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Methods for Measuring Rates of Synthesis of Albumin by the Isolated Perfused Rat Liver

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Although the ability of the isolated perfused rat liver to synthesize plasma proteins was clearly demonstrated in 1951 by Miller, Bly, Watson & Bale, few such experiments have subsequently been reported. The potential use of this system for studies on protein synthesis was, however, recognized by Jensen & Tarver (1956). These authors added ^{14}C-labelled histidine and lysine to the blood used for perfusing a liver, and then measured the specific activity of the free amino acids in the plasma at regular intervals. With these data, and by measuring the corresponding amounts of the [^{14}C]amino acid which had become protein-bound, they were able to calculate a rate of protein synthesis.

However, their measurements of the specific activities of the free amino acids in the plasma, which were obtained by means of the bacterial decarboxylase method, may have been subject to considerable experimental error. For this reason it seemed worth while to repeat some of their measurements with the aid of a more accurate estimation for the specific activity of the free amino acids in the plasma.

The results thus obtained could be compared with the rate of albumin synthesis *in vivo* found by Campbell, Cuthbertson, Matthews & McFarlane (1956). Since it seemed possible that the perfused liver may on average synthesize albumin either faster or slower than it does *in vivo*, it seemed desirable also to measure albumin synthesis directly in the perfusion system. To achieve this, isolation of the newly formed albumin was necessary. Newly formed rat albumin cannot be separated from the rat albumin initially present in the system when the perfusion is conducted with rat blood. Thus the expedient of perfusion with a heterologous blood became necessary. Human blood from which most of the white cells and platelets had been removed proved satisfactory for liver perfusions and permitted the isolation of newly formed rat albumin by means of an appropriate antiserum. Although such a system suffers from certain disadvantages mentioned below, its use has permitted direct comparison between rates of synthesis of albumin calculated from changes in plasma-amino acid specific activity and from the amount of albumin obtained by isolation in the same experiment.

METHODS

Perfusion technique. Perfusions were carried out as described by Cohen & Gordon (1958), except for the use of human blood in certain cases. Carrier lysine was used in all experiments except rat-liver perfusion (RLP) 87 (for amounts see Table 1). When human blood was used the white cell and platelet layer, which separated from the heparinized blood during preliminary centrifuging at 1800 *g* for 20 min. at 20°, was removed as completely as possible by suction and the plasma was re-mixed with the red cells. Warm Ringer's solution was allowed to flow through the liver for 1–2 min. before its removal from the

donor rat, in order to remove as much as possible of the rat blood initially present.

Injection of liver-donor rat with rat [^{131}I]*albumin.* In order to estimate the quantity of rat albumin remaining in the liver, despite the preliminary perfusion with Ringer's solution, rat [^{131}I]albumin (approx. 5 mg. containing 150 μc of ^{131}I) was injected into the donor rat on the day before the perfusion experiment. It was assumed that by the time the liver was removed all extracellular albumin present would have become mixed with ^{131}I-labelled albumin to the same extent as that in the serum, thereby enabling it to be recognized and measured. The principle involved here was used by Humphrey & Sulitzeanu (1958) for isolation of intracellular antibody from the tissues of hyperimmunized rabbits. Labelling with ^{131}I was carried out by the ICl method of McFarlane (1958) at a level of not more than 1 atom of iodine/molecule of albumin.

