important light on the purity of the biochemical specimen.

Against these advantages has to be set the fact that the apparatus described requires the close attention of a skilled operator; unlike solid-sample counting, the technique does not lend itself to automatic operation.

SUMMARY

A technique is described for the assay of ^{14}C using carbon dioxide. The gas obtained by micro-analytical combustion is purified by contact with a hot copper filament and its radioactivity assayed in the proportional region without admixture.

We owe the construction of the counting tubes and of several gas lines to the skill of Mr D. Hart, whose services we gladly acknowledge.

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Fate of Homologous Antibody in the Rabbit

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In the preceding paper (Humphrey & McFarlane, 1954) it was shown that ^{14}C -labelled antibody and ^{14}C -labelled normal γ -globulin disappear from the circulation of the rabbit at similar rates. Evidence was produced to support the view first put forward by Heidelberger, Treffers, Schoenheimer, Ratner & Rittenberg (1942) that antibody molecules in the circulation do not exchange their constituent amino acids, and it was suggested that normal globulin molecules probably also do not do so. Antibody molecules may therefore be permissible tools for use in the study of the ultimate fate of plasma globulins, the important advantage being that they can be identified and recovered from the tissues by means of specific precipitation.

The fate of transfused homologous whole-plasma proteins labelled with [^{14}C]lysine has been studied by Yuile, Lamson, Miller & Whipple (1951) in dogs. Although these workers were able to account for a high proportion of the original activity transfused, their results do not reveal how much of the radioactivity found in the tissues was in the form of unchanged transfused protein and how much was represented by newly formed tissue proteins. In addition, they did not distinguish between labelled intracellular protein and labelled plasma protein in the lymph. By transfusing ^{14}C -labelled antibody globulin and using specific antigen-antibody precipitation, it may be possible to determine how

much of the labelled protein found in the tissues is unchanged antibody. Furthermore, if Evans Blue dye (T 1824) is injected at least 12 hr. before death, the dye content of the perfused organs provides an estimate of the maximum amount of residual lymph protein—with certain reservations which are discussed below.

There follow results of a study of this kind on a rabbit killed 8 days after receiving a transfusion of [^{14}C]phenylalanine-labelled plasma globulins taken from another rabbit immunized against the capsular polysaccharide of pneumococcus type III. It would obviously be preferable to use pure labelled antibody, but since it was impracticable to separate pneumococcus antibody from other γ -globulins in sufficient amount we were obliged to use the total γ -globulins (called hereafter ^{14}C -labelled globulin) from the plasma of an immunized rabbit. Thus, although all the transfused material had a radioactive label, only 40% was labelled serologically. We assume throughout that the ratio of ^{14}C -labelled antibody to ^{14}C -labelled normal globulin is not altered during the passage of the transfused proteins throughout the body fluids of the recipient, and that both antibody and normal globulin are broken down and/or incorporated into tissue protein at the same rate. It is a corollary of this assumption that when labelled antibody is extracted from tissues by specific precipitation, a proportionate

amount of radioactivity due to transfused labelled normal globulin is left behind, and this has to be taken into account in estimating the true radioactivity of the tissue carbon.

METHODS

[¹⁴C]Phenylalanine-labelled antibody-containing globulins.

The preparation of these has been described in the preceding paper (Humphrey & McFarlane, 1954), the mixture comprising 40% specifically precipitable antibody with activity 6320 counts/min./mg. C and 60% other globulins giving 4840 counts/min./mg. C. β -Globulins were less than 5%, and radioactive free amino acids were absent.

Estimation of radioactivity. Radioactivity was measured by combustion to CO₂, and counting in the gas phase as described by Bradley, Holloway & McFarlane (1954). An advantage of the method for the present work lies in the fact that it furnishes also the carbon content of the dried sample and therefore permits specific activity values to be related to the organic matter in the material (assumed to contain 50%, w/w, carbon).

