradley for help with the use of the automatic counter, and Dr J. J. Scott, with whom we discussed many of the theoretical problems in this work.

REFERENCES

Abdou, I. A. & Tarver, H. (1951). *J. biol. Chem.* **190**, 769.

Anderson, E. I. & Mosher, W. A. (1951). *J. biol. Chem.* **188**, 717.

Åquist, S. E. G. (1951). *Acta chem. scand.* **5**, 1065.

Arnstein, H. R. V. (1954). *Advanc. Protein Chem.* **9**, 1.

Arnstein, H. R. V. & Neuberger, A. (1951). *Biochem. J.* **50**, 154.

Arnstein, H. R. V. & Neuberger, A. (1953). *Biochem. J.* **55**, 271.

Barcroft, H. & Swan, H. J. C. (1953). *Sympathetic control of human blood vessels*, 1st ed. London: Arnold.

Barton, A. D. (1951). *Proc. Soc. exp. Biol., N.Y.*, **77**, 481.

Borsook, H., Deasy, C. L., Haagen-Smit, A. J., Keighley, G. & Lowy, P. H. (1950). *J. biol. Chem.* **187**, 839.

Brunish, R. & Luck, J. M. (1952). *J. biol. Chem.* **198**, 621.

Campbell, P. N. & Work, T. S. (1952). *Biochem. J.* **52**, 217.

Christensen, H. N., Cooper, P. F., Johnson, R. D. & Lynch, E. L. (1947). *J. biol. Chem.* **168**, 91.

Dovey, A., Holloway, R. C., Piha, R. S., Humphrey, J. H. & McFarlane, A. S. (1954). *Proc. 2nd Radioisotope Conference*, Oxford, **1**, 337. Ed. J. E. Johnston. London: Butterworth.

Dubreuil, R. & Timiras, P. S. (1953). *Amer. J. Physiol.* **174**, 20.

Fager, E. W. (1947). Report of a Symposium on the 'Use of Isotopes in Biological Research', Chicago, 3–4 March. Quoted from Calvin, M., Heidelberger, C., Reid, I. C., Tolbert, B. M. & Yankwich, P. F. (1949). *Isotopic Carbon*, p. 107. New York: John Wiley.

Fisher, R. B. (1954). *Protein Metabolism*, 1st ed. p. 117. London: Methuen.

Fodor, P. J., Miller, A., Neidle, A. & Waelsch, H. (1953). *J. biol. Chem.* **203**, 991.

Friedberg, F. & Greenberg, D. M. (1947). *J. biol. Chem.* **168**, 411.

Friedberg, F., Tarver, H. & Greenberg, D. M. (1948). *J. biol. Chem.* **173**, 355.

Grayson, J. & Johnson, D. H. (1953). *J. Physiol.* **120**, 73.

Green, H. D., Lewis, R. D., Nickerson, N. D. & Heller, A. L. (1944). *Amer. J. Physiol.* **141**, 518.

Greenberg, D. M. & Winnick, T. (1948). *J. biol. Chem.* **173**, 199.

Gregory, P. W. & Goss, H. (1939). *Growth*, **3**, 159.

Grindley, J. H., Herrick, J. F. & Mann, F. C. (1941). *Amer. J. Physiol.* **132**, 489.

Harkness, R. D., Marko, A. M., Muir, H. M. & Neuberger, A. (1954). *Biochem. J.* **56**, 558.

Hopkins, F. G. (1929). *J. biol. Chem.* **84**, 269.

Hultin, T. (1950). *Exp. Cell Res.* **1**, 376.

Humphrey, J. H. & McFarlane, A. S. (1954). *Biochem. J.* **57**, 186.

Keller, E. B., Zamecnik, P. C. & Loftfield, R. B. (1954). *J. Histochem. Cytochem.* **2**, 378.

Krol, S. (1952). *Biochem. J.* **52**, 227.

Krueger, R. (1949). *Helv. chim. acta*, **32**, 238.

Krueger, R. (1950). *Helv. chim. acta*, **33**, 429.

Leblond, C. P. & Stevens, C. E. (1948). *Anat. Rec.* **100**, 357.

Linzell, J. L. (1953). *J. Physiol.* **121**, 390.

McMinn, R. M. W. (1954). *J. Anat., Lond.*, **88**, 527.

Miller, L. L., Beale, W. F., Yuile, C. L., Masters, R. E., Tishkoff, G. H. & Whipple, G. H. (1949). *J. exp. Med.* **90**, 297.

Miller, L. L. & Beale, W. F. (1954). *J. exp. Med.* **99**, 125.

Miller, L. L., Bly, C. F. & Beale, W. F. (1954). *J. exp. Med.* **99**, 135.

Neuberger, A. (1952). *Brit. med. Bull.* **8**, 210.

Neuberger, A., Perrone, J. C. & Slack, H. G. B. (1951). *Biochem. J.* **49**, 199.

Peters, T. & Anfinsen, C. B. (1950*a*). *J. biol. Chem.* **182**, 171.

Peters, T. & Anfinsen, C. B. (1950*b*). *J. biol. Chem.* **186**, 805.

Popják, G. (1950). *Biochem. J.* **46**, 560.

Reiner, J. M. (1953*a*). *Arch. Biochem. Biophys.* **46**, 53.