Isolation and estimation of rat albumin. This was carried out by precipitation with an antiserum, prepared by injection of purified rat albumin into rabbits, and containing about 3 mg. of anti-rat albumin/ml. Since the original antiserum precipitated some of the α-globulins as well as the albumin it was pretreated by addition of rat α-globulin (60 mg./100 ml.). This material was kindly prepared by Dr S. Cohen by chromatography on carboxymethylcellulose prepared according to Peterson & Sober (1956). A portion (5 ml.) of the pretreated antiserum was added to a sample (2 ml.) of the human plasma obtained at the end of the perfusion. Sodium ethylenediaminetetra-acetate (0·4 ml. of 1% solution), to prevent coagulation, carrier lysine (10–20 mg.) and heparin (0·05 ml., 250 i.u.), were also present. After 2 hr. at 37° the precipitate was removed on the centrifuge and washed three times at 2° with 0·9% NaCl. It was decomposed, and the albumin was recovered in solution, by addition of 2 ml. of 1% trichloroacetic acid in aq. 96% (v/v) ethanol (Delaville, Delaville & Delaville, 1954; Schwert, 1957). The albumin-containing solution was dialysed against water for 48 hr. in Visking dialysis tubing which had previously been soaked and rinsed in water to remove preservatives. The recovered rat albumin was freeze-dried and redissolved in 1 ml. of water. From this solution samples were taken for estimation of protein by the method of Lowry, Rosebrough, Farr & Randall (1951) and measurement of ^{131}I-radioactivity and of ^{14}C-radioactivity after combustion to CO_2 (Bradley, Holloway & McFarlane, 1954). The efficiency of this method for the recovery of pure rat albumin was tested by adding ^{131}I-labelled rat albumin of known specific radioactivity to human plasma, recovering the albumin in the way described and comparing the specific radioactivity of the recovered material with that of the original. Since rat α-globulin was the most probable contaminant unlabelled α-globulin was also added to the mixture. The procedure was as follows: 2·37 mg. of rat [^{131}I]albumin + 2 mg. of rat α-globulin were added to 2 ml. of human plasma. This was mixed with 15 ml. of rabbit antiserum against rat albumin, containing heparin (0·1 mg./ml.) and left overnight at 2°. The amount of antibody present was sufficient to precipitate 5 mg. of rat albumin, but the antiserum also contained some antibodies against other rat-plasma proteins. After washing the antigen–antibody precipitate twice on the centrifuge with 15 ml. of cold 0·9% NaCl 96% of the radioactivity was recovered in the complex. The washed precipitate was stirred with 6 ml. of 1% (w/v) trichloroacetic acid in 96% (v/v) ethanol at 2° and the undissolved protein separated after 10 hr. by centrifuging; the precipitate was extracted with a further 2 ml. of trichloroacetic acid–ethanol mixture, and the clear supernatants were combined. To this solution, which contained 35% of the radioactivity of the original antigen–antibody precipitate, was added 2 vol. of ether at 2°, and albumin precipitate, which formed in a few minutes, was separated by centrifuging. It was dissolved in 1 ml. of water containing a drop of M-$NaHCO_3$, and samples were taken for estimation of protein radioactivity. The specific radioactivity of the recovered rat albumin was 86% of that of the starting material, measured at the same time. This indicates that the rat albumin recovered by this technique was largely, but not quite, free from contaminating protein. The conditions of the experiment were designed to be rather more exacting than in the actual liver-perfusion experiments, since the ratio of rat α-globulin to albumin was several times higher than that in normal plasma, and the technique was judged sufficiently specific to warrant its use in the work described below.

Estimation of [^{14}C]*lysine specific activity.* Samples of plasma of 2–10 ml. (depending on the expected lysine concentration) were stirred with 2 vol. of 10% trichloroacetic acid and centrifuged. The whole of each supernatant, less 0·5 ml. which was kept for amino acid estimation by the ninhydrin method (Jacobs, 1956), was allowed to run through a 0·3 cm. × 7·0 cm. column of Zeo-Karb 225 (4½% cross-linked, passing 200 mesh). The resin was pretreated with hot NaOH and HCl according to Moore, Spackman & Stein (1958), and was used in the acid form after extensive washing with water. After removal of all the trichloroacetic acid from the column by passage of water the amino acids were eluted with 5 ml. of aq. 3N-NH_3 soln. Complete removal of the NH_3 was next carried out by repeated evaporations on the steam bath. Finally the solid material was dissolved in approx. 0·05 ml. of water and transferred, as completely as possible, to a 3 cm. wide strip of Whatman no. 3 MM filter paper arranged for paper electrophoresis. Because such paper may contain large quantities of material reacting with ninhydrin, the strips were prewashed in several litres of dilute Na_2CO_3 solution, water and finally 0·01 M-Na_2CO_3; 0·01 M-Na_2CO_3 was also used as the buffer for the subsequent paper electrophoresis. The strips were not dried before use for the paper electrophoresis [which was carried out for 21 hr. at 500 v in the apparatus described by Flynn & de Mayo (1951)]. Next the strips were dried at a temperature not exceeding 100° and scanned for radioactivity. Adequacy of separation of lysine and arginine was checked by means of the ninhydrin colour which was developed on control strips. Possibly because very dilute Na_2CO_3 was used, the absolute positions of the amino acids varied considerably, but the relative positions of lysine and arginine remained reasonably constant. It was therefore possible to cut off a part of each strip containing only lysine. Care was necessary, however, to obtain cuts containing only lysine, since not all of the radioactivity appeared in the lysine band (see below). Elution of the lysine from the selected part of each strip yielded solutions which were divided for ^{14}C-counting and amino acid estimation. Because volumes of less than 0·1 ml. were sufficient for accurate estimation of radioactivity, and because it was necessary to add carrier carbon for this purpose, the solutions were allowed to soak into small pieces of filter paper placed ready in the platinum boats.