Estimation of antibody. The amounts of antibody present in the various organ samples were too small to allow direct precipitation from the supernatant fluids obtained after disintegration of the tissues, especially because of the particulate nature of the tissue extracts. An 'antibody dilution' method was therefore adopted in which a known amount of inactive carrier antibody was added to the tissue, and the amount of radioactive antibody estimated by measurement of the radioactivity in the mixed precipitate. After disintegration of the tissues, as outlined below, a solution of inactive antibody containing roughly 1 mg. specifically precipitable material/g. wet wt. of tissue was added and stirred well in. The antibody was in the form of a γ -globulin concentrate prepared from a hyperimmune serum by the method used to prepare ¹⁴C-labelled globulins. After addition of carrier antibody, the disintegrated tissues were frozen to -70° and stored overnight at -5°, thawed out and stirred again. By this means carrier should come into equilibrium with any radioactive antibody present. The mixtures were then centrifuged at 2000 g for 2 hr. at 4° and the supernatants removed. Each deposit was stirred with a smaller volume of 0.9% (w/v) NaCl, and the mixture

centrifuged as before. The washed deposits were stored at -5° and the combined supernatants centrifuged for a further period of 2 hr. at 2500 g at 4°, to remove any material which might interfere with subsequent precipitation of the antigen-antibody complex. The supernatants at this stage were clear or faintly opalescent, and were tinged with varying amounts of blue (for use of Evans Blue see below). To them were added solutions of antigen calculated to be in slight excess of optimum proportions for the carrier antibody. After standing overnight at 2°, the precipitates were collected by centrifuging for 15 min. at 2000 g at 4°, and washed twice with 0.9% NaCl solution followed by 50% (v/v) aqueous methanol, absolute methanol and ether. Drying was completed overnight at 37°, and the residues subsequently weighed and used for estimation of radioactivity. The supernatants, from which antibody had been removed, were stored at -5° until they were dried from the frozen state, and later used for estimation of radioactivity and dye content as described below.

Calculation of the amount of radioactive antibody present in the disintegrated tissue was carried out as follows: if *C* mg. of added carrier antibody come into equilibrium with *A* mg. already present, and the specific radioactivities of the originally transfused antibody and of the specific precipitate are *R*^a and *R*^p, respectively, then

$$R^a \times A = R^p(C + A).$$

Hence

$$A = \frac{C \times R^p}{R^a - R^p}.$$

Preliminary control experiments were made in which small quantities of radioactive globulin containing known amounts of antibody were added to the minced tissues of a perfused normal rabbit. The tissues were then disintegrated as in the main experiment, and the procedure described above was followed. The results in Table 1 show that the labelled-antibody content estimated by the antibody-dilution technique agrees with the amount added. Furthermore, when supernatants before precipitation of antibody were treated with anti-ovalbumin serum containing 5 mg. antibody followed by ovalbumin at optimum proportions, the anti-ovalbumin precipitates contained no radioactivity, indicating that precipitation in this antigen-antibody system was specific.

Table 1. Control of recovery of radioactive antibody added to tissues (5 g. wet weight)

Tissue	Radioactive antibody added (mg.)	Carrier antibody added (mg.)	Specific activity of recovered antibody	Calculated amount of radioactive antibody recovered
Kidney	0.69	4.3	861	0.745
	1.31	4.3	1321	1.26
Lung	0.086	6.0	43.2	0.0352
	0.018	6.0	28.7	0.023
	0.018	6.0	20.3	0.0166
Muscle*	0.18	6.0	196	0.16
	0.18	6.0	192.5	0.159
	0.018	6.0	37	0.022

* If the muscle extract remained at 2° for 3-7 days after the first antigen-antibody precipitate had formed, a further precipitate occurred, composed of some unstable muscle proteins. One precipitate was collected and found to have no radioactivity, although radioactive globulin not precipitable with specific pneumococcal polysaccharide was still present in the fluid, demonstrating that non-specific coprecipitation did not occur.

Estimation of Evans Blue in tissues. Evans Blue (T1824, British Drug Houses Ltd., Poole) was used as described below to estimate the amount of residual plasma proteins in the perfused tissues and organs. This dye is known to combine rapidly and completely with plasma albumin (Rawson, 1943) and the amount used was estimated to be approx. 1 mole/mole albumin. Since the disintegrated tissues contained varying amounts of haemoglobin and other haem compounds, and of lipid, it was not possible to estimate the amount of dye present in the usual way by measurement of light absorption at 620 m μ . It was found, however, that the dye could be quantitatively extracted from freeze-dried tissues or supernatant fluids by heating with acid alcohol (6 vol. ethanol and 1 vol. conc. HCl) until the alcohol just boiled, followed by immediate cooling in ice. Such extracts also contained acid haematin as the principal interfering substance. Control experiments on dye solution and on tissue extracts prepared from the organs of perfused normal rabbits showed that, whereas the absorption by the dye at 400 m μ . was only one-third that at 620 m μ ., the absorption of tissue extracts was 12 to 20 times greater at 400 than at 620 m μ . (depending on the tissue). Hence the absorption values at the shorter wavelength were used to correct those at 620 m μ . The dye concentrations in the extracts were compared with that in a freeze-dried sample of serum taken 1 hr. before death. An interval of 25 hr. was allowed between giving the dye and killing the rabbit, since a study of the rate of disappearance of Evans Blue from the plasma in other rabbits (Fig. 1) showed that this interval was