Reiner, J. M. (1953*b*). *Arch. Biochem. Biophys.* **46**, 80.

Sakami, W. (1948). *J. biol. Chem.* **176**, 995.

San Pietro, A. & Rittenberg, D. (1953). *J. biol. Chem.* **201**, 445.

Schoenheimer, R., Ratner, S. & Rittenberg, D. (1939). *J. biol. Chem.* **130**, 703.

Schoenheimer, R. & Rittenberg, D. (1940). *Physiol. Rev.* **20**, 218.
Shemin, D. (1946). *J. biol. Chem.* **162**, 297.
Shemin, D. & Rittenberg, D. (1944). *J. biol. Chem.* **153**, 401.
Shemin, D. & Rittenberg, D. (1946). *J. biol. Chem.* **166**, 621.
Snoke, J. E. & Bloch, K. (1952). *J. biol. Chem.* **199**, 407.
Solomon, A. R. (1949). *J. clin. Invest.* **28**, 1297.
Sprinson, D. B. & Rittenberg, D. (1949). *J. biol. Chem.* **180**, 715.
Tarver, H. & Schmidt, C. L. A. (1942). *J. biol. Chem.* **146**, 69.
Ussing, H. H. (1943). *Acta physiol. scand.* **5**, 335.
Waelsch, H. & Rittenberg, D. (1941). *J. biol. Chem.* **139**, 761.
Waelsch, H. & Rittenberg, D. (1942). *J. biol. Chem.* **144**, 53.
Wakin, K. G. & Mann, F. C. (1953). *Proc. Mayo Clin.* **28**, 218.
Winnick, T., Friedberg, F. & Greenberg, D. M. (1948). *J. biol. Chem.* **175**, 117.
Zilversmit, D. B., Entenman, C. & Fishler, M. C. (1943). *J. gen. Physiol.* **26**, 325.

Some Non-protein Constituents of the Tissues of the Lobster

By W. O. KERMACK, H. LEES and J. D. WOOD
Department of Biological Chemistry, University of Aberdeen

(*Received* 24 *December* 1954)

The object of this work has been to obtain information about some non-protein constituents, more especially the amino acids, of muscle, hepatopancreas, and blood of the lobster (*Homarus vulgaris*). Some preliminary results were included in an earlier communication (Fraser, Kermack, Lees & Wood, 1952).

MATERIALS AND METHODS

Removal of tissue. The lobster was killed by a spike inserted below and just behind its head; the blood that dripped from the wound was collected in a beaker. When the flow of blood had ceased, the shell was cut open and the tail muscle and hepatopancreas removed.

Extraction with ethanol. The tissue was macerated in an Atomix homogenizer in 4 vol. 96% (v/v) ethanol for 5 min. at 12000 rev./min. The suspension was kept at room temperature overnight and then filtered on a Büchner funnel. The ethanol was removed as far as possible from the filtrate by distillation under reduced pressure at 60°. Carotenoid material was extracted by light petroleum, b.p. 60–80°.

Extraction with water. The tissue was macerated with an equal volume of water for 5 min. in an Atomix homogenizer. After standing for a further 5 min. the suspension was treated with an equal volume of 10% (w/v) trichloroacetic acid and the precipitated proteins were filtered off.

Water and ethanol both extracted non-protein nitrogenous constituents of lobster muscle and hepatopancreas. Successive extractions with both water and ethanol were carried out on muscle samples from the same lobster, and the total N of the individual extracts was measured (Table 1). If a sufficient number of extractions are carried out, the same total amount of nitrogenous material is brought into solution by both ethanol and water, but extraction with water is more rapid. Analyses, unless otherwise indicated, were made on solutions obtained by combining the filtrates from two successive aqueous extracts.

Autolysis. In order to study the effects of autolysis, lobster muscle or hepatopancreas (25–50 g.) was cut into small pieces and incubated in presence of 1 ml. toluene in a stoppered flask.

Blood. Lobster blood very rapidly congeals on shedding. The clot was extracted with ethanol in the same way as muscle. The extract from the blood clot was desalted by passing it through a column of sulphonated polystyrene resin and eluting the amino acids from the column with N-NH_4OH. The displaced material was concentrated under reduced pressure and the solution chromatographed.

Table 1. *The nitrogenous material removed from lobster muscle and hepatopancreas by successive aqueous and ethanol extractions*

All figures are given in mg./100 g. wet muscle.

Extraction no.	Total N	
	Ethanol	Water
1	500	644
2	176	105
3	54	28
4	20	8
5	18	3
6	7	—
Total	775	788

Chromatographic solvents. Phenol (400 g.) was treated with 100 ml. water. A solution of 0·5 N ammonia was placed in the tank, the atmosphere of which was saturated with coal gas. Collidine–water (3:1, by vol.) and *n*-butanol–water–glacial acetic acid (5:4:1, by vol.) were used.

Two-dimensional paper chromatograms of muscle and hepatopancreas aqueous extracts were run in phenol and collidine, and the relative amounts of the individual amino acids were estimated approximately by visual examination of the size and intensity of the spots.

The procedure for ion exchange chromatography was that described by Partridge & Brimley (1952), using a sulphonated polystyrene resin; displacement was carried out by 0·075 N-NaOH. Fractions from the columns were examined for amino acids by one-dimensional chromatograms run in phenol or butanol–water–acetic acid.