Care was taken during the ^{14}C estimations to avoid loss of $^{14}CO_2$, so that the count obtained, appropriately corrected for transfer losses, represented the whole of the radioactivity in the sample. The values for total radioactivity thus obtained, divided by the amounts of lysine present, gave the specific activity of the free lysine in each plasma sample. The accuracy of this method for measurement of lysine specific activity was tested by isolation of [^{14}C]lysine from a synthetic mixture of amino acids including arginine, sufficient lysine to give amounts of approx. 50 μg. of amino acid for each ninhydrin estimation being employed. The mean specific activity of the isolated [^{14}C]lysine thus found was $95 \pm 4{\cdot}8$ % of the original sample.

Extraction of intracellular lysine. Immediately after perfusion the livers were chopped coarsely with scissors and rapidly frozen in liquid air. After transfer to a cooled mortar and addition of 25 ml. of 20% trichloroacetic acid the liver was ground thoroughly as soon as the rising temperature permitted. Contamination with extracellular lysine was neglected in view of the much higher concentration of all amino acids within the liver cells than in lymph or plasma.

Estimation of radioactivity in proteins. ^{131}I-Radioactivity was measured in a standard volume, in a well-type scintillation counter.

^{14}C-Radioactivity was measured after combustion to CO_2 (Bradley *et al.* 1954), thereby permitting ^{14}C to be measured in samples containing ^{131}I. To ensure removal of non-protein ^{14}C in plasma samples they were first treated as described by Miller *et al.* (1951).

Calculations. The rate of synthesis of albumin has been calculated in three different ways. In method *A* the calculation is based on the specific activity of the ^{14}C-labelled rat albumin isolated from the plasma at the end of each experiment and the total ^{14}C-radioactivity of this plasma. Since the proportion of radioactivity present as albumin was also measured in RLP 90 and in a subsequent experiment (see below) the total ^{14}C present as albumin in the final plasma could be calculated. Division of the total albumin radioactivity by the albumin specific activity (Table 2) gives the amount of newly synthesized albumin together with the amount transferred from the liver donor. This latter quantity can be estimated, since the donor albumin was labelled with ^{131}I, and can be subtracted to give a value for the amount of albumin newly synthesized during the perfusion.

In method *B* (Table 3) the calculation is based on the amount of rat albumin isolated by means of the antiserum, estimated by the method of Lowry *et al.* (1951). Losses occurring during the isolation are measured and corrected by estimation of ^{131}I-radioactivity at each stage, since rat albumin was the only ^{131}I-labelled protein present. As in method *A*, it is necessary to subtract the amount of albumin transferred from the liver donor.

In method *C* (Table 4) the rate of synthesis of albumin has been derived from data for the increases in the ^{14}C-radioactivity of the plasma albumin during each hour of the experiment, together with the average specific activities of the free lysine in the plasma during each corresponding time interval. From these can be calculated the weight of lysine incorporated into plasma proteins during each hour, on the assumption that the plasma-lysine specific activity was the same as the specific activity of the lysine at the site of synthesis. How nearly this assumption is valid is considered below.

RESULTS

Ratio of [^{14}C]*lysine incorporated into albumin and total plasma protein by the perfused liver.* A portion (3 ml.) of the final plasma (human blood) from RLP 90, in which [^{14}C]lysine was present, was subjected to electrophoresis on a column of treated cellulose (Porath, 1954). When the protein was eluted the peak of human albumin (detected by absorption at 280 mμ) was, as expected, somewhat ahead of the peak of rat albumin, which was estimated by scintillation-counting. The fractions corresponding to the u.v.-absorption peaks were pooled and concentrated. Their ^{14}C-radioactivities were measured and protein content was estimated by the biuret method (Gornall, Bardawill & David, 1949). The proportion of the radioactivity appearing in the leading fraction was 60%, which was shown by means of electrophoresis on paper to

Table 1. *Details of individual perfusions*

Blood donors had not been starved before use. [^{14}C]Lysine (uniformly labelled; 25 μc) was added at the start of each perfusion. Bile was collected from the cannulated bile duct in all perfusions except nos. 87 and 92.