Fig. 1. Disappearance of Evans Blue from rabbit plasma. 40 mg. Evans Blue in 2 ml. 0.9% NaCl solution injected intravenously into a rabbit weighing 2.35 kg. Blood samples of 4 ml. were taken at intervals, avoiding haemolysis, and the clear plasma was diluted 1:40 before measurement of $E_{1 \text{ cm.}}$ at 620 m μ .

sufficient to allow complete mixing of the dyed proteins of the plasma with the proteins in the lymph. The use of a dye bound exclusively to albumin to follow the concentration of γ -globulins (including antibody) in the lymph is justified only if the albumin:globulin ratios in plasma and lymph are similar. The available evidence (see Drinker & Yoffey, 1941) shows that the albumin:globulin ratio in lymph is not more than 10–20% greater than that in the plasma, and this conclusion is supported by results of experiments, which will be published shortly from this laboratory, showing that transfused ^{14}C -labelled homologous albumin and globulin have their specific radioactivities diminished in approximately the same proportion on passing out of a recipient rabbit's plasma into the lymph. Unfortunately, in the experiments to be described here, significant adsorption of dye by certain tissues (notably kidney, liver, spleen, bone marrow and, to a lesser extent, vascular endothelium, gut and lymphoid tissue) was observed. Furthermore, when disintegrated tissues from a perfused normal rabbit were mixed with small amounts of serum taken from a rabbit which had received Evans Blue, some degree of adsorption of the dye on to particulate material occurred, even *in vitro*. This creates a difficulty in determining the *ante mortem* distribution of the dye, which we propose to resolve by considering the total amount of dye as a measure only of the maximum total lymph protein.

Perfusion of rabbit. Blood was removed practically completely in the following way. The rabbit was injected intravenously with 5 mg. heparin, and then the chest was opened under Nembutal anaesthesia and a large cannula tied into the left auricular appendix. The right auricle was removed, and fluid run in at about 150 cm. water pressure through the cannula, while the ventricles were alternately filled and emptied by manual compression so as to imitate the action of the beating heart. 3 l. of Ringer solution were perfused, followed by 2 l. citrate saline, warmed to 37°. After the first 3 l. had been perfused the fluid issuing from the right auricle was practically blood-free. It is probable that some extracellular tissue constituents will have been washed out since lymph flow is quite marked when perfusion with a pulsating pressure is used. After perfusion no blood could be obtained from the cut surfaces of the organs, which appeared to be quite bloodless although stained varying depths of blue.

Disintegration of the tissues. After removal from the animal, the organs were weighed, mixed with crushed ice and stored at 2°. Weighed portions of the soft tissues were coarsely minced, mixed with an equal weight of 0.9% NaCl solution, and ground for 20–30 sec. first in a coarse and then in a fine glass Potter-type homogenizer rotating at 200–300 rev. min.⁻¹ and cooled in ice so that no appreciable rise in temperature occurred during the grindings. The disintegrated tissues were then cooled to –70° and stored overnight at –5° after which they were thawed out and carrier antibody was added as described above. Skin and muscle could not be disintegrated in the homogenizers and, before adding the saline solution, were shattered by repeated banging with a hammer in a stainless-steel block cooled with liquid air.

No attempt is made to define the degree of cell destruction produced by these means, although the combination of grinding with repeated freezing and thawing is generally presumed to break up cells and to release soluble constituents. It is probable that intact mitochondria and larger particles were in the deposits obtained in the subsequent centrifugation (cf. 'Estimation of antibody', above) since the supernatants were clear or only moderately opalescent.

Table 2. *Distribution of radioactivity in the tissues of a rabbit 8 days after receiving ¹⁴C-labelled normal and immune rabbit globins*

Organ	Measured specific activity of carbon in					Distribution of radioactivity in tissues		
	mg. organic matter/g. wet wt.		mg. organic matter/g. total organic matter present as			Total radio-activity*/g. organic matter (8)	Radio-activity due to antibody/g. organic matter (9)	% Total radio-activity due to antibody (10)
	(1)	(2)	Super-natant (3)	Antibody† (4)	Deposit‡ (5)			
Liver	200	590	410	0.117	12.1	8070	370	4.6
Kidney	145	720	280	0.06	11.1	5730	190	3.3
Spleen	125	340	660	0.08	12.9	5423	253	4.7
Lymph glands	120	440	560	0.34	9.5	5900	1070	15.2
Appendix	120	600	400	0.078	11	4046	246	6.1
Ileum	115	730	270	0.071	10.4	5945	250	4.2
Skin	250	950	50	0.2	2.5	2445	630	25.6
Skin + connective tissue	240	970	30	0.23	2.7	2500	725	29
Femoral bone marrow	270	890	110††	0.024	10.5	5750	75	1.3
Adrenal	170	650	350	0.25	11	7215	790	11
Thigh muscle	260	830	170	0.02	2.7	2353	63	2.7
Lung	210	870	130	0.23	9.4	6645	725	11
Heart	210	880	120	0.038	6.1	3287	120	3.6
								6.05
								11.17
								11.03
								4.43
								9.95
								6.05

* Calculated from carbon recovered after combustion, on assumption that C content is 50%. The C content of proteins was found in our experiments to be 46-48%.

† Calculated as described under *Estimation of antibody*. The specific activity of the transfused antibody C was 6320 counts/min./mg. Carrier antibody is not included.

‡ After extraction with acetone to remove lipids.

§ Figures given are for the antibody precipitate obtained after adding carrier antibody. In parentheses are given the weights (mg.) of carrier antibody added to the tissue samples (which were of arbitrary amounts). The proportion of carrier antibody recovered as specific precipitate with antigen from the supernatant fluid of the disintegrated tissues averaged 70%.

|| Expressed as counts/min. Values obtained by summing the products of specific radioactivities and quantities of material present in 1 g. tissue organic matter.

¶ In the globulins originally transfused for every count due to antibody there were 1.17 counts due to normal globulin. Assuming that normal globulin and antibody behaved alike, the percentage of the tissue radioactivity due to transfused antibody + normal globulin is obtained by multiplying by 2.17.

** Derived by compounding specific activities of deposits (column 5) and supernatants (column 6) and disallowing the contributions (in most cases small) of transfused radioactive normal globulin and of unrecovered radioactive and carrier globulin in the deposits.

†† Most of the radioactivity of these samples was accountable as unchanged transfused normal globulin (see Table 3).

‡‡ Owing to its high fat content the solid matter rose to the surface on centrifuging, and only part of the fluid could be separated cleanly. The proportion of supernatant is therefore underestimated.

Table 3. *Corrected tissue-protein specific radioactivities*

The values shown here are derived from the measured values shown in Table 2 by allowing for the fact that the crude deposits and supernatants both contained, in addition to tissue protein, some transfused active globulin and some inactive carrier globulin. The deposits also contained antibody (both radioactive and carrier) which was not recovered in the supernatant.

	Specific radioactivity of carbon in		Total organic matter found in supernatant (%)
	Deposit	Supernatant (after antibody pptn.)	
Liver	11.0	20.0	41
Kidney	10.6	11.0	28
Spleen	12.0	9.5	66
Lymph glands	7.8	9.1	56
Appendix*	10.2	1.8	40
Ileum	10.1	15.0	27
Skin	1.8	10.0	5
Skin and connective tissue	2.0	13.0	3
Femoral bone marrow	10.4	19.3	11†
Adrenal	9.1	16.3	35
Thigh muscle	2.6	14.0	17
Lung	8.3	22.5	13
Heart	6.0	10.0	12

* Supernatant contained a considerable amount of mucus, which may have lowered the specific activity.

† Organic matter in supernatant probably underestimated (see note in Table 2).