			Perfusion blood						
Blood used	Perfusion no.	Liver donor body wt. (g.)	Vol. before sampling (ml.)	Initial haemato-crit value (%)	Initial concn. of plasma protein (g./100 ml.)	Concn. of free amino acid (mg./100 ml. of plasma) Initial	Final	Time perfused after addition of [^{14}C]lysine (hr.)	L-Lysine added (mg.)
Rat	93	290	74	38	7·0	73	138	4·33	7·5
	97	345	75	37	7·3	62	132	4·33	6·8
	1	295	64	40	7·4	—	—	4·33	6·2
	8*	315	72	41	7·2	68	134	4·42	5·6
Human	87	315	50	39	8·8	58	176	3·66	0
	88	360	61	44	9·0	54	177	4·0	6·8
	90	300	72	43	7·9	88	210	4·25	6·8
	92	280	74	44	8·0	80	172	4·25	6·5

* Penicillin and streptomycin sulphate (2·5 mg. each) were added initially.

Table 2. *Calculations of rate of synthesis of albumin from amounts isolated after perfusion with human blood*

Method *A*: not depending on recovery of ^{131}I

Perfusion no.	87	88	90	92
Plasma in circuit minus samples (ml.)	26·6	28·1	26·6	27·5
Final plasma-protein ^{14}C (μc/ml.)	$8{\cdot}41 \times 10^{-3}$	$5{\cdot}87 \times 10^{-3}$	$6{\cdot}50 \times 10^{-3}$	$4{\cdot}73 \times 10^{-3}$
Total protein ^{14}C in circuit, including samples (μc)*	$2{\cdot}41 \times 10^{-1}$	$1{\cdot}84 \times 10^{-1}$	$2{\cdot}12 \times 10^{-1}$	$1{\cdot}57 \times 10^{-1}$
(1) Albumin ^{14}C-radioactivity at 64% of total protein radioactivity (μc)†	$1{\cdot}54 \times 10^{-1}$	$1{\cdot}18 \times 10^{-1}$	$1{\cdot}36 \times 10^{-1}$	$1{\cdot}00 \times 10^{-1}$
(2) ^{14}C specific activity of isolated rat albumin (μc/g.)	3·18	1·96	2·04	2·18
Albumin synthesized (uncorrected), i.e. (1)/(2) (mg.)	48·4	60·0	66·2	46·1
Albumin transferred from liver donor (mg.)‡	11·0	11·6	10·5	10·8
Therefore, by subtraction, albumin synthesized/4 hr. (mg.)	37·4	48·4	55·7	35·3
Albumin synthesized/hr. (mg.)	9·3	12·1	13·9	8·8
Average rate of albumin synthesis (mg./hr.)		11·0		

* No correction has been made for free [^{14}C]lysine removed in samples, on the grounds that it represents a relatively small fraction of the total in the system, including the liver itself.

† See Results section.

‡ Calculated from ^{131}I in donor-rat albumin.

Table 3. *Calculation of rate of synthesis of albumin from amounts isolated*

Method *B*

Perfusion no.	87	88	90	92
Rat albumin in circuit recovered by specific precipitation with antibody and estimated by method of Lowry, Rosebrough, Farr & Randall, 1951 (uncorrected) (mg.)	32·0	44·8	31·4	25·8
Correction factor from ^{131}I recovery	0·552*	0·469	0·575	0·616
Rat albumin in circuit corrected by ^{131}I recovery (mg.)	58·0	95·6	64·6	41·8
Albumin transferred from liver donor (mg.)	11·0*	11·6	10·5	10·8
By subtraction, albumin synthesized/4 hr. (mg.)	47·0	84·0	44·1	31·0
Albumin synthesized/hr. (mg.)	11·7	21·0	11·0	7·8
Average rate of albumin synthesis (mg./hr.)		12·9		

* No ^{131}I label was used in this experiment. The correction factor used is the average of that in the other three experiments.