Table 4. *Recovery of radioactivity in the tissues of a rabbit 8 days after transfusion of 1 g. homologous [¹⁴C]phenylalanine-labelled γ -globulins*

Organ	Weight (wet) (g.)	Transfused radioactivity (%)
Liver	80	5.0
Kidneys	19	0.56
Spleen	1.3	0.03
Lymph glands	5.0*	0.13
Appendix	10	0.19
Intestine (as ileum)	169	4.35
Skin + connective tissue	360	8.0
Bone marrow (as femoral)	10†	0.61
Adrenals	0.36	0.016
Skeletal muscle (as sartorius)	1200‡	28.6
Lung	8	0.41
Heart	5.5	0.13
Plasma samples during experiment		12.4
Plasma + perfusion fluid at death (including about half the tissue fluid)		22
Total		82.3

* Arbitrary figure—actual wt. used, 1.4 g.

† Arbitrary figure—actual wt. used, 2.7 g.

‡ Estimated by complete dissection of a similar control animal.

PROCEDURE AND RESULTS

The experiment was conducted as follows. 1 g. of [¹⁴C]phenylalanine-labelled globulins, containing 40% (w/w) of antibody, was transfused intravenously to a 2.5 kg. rabbit. The disappearance of antibody from the circulation of this animal is illustrated in Fig. 2 of the previous paper (Humphrey & McFarlane, 1954). An intravenous injection of 30 mg. Evans Blue in 1.5 ml. 0.9% NaCl solution was given at 168 hr. and 25 hr. later, when 80% of the transfused protein had left the plasma and extracellular tissue fluids, the animal was killed and

Fig. 2. Specific activities of carbon (largely protein) in various organs after perfusion. Open columns, specific activity of total tissue carbon; filled columns, specific activity after allowing for contribution of transfused labelled globulins. The dotted line shows the specific activity of the carbon in the plasma free amino acid fraction at death. This fraction was obtained by adsorption of the diffusate from 20 ml. serum on to Zeokarb, followed by washing and elution of the amino acids. The significance of this measurement is considered in the Discussion.

perfused. The organs and tissues were disintegrated and suitable amounts of carrier antibody allowed to equilibrate with the tissue constituents as described. After centrifuging, antibody was precipitated from the supernatants and the total weights and specific radioactivities of the carbon in the supernatants, deposits and antibody precipitates were measured.

The main findings are summarized in Tables 2–5 and in Fig. 2. In the second and third columns of

Table 5. *Evans Blue* in tissues 25 hr. after injection compared with antibody 8 days after injection

The dye content of serum taken 1 hr. before death was 0.11 mg./ml.

Tissue	Naked-eye intensity of staining	mg. dye/g. whole tissue organic matter		mg. dye calculated* to be equivalent to amount of antibody found/g. whole tissue organic matter (cf. Table 2, col. 4)
		Supernatant	Deposit	
Liver	++	0.025	0.099	0.022
Kidney	+++	0.041	0.198	0.011
Spleen	++	0.032	0.198	0.015
Lymph gland	±	0.044	0.075	0.062
Appendix	+	0.012	0.055	0.014
Ileum	± to +	0.014	0.037	0.013
Skin	- or trace	0.0047	0.03	0.037
Skin + connective tissue	- or trace	0.0035	0.018	0.04
Femoral bone marrow	++	0.0018	0.092	0.0042
Adrenal	Trace to ±	0.01	0.03	0.045
Thigh muscle	-	0.0008	0.0015	0.0037
Lung	+±	0.0014	0.104	0.042
Heart	Trace	0	Sample lost	0.0067

* Assuming ratio dye:antibody to be that found in serum 1 hr. before death.

Table 2 will be seen the proportions of cellular organic matter which were present in the deposits and supernatants after the disintegrated tissues had been centrifuged at 2500 *g* for 2 hr., and from them may be obtained an idea of the efficiency of the disintegrating process. The fourth column gives the amounts of radioactive antibody with which the added carrier antibody came into equilibrium, calculated as described above. The fifth and sixth columns show experimental values obtained for the specific radioactivities of carbon in the tissue deposits and supernatants after antibody precipitation. These values include contributions from unchanged radioactive normal γ -globulin not precipitated along with the antibody, and from inactive normal globulin added with the carrier antibody, as well as from antibody not recovered in the supernatant fluid. Data are available to allow for these contributions and the corrected values are shown in Table 3. It is clear that a substantial conversion of transfused radioactive carbon into labelled tissue carbon has taken place.

Fig. 2 shows graphically the distribution of specific radioactivities among the total carbon of the organs before and after allowing for contributions not specifically due to tissue protein. The uncorrected values are remarkably similar to those found by Yuile *et al.* (1951) after transfusion of total serum proteins to the dog, and in most instances the correction for unchanged labelled globulins is quite small.

Table 4 is an analysis of the overall recovery of radioactivity, and again the similarity to a corresponding table by Yuile *et al.* is striking—particularly when the variation in relative size of different organs in the two species is considered.

Unlike Yuile *et al.* (1951) we did not measure the radioactivity in the skeleton, brain, red cells, excreta or expired air, but according to their results the contribution which this represents is less than 10% of the total body radioactivity. Since our overall recovery was 82%, a balance remains to be accounted for which probably arises from failure to recover all radioactive materials from mincing and other processes.

Table 5 compares the amounts of dye recovered from the disintegrated tissues with the amounts of dye which would be expected on the basis of the observed antibody content—assuming that the antibody was present entirely in lymph, and that the relative proportions of antibody and albumin in the lymph were the same as in the serum at death. It is noticeable that where the tissues were visibly blue, the dye in the combined supernatants and deposits is considerably more than the dye equivalent of the antibody. Where the tissues are not stained the amounts of dye found roughly correspond to the antibody content. In any case, the estimated residual lymph proteins are quite insufficient to account for the radioactivity present. The findings are consistent with the view that recognizable antibody of the tissues is largely, if not wholly, present in the lymph, the balance of the injected antibody which enters cells being converted into another kind of protein, while the dye which penetrates at the same time is stored as such.

DISCUSSION

A striking aspect of the results described is the way in which they agree with those of Yuile *et al.* (1951), although these workers used lysine as label instead

of phenylalanine, whole-serum protein instead of an antibody-normal globulin mixture, and dog instead of rabbit. The results demonstrate that the fate of antibody in the rabbit is closely similar to that of serum protein in the dog, thus supporting the view (Humphrey & McFarlane, 1954) that antibody is a permissible substance to use in deducing the rate of elimination and fate of total serum proteins.

Yuile *et al.* claimed that labelled serum proteins were converted into tissue proteins. Their animals were bled out without perfusion, however, and it was not possible to assess the proportion of tissue radioactivity due to residual blood and lymph proteins. When the tissues are perfused, and a maximum value for the lymph proteins in each tissue is assessed on the basis of the residual Evans Blue content (Table 5), it is evident that their radioactivity generally represents only a small proportion of the whole and that the conclusion of Yuile *et al.* is amply justified.

By transfusing labelled antibody and adding inactive carrier antibody to the disintegrated tissues—a technique used by Keston & Dreyfus (1951) to demonstrate labelled-antibody synthesis by tissue slices *in vitro*—we have attempted to find out to what extent the total protein radioactivity of the tissues after 8 days is still in the form of antibody protein. Table 2 shows that only in skin, which is recognized to be rich in extracellular tissue fluid, is the proportion of radioactivity due to recognizable antibody large and it becomes insignificant in the more highly labelled organs viz. liver, kidney, spleen and bone marrow. We conclude that labelled antibody is converted for the most part into some other kind of protein. This conclusion presupposes that the treatment of the disrupted tissue after carrier antibody was added was adequate to bring the inactive antibody into equilibrium with a substantial part of any labelled antibody present. It is necessary to consider experiments of Khesin (1952) showing that extraction of rat-liver cytoplasmic granules with water for 10 min. releases only one-third of the amounts of serum albumin which can be extracted after treatment with butanol and ethanol. We have tried to avoid such a discrepancy by freezing and thawing in the presence of a large excess of carrier antibody. Nevertheless, even though we have shown that small amounts of labelled antibody added to the tissues at mincing can be recovered quantitatively by this means, the possibilities remain that intracellular labelled antibody is in some way isolated from contact with carrier antibody or becomes denatured in the course of disrupting the cells.

The mechanism by which the amino acids of transfused labelled globulins could be incorporated into tissue proteins is of some interest. Yuile *et al.* (1951) inclined to the view that transfused proteins

are ingested as such by a wide variety of cells, and modified by them to form tissue protein, in such a way that their constituent amino acids do not come into equilibrium with the free amino acids of the plasma. We would like to put forward the alternative view that labelled proteins are broken down to free amino acids somewhere in the body, and that the free amino acids, after liberation into the plasma, are used by the tissue cells for protein synthesis.