Table 4. *Example of method of calculating rate of synthesis of albumin from plasma-lysine ^{14}C specific activities and incorporation of lysine ^{14}C into total plasma proteins at hourly intervals (RLP 92; human blood)*

Method *C*

Time (hr.)	1	2	3	4
Protein C in perfusion circuit (mg.) (*a*)	1400	1297	1178	1075
μc of ^{14}C/mg. of C entering all plasma proteins (*b*)	$7{\cdot}10 \times 10^{-5}$	$6{\cdot}55 \times 10^{-5}$	$4{\cdot}82 \times 10^{-5}$	$2{\cdot}73 \times 10^{-5}$
Mean plasma lysine C specific activity during each hour (μc/mg. of C) (*c*)	0·531	0·312	0·159	0·108
Lysine C incorporated (mg.) into all plasma proteins (*ab*)/*c*	0·19	0·28	0·36	0·28
Albumin (mg.) synthesized* $\left(\frac{ab}{c} \times \frac{10 \times 0{\cdot}64 \times 100}{\text{C (\%) in albumin}}\right)$	2·42	3·58	4·62	3·58
Rate of albumin synthesis				
Average over 4 hr. (mg./hr.)		3·54		

* Assuming (*a*) that lysine C composes 10% of all plasma-protein C (this figure is calculated from corresponding figures for human-plasma proteins, taken from Spector, 1956); (*b*) that albumin ^{14}C composes 64% of all plasma-protein ^{14}C (experimental result); (*c*) that the rate of labelling of albumin and of the total plasma proteins is the same.

consist only of albumin. The remaining 40% of the radioactivity was in later fractions containing mostly α- and β-globulin, but also a trace of albumin. In order to allow for the radioactivity of the albumin which had trailed, the percentage of total radioactivity incorporated into albumin was taken as 64%.

Comparison of livers perfused with human and with rat blood. The main information is contained in Tables 1–4. It is evident that the rat liver when perfused with human blood still retains its ability to synthesize albumin and at least some of the plasma globulins. However, those livers which had been perfused for 4 hr. with human blood could

Table 5. *Comparison of rates of synthesis of albumin*

Blood used	Perfusion no.	Average plasma free-amino acid concn. (mg./100 ml.)	Percentage of dose incorporated into all plasma proteins in 4 hr.	Albumin synthesized (mg./hr./300 g. rat): Isolation of newly formed albumin (method *A*)	Albumin synthesized (mg./hr./300 g. rat): Plasma lysine specific activity (method *C*)
Rat	93	105	10·1	—	—
	97	97	12·0 (With samples)	—	—
	1	—	16·1	—	4·8 | 5·0
	8	101	15·2	—	5·2 (average)
Human	87	135	16·1*	9·3	—
	88	133	11·9 (With samples)	12·1	—
	90	149	15·0	13·9	6·4 | 4·9
	92	126	11·3	8·8	3·5 (average)
Rat	Jensen & Tarver (1956)	—	—	—	13·1†

* No extra lysine was added to perfusion.

† Assuming that 64% of the plasma-protein radioactivity at the end of the perfusion is in the form of albumin, and that the mean lysine content of rat-plasma proteins is 10%.

usually not be freed of blood so completely by a final perfusion with 0·9% NaCl as could livers which had been perfused similarly with rat blood. Furthermore, although the average initial total free amino acid level of the human blood used in these experiments was close to that of the rat blood, this level rose considerably higher at the end of the experiments with human blood (cf. Table 1). Despite these differences certain comparisons between the two systems are worth making. The average percentage of the added [^{14}C]lysine which became incorporated into plasma protein was almost the same in the perfusions with rat (13·3%) or human (13·6%) bloods. Comparison of the specific activities of the free lysine of plasmas obtained during perfusion with human and with rat blood reveals no differences attributable to the bloods used. Furthermore, the average rate of albumin synthesis calculated (method *C*) from the specific radioactivities of the free lysine of plasma are very similar for the livers perfused with the two kinds of blood (Table 5). In fact the difference calculated in this same way between RLP 90 and 92, both of which were perfused with human blood, was much greater than the difference between the average for human and the average for rat blood. With only a pair of experiments of each kind this may be accidental, but the ratios between the rates of albumin synthesis in experiments RLP 90 and 92, calculated by methods *A* and *C*, and the ratio of the percentages of the dose of [^{14}C]lysine incorporated into total plasma protein in the same two perfusions, are rather similar.

Rates of synthesis of albumin. The amounts of rat albumin isolated by means of the antiserum from the perfusions with human blood have been calculated in Tables 2 and 3 by methods *A* and *B* given above. Assuming that the albumin isolated was pure (the impurities amounted almost certainly to less than 10%; see under Methods) the accuracy of these calculations should depend only on the accuracy of the methods for estimation of (1) ^{14}C-radioactivity (Bradley *et al.* 1954), (2) protein concentration of the isolated albumin (Lowry *et al.* 1951), (3) ^{131}I-radioactivity, together with (4) the estimate of the proportion of ^{14}C occurring as albumin in the final plasma.

The calculated amounts of albumin synthesized in perfusions 87, 88, 90 and 92 calculated by methods *A* and *B* are more variable by method *B* than *A*. It seems likely that the figures obtained by method *A*, which are based on direct measurements of total ^{14}C-activity in the perfusing blood, are the more accurate. In any case the average rates of albumin synthesis calculated by the two methods are in substantial agreement (cf. Tables 2 and 3).

The specific activities of the free lysine of plasma measured at various times during four of the liver perfusions are shown in Fig. 1. These values have been used to calculate the rate at which lysine is incorporated into plasma albumin, and so to estimate the rate of albumin synthesis, with the results shown in Table 5. In perfusions 90 and 92 the average rate thus calculated is approximately two-fifths of that obtained by the method of isolating the albumin. As mentioned above, calculation by method *C* takes no account of any dilution of the [^{14}C]lysine with the unlabelled lysine which is continuously produced from preformed proteins inside the liver cell. The true specific activity of intracellular lysine is difficult to measure with certainty, both because the liver must first be freed of blood, which process may itself

alter the intracellular-amino acid specific activity, and also because any abnormality of environment or partial damage to the liver may accelerate catabolism of all kinds of intracellular proteins. As shown by Fig. 1, the specific activity of free lysine in plasma varies considerably from one perfusion to another. Measurements of intracellular-lysine specific activity were at least as variable; an average of six experiments gave a value 55% (range 17–73%) of the corresponding average for the specific activity of free lysine in plasma. If the rates of albumin synthesis calculated by method *C* are corrected to allow for intracellular-lysine specific activities half those found in the plasma, values more nearly in agreement with, but still lower than, those obtained by isolation are obtained.

Appearance of lysine-degradation product during perfusion. Rather surprisingly the paper-electrophoresis method used for the separation of lysine from the other amino acids present in the plasma demonstrated the presence among the bases of a radioactive substance not corresponding with either lysine or arginine. The amount of this substance in the plasma increased during the course of each perfusion. Under the conditions for electrophoresis used its mobility was sufficiently greater than that of lysine to permit nearly complete separation. In most perfusions, even after 4 hr., the amount present did not exceed 20% of the total radioactivity of the isolated lysine. Attempts to detect such a component in the liver itself were unsuccessful. Some further data on the properties of this substance, which suggest that it may be an amine, will be presented in a separate paper. Whether it is formed by the liver itself or by the anaerobic bacteria usually found in this organ in the rat is not known. The latter possibility seems unlikely, since the material was formed in perfusion 8, to which both penicillin and streptomycin had been added.

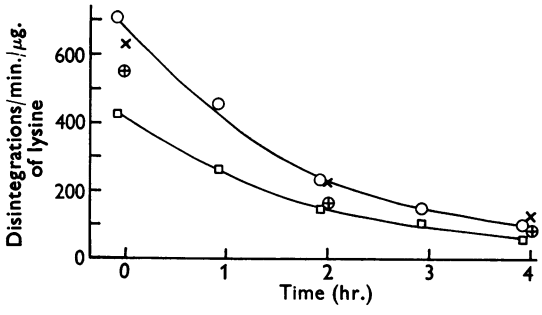

Fig. 1. ^{14}C specific activity of plasma lysine at various times after addition to perfusion blood. ○, □, Human blood; ×, ⊕, rat blood. $2{\cdot}2 \times 10^6$ disintegrations/min. $\equiv 1\,\mu c$.

DISCUSSION

The first aim of the present investigation was to attempt to confirm the results of Jensen & Tarver (1956) on the rate of synthesis of plasma protein by the perfused liver.