As was pointed out in the preceding paper (Humphrey & McFarlane, 1954), homologous globulins are not changed so long as they remain in the circulation, and their breakdown to free amino acids therefore requires that they be ingested by certain cells in one or more tissues. Thus both views postulate that plasma proteins can enter tissue cells, but disagree on the number and variety of cells in which this occurs.

The possibility that direct conversion of plasma protein into tissue protein takes place without the intermediacy of free plasma amino acids would be established if it could be shown, in any experiment of the kind described here, that in certain cells or tissues the degree of labelling of tissue-protein phenylalanine exceeded the maximum labelling of the free plasma phenylalanine during the period of the experiment. Since it is necessary to deal with whole tissues, which contain a variety of grosser elements such as connective tissue or resting nuclei whose amino acid turnover is known to be relatively slow, it will probably be among the soluble proteins that any high degree of labelling should be sought. Technically, such a demonstration will always be a difficult matter, since it involves the isolation and measurement of the activity of a free amino acid in plasma at intervals during the experiment and of the same amino acid in tissue proteins obtained post mortem.

As a step in this direction, we took a large blood sample just before killing the animal. After pressure dialysis of 20 ml. serum against water, the amino acids of the diffusate were adsorbed on Zeokarb (cf. Dovey, Holloway & McFarlane, 1952). This was washed to get rid of other labelled metabolites, the total amino acid fraction was eluted, and the specific activity found to be 12.0 counts/min./mg. carbon. The size of our earlier blood samples did not permit similar measurements, and unfortunately there appears to be no direct experimental evidence in the literature on specific activities of free amino acids after transfusion of labelled protein. If we assume that no considerably higher value of plasma free amino acid radioactivity occurred during the preceding 8 days, consideration of Table 3 and Fig. 2 shows that some part at least of the carbon in several organs (e.g. liver, bone marrow and lung) has a higher specific activity than the plasma free

amino acid fraction and could not have arisen from plasma free amino acids. That of some other tissues, however, such as muscle fibre, is much less, and the significance of this is discussed below. Since we know neither the exact proportion of phenylalanine in the plasma free amino acid fraction compared with that in the tissue proteins (collagen, for example, has a very low phenylalanine content) nor the extent to which non-protein or non-amino acid carbon is included in our determinations of specific activities, we may not claim to have substantiated this conclusion. Our results are, however, compatible with the idea that some of the more actively labelled tissues may have used transfused globulins directly for protein synthesis. This view does not necessarily imply that free amino acids were not formed as intermediates, but only that the intracellular amino acid pool was not in immediate (and complete) equilibrium with the plasma free amino acids, and consequently that the breakdown products of ingested plasma protein were used preferentially for protein synthesis by these cells.

If we consider the tissues in which part of the carbon has a radioactivity substantially lower than that of the plasma free amino acid fraction, it is permissible to conclude (while still remembering the qualifications given above) that this labelled carbon could have been derived from plasma free amino acids. Before doing so, however, attention must be paid to the turnover rate of the proteins involved, since a slow turnover rate might lead to a low degree of labelling at 8 days whatever the operative mechanism. In the case of muscle fibre, for example, it has been found (private communication from Dr A. Neuberger) that in rabbit muscle, radioactivity of combined glycine is still only 20 % of that of the free muscle glycine at 18 hr. after injection of [^{14}C]glycine, and the turnover rate must therefore be slow. Provided that maximum radioactivity of muscle glycine is attained inside 8 days, we feel justified in inferring from Fig. 2 that the labelling of this tissue could have come predominantly or even exclusively from the re-incorporation of labelled free amino acids of plasma. Similar considerations may well apply to the other poorly labelled tissues, e.g. skin.

Those tissues which showed high specific radioactivities were also visibly stained with Evans Blue, while perfused muscle was quite colourless. The dye was also given to another rabbit 145 hr. before killing and perfusing, and, as anticipated, the liver, kidney and spleen were deeply stained and the organs contained 2-3 times more dye/g. dry weight than the same organs 25 hr. after giving the dye. Perfused muscle, however, was still practically colourless and contained no significant weight of dye. Since at no time is there free dye in the plasma and lymph, it is almost certain that the dye enters

the tissues in association with plasma protein and that the amount in the perfused tissue after allowing for that contained in residual lymph reflects the uptake of protein. The fact that none is taken up by muscle at the end of a week when the protein specific radioactivity is nearly one-third that of liver, kidney or spleen suggests strongly that the radioactivity of this and some other relatively inert tissues is mainly the result of re-incorporation of labelled amino acids liberated into the plasma by the steady breakdown of labelled protein elsewhere.

Although Keppie & Macfarlane (1948) found traces of antibody to *Clostridium welchii* in washed and perfused muscle from actively immunized animals, our calculations show that the amount found can be attributed to extracellular fluid remaining in the muscle. Skin occupies a peculiar position in providing such a small proportion of its total organic matter in the supernatant after disintegration (Table 2) and in having such a high proportion of its total radioactivity attributable to lymph protein (Fig. 2). This is presumably because it consists largely of structural fibrous protein, but nevertheless occludes a considerable amount of lymph.

Table 5 shows that in no tissue was recognizable antibody found in marked excess of that attributable to the contained lymph. It is important to bear in mind, as already stated, that our estimates of antibody content are valid only if the method of extraction from disintegrated tissues was reasonably quantitative, and that the estimates of lymph protein are maximum figures. If these assumptions are valid, it appears that homologous transfused antibodies (pneumococcus antibodies, at any rate) do not reside to any great extent, or for any length of time, unchanged within the tissue cells. There is, of course, one class of cells within which all plasma proteins may be expected to be found, namely the vascular endothelium, through which the proteins effectively pass on their way in and out of the lymph. In this case, however, comparison of recoverable antibody with Evans Blue would give no clue as to whether the antibody was within the cells or not.

SUMMARY

1. The distribution of radioactivity in the tissues of a rabbit killed and exsanguinated by perfusion 8 days after receiving a transfusion of [^{14}C]phenylalanine-labelled rabbit pneumococcus antibody has been studied, and the radioactivity found to be predominantly still in the form of labelled protein.

2. By extracting the disintegrated tissues with carrier antibody and by measuring the amounts of residual lymph by means of Evans Blue injected before death it is shown that practically all the precipitable radioactive antibody could be ac-

counted for in residual lymph, implying that the remainder of the radioactivity present was in the form of protein which had lost its original specific immunological configuration.

3. It is concluded that transfused antibody entered cells in certain organs which became highly labelled, notably in liver, kidney, spleen, bone marrow, lung and gut, without being broken down to the amino acid level, and was rapidly changed into some other kind of intracellular protein. The level of radioactivity in the plasma free amino acid fraction at death, however, was high enough to be consistent with the idea that the protein radioactivities of other poorly labelled tissues, notably muscle, could be due to incorporation of labelled free amino acids.

4. Evans Blue uptake may be a convenient guide to plasma protein assimilation by tissues. This dye is found in high concentration in the highly labelled tissues and hardly at all in the poorly labelled ones, supporting the view that the cells of the latter are impervious to proteins.

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Some Properties of Creatine Phosphokinase

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The enzyme creatine phosphokinase responsible for catalysing the reaction

was first described by Lohmann (1934), who showed that in extracts of skeletal muscle the two labile phosphate groups of adenosine triphosphate were transferred to creatine. The reversibility of the reaction was demonstrated by Lehmann (1935), and it was claimed by both of these authors that the dephosphorylation of adenosine triphosphate proceeded to adenylic acid with the result that 2 molecules of phosphocreatine were formed. Banga (1943) effected a partial purification of the enzyme, and the preparation was able to transfer only the terminal phosphate group of adenosine triphosphate to creatine with the formation of phosphocreatine. In addition, Banga (1943) claimed that another enzyme was responsible for the transfer of phosphate from

adenosine diphosphate to creatine. This was named adenosine diphosphate-creatine phosphotransferase in contrast to adenosine triphosphate-creatine phosphotransferase. Later Soreni & Degtyar (1948) achieved a partial purification of creatine phosphokinase and described certain of its properties. Recently Askonas (1951) has described a purification technique depending upon propanol fractionation. The properties of creatine phosphokinase present in cerebral cortical tissue were described by Narayanaswami (1952), but in this work no attempt was made to effect any purification. The present communication is concerned with a more detailed investigation into the properties of creatine phosphokinase than has hitherto been reported.

Abbreviations

The following abbreviations will be used: ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine 5'-phosphate; PC, phosphocreatine; CPK, creatine phosphokinase; THAM, aminotris(hydroxymethyl)methane.

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