On the basis of experiments in which [^{14}C]lysine was used these authors reported a rate of synthesis of 31·4 mg. of total plasma protein/hr. by the liver of a 322 g. rat. In the present work the average rate of synthesis of total plasma protein by the livers of two only slightly smaller rats (RLP 1 and 8), which were perfused with rat blood, was found to be 7·8 mg./hr. The method of calculation used to obtain this figure is the same as that given for albumin in Table 4, but omitting the correction for the proportion of albumin to total protein synthesized. Recalculation of Jensen & Tarver's data on lysine incorporation with the assumptions and by the method shown in Table 4 (method *C*) gives 20·6 mg. of total plasma protein/hr., which is nearly three times the value calculated by this method from our results. In view of the considerable discrepancy between these two sets of results it is of special interest to compare each with the rate of plasma-protein synthesis *in vivo* reported for the strain of rats in question. Thus Jensen & Tarver (1956) gave the rate of synthesis of the plasma proteins observed in the perfused liver as only slightly below (95%) the maximum rate observed *in vivo* in an experiment in which ^{14}C-labelled plasma was given to rats maintained on diets containing three different levels of protein. The rate of synthesis is taken as the biological half-life of plasma protein after transfer. However, as shown by Freeman & Matthews (1958), this involves certain rather unlikely assumptions about the rate of loss of albumin from the intra- and extra-vascular pools. Dr S. Cohen (unpublished observations) has compared the rate of catabolism of albumin in rats calculated in this way with the results of calculations by the equilibrium–time method (Campbell *et al.* 1956). The latter method was found to give a rate of only 63% of that derived from the apparent biological half-life. Thus it seems reasonable to conclude that the actual maximum rate of plasma-protein synthesis of the strain of rats used by Jensen & Tarver may have been nearer 20 mg. than the suggested 33 mg./hr. In addition it would appear from the data of Steinbock & Tarver (1954) that the rate of protein catabolism is greatly increased in rats on a 65% protein diet compared with that in rats on a normal diet. Although these findings have not been confirmed by D. P. Cuthbertson & A. S. McFarlane (unpublished work), such a difference would imply that had Jensen & Tarver's rats been maintained on a diet similar to that of our rats their protein

turnover would have been substantially lower than the rate which they quote. These considerations greatly reduce the difference between the synthesis rates *in vivo* of the two strains of rats under comparison but make Jensen & Tarver's (1956) observations of a rate of synthesis of plasma protein of 31·4 mg./hr. by the perfused liver unaccountably high. For the strain of rats used in the present work more accurate figures are available for the rate of synthesis *in vivo* of albumin than for the synthesis of the plasma proteins taken together (Campbell *et al.* 1956). As shown in Table 5, RLP 1 and 8, in which rat blood was used, gave rates of albumin synthesis of 4·8 and 5·2 mg./hr. when calculated from the plasma-lysine specific activities, i.e. only an average of 55 % of the rate of synthesis *in vivo* of this protein (8·9 mg./hr.). That our results are nearer to the true rate than those of Jensen & Tarver (1956) is, however, suggested by the findings with those livers which were perfused with human blood. Thus the amounts of albumin isolated from four such experiments represent an average rate of synthesis of 11 mg./hr. or 128 % of the rate observed *in vivo*. If it is therefore assumed that in these perfusions synthesis is taking place at least as fast as *in vivo* it is interesting that the rate of albumin synthesis calculated from the plasma-lysine specific activities is again very low. In fact the average for perfusions 90 and 92 is once again 55 % of the rate of albumin synthesis *in vivo*. Since, however, tissue intracellular-amino acid specific activities *in vivo* are always found to be below the corresponding values for plasma [cf. Loftfield & Eigner (1958) for rat liver and Askonas & Humphrey (1958) for rabbit lung] and similar results now obtained for lysine in the perfused rat liver, the synthesis rates should probably be corrected for this factor. It is in any case difficult to be sure that the specific activities of intracellular amino acids obtained from a whole tissue reflect the specific activities of the amino acids at the sites of protein synthesis, and thus an additional element of uncertainty is inevitable in calculations made by this method.

On the basis of our mean figure (intracellular free-lysine specific activity 55 % of that in plasma after 4 hr. perfusion), the correction would be about twofold. Our results would then fall more nearly into line with those obtained by isolation of the albumin. On the other hand the rate of synthesis of the total plasma proteins given by Jensen & Tarver (1956) when treated in the same way becomes 44 mg./hr. for a rat of 322 g. It should be noted that in order to make the above comparison with the results of Jensen & Tarver (1956) it has been necessary to multiply the rate of protein synthesis of the perfused liver given by them by a factor of 0·7, to compensate for their assumption that rat plasma contained 7 % of lysine instead of 10 % of lysine as assumed in the present work.

As shown in Table 5 the average plasma free-amino acid concentrations in the perfusions with human blood were somewhat higher than when rat blood was used. This fact may account for the ability of the livers under these conditions to synthesize albumin somewhat faster than has been observed *in vivo* for the same strain of rats. On the other hand, neither the specific activity of the plasma-free lysine nor the rate of albumin synthesis calculated on this basis (method *C*) differed much between the experiments with rat and with human blood. Thus livers perfused in these two ways apparently behave similarly, which appears to justify the use of heterologous blood for experiments aimed at studying variation of rates of synthesis by isolation of newly synthesized albumin.

The present work indicates that part of the lysine remaining free in the plasma is progressively transformed under the conditions of our experiments into some other substance, which is presumably unavailable for protein synthesis. This fact will need to be taken into account in any final comparisons between rate of albumin synthesis as measured by incorporation of lysine by the perfused liver and *in vivo*, but does not seem to constitute a likely explanation for the discrepancy between our results and those of Jensen & Tarver (1956). Evidently the accumulation of a radioactive degradation product from [^{14}C]lysine in the blood during liver perfusion is a reason against the use of this amino acid in such work. Thus repetition of the present work with another precursor such as [^{14}C]histidine as was used by Jensen & Tarver (1956) would seem to be worth while.

Our work, together with that of Jensen & Tarver, indicates that the rate of protein synthesis by the isolated perfused liver is sufficiently near to that in the living animal to warrant the use of such a system for further study of the factors governing protein synthesis *in vivo*.

SUMMARY

1. The rate of synthesis of albumin by the isolated perfused rat liver has been calculated from estimates of the free [^{14}C]lysine specific activities in the plasma.

2. Livers have been perfused both with rat blood and with human blood. Newly synthesized albumin has been isolated from the latter experiments by means of an antiserum.

3. Liver-donor rats were previously injected with ^{131}I-labelled rat albumin to allow measurement of the amount of rat albumin transferred to the perfusion circuit with the liver.

4. In perfusions with human blood the average rate of synthesis of albumin, measured by direct isolation, was 12·9 mg./hr. for a liver from a 300 g. rat. By an indirect method of calculation, based on the specific radioactivity of isolated albumin and the total radioactivity incorporated into plasma protein, it was 11 mg./hr. In perfusions with rat or human blood the average rate of synthesis of albumin, calculated from the amounts of [^{14}C]-lysine incorporated into plasma protein and the specific activities of free lysine in the plasma, was 5 mg./hr. The possibility is discussed that the difference is due to the fact that the specific activity of lysine at the site of synthesis was less than that in the plasma.

5. A non-protein ^{14}C-labelled basic substance other than lysine has been found to accumulate during these perfusions.

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The Extraction of Pigments from *Euglena gracilis*

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Euglena gracilis is a flagellated green alga commonly employed in the microbiological assay of vitamin B_{12}. As a preliminary to assay, biological fluids are ideally diluted to contain about 10 $\mu\mu$g. of vitamin B_{12}/ml. and a series of standards covering a range of 0·25–50 $\mu\mu$g./ml. is in general use. Growth is usually assessed in a colorimeter 6–7 days after commencement of the assay (Ross, 1952; Hutner, Bach & Ross, 1956). The response thus measured is dependent both on turbidity and colour of the *Euglena* suspension.

In the course of experiments on potential vitamin B_{12} antimetabolites (Timmis & Epstein, 1959; Epstein & Petrow, 1960), a series of compounds was screened for inhibitory activity against *E. gracilis* grown at low concentrations of vitamin B_{12}. Compounds found active on screening were subsequently tested again to determine whether their antagonism was competitive in nature and therefore susceptible to reversal by massive vitamin concentrations. In this type of experiment, however, both growth and pigment production, particularly in the standards, became so prolific that the ensuing response could no longer be assessed by the methods referred to above (i.e. measuring both turbidity and colour). The object of this paper is to present a simple and sensitive pigment-extraction technique by which this difficulty may be obviated.

EXPERIMENTAL

Assay of vitamin B_{12}. The test organism was *Euglena gracilis* var. *z*. The method, including basal growth medium, was based on that described by Hutner *et al.* (1956).