The Catabolic Rate of Albumin Doubly Labelled with ^{131}I and ^{14}C in the Isolated Perfused Rat Liver

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The ability of the isolated perfused rat liver to catabolize plasma proteins has been investigated both with blood containing ^{131}I-labelled isolated proteins (Gordon, 1957; Cohen & Gordon, 1958) and with blood containing whole plasma labelled with ^{14}C (Green & Miller, 1960). Since the rate of catabolism obtained for albumin by the former method (1·1–1·4 mg. of albumin/hr.) is very much below the value which might be expected from the experiments with ^{14}C-labelled whole plasma [approximately 7 mg. of protein/hr./300 g. rat as calculated from the data given by Green & Miller (1960), assuming that the catabolic rate of albumin in the liver is an average of that for the whole plasma], further work was needed to show whether the low results with [^{131}I]albumin were due to the relatively slow catabolism of this protein in the liver compared with other plasma proteins, or to some discrepancy between the labels.

In vivo the many different proteins in whole plasma are catabolized at different rates and a different proportion of each may be expected to be broken down by the liver. Thus in order strictly to compare the two labels, single proteins are required, preferably in fully native form, labelled both with ^{14}C and with ^{131}I. For the present work rat albumin doubly labelled in this way has been used. The results show that there is fair agreement between the rates of catabolism calculated from liberation of non-protein ^{131}I and from the amounts of ^{14}C appearing in the liver, the plasma free amino acids and in the carbon dioxide. However, although the method of isolation was selected because of its mildness, the rate of catabolism as measured by ^{131}I of the doubly labelled albumin was found to be considerably above that previously found for ^{131}I-labelled fully native protein. The present data cannot therefore be used directly to calculate the proportion of native albumin catabolized by the liver; however, they are considered to validate previous results obtained by means of ^{131}I-labelled proteins.

METHODS

Perfusion apparatus and technique. The apparatus and surgical technique were the same as described by Cohen & Gordon (1958) except that, for reasons given below, after 2 hr. the liver undergoing perfusion was taken out of the apparatus and replaced, with the minimum of delay, by a second rat liver. In order to expedite the change, the operation on the second liver donor was carried almost to completion before the removal of the first liver from the circuit. Great care was taken to avoid loss of blood, which contains labelled albumin, during cannulation of the second liver. The blood contained by the first liver was returned to the circuit by perfusion of the organ with 4 ml. of 0·9% NaCl. Heparin (500 i.u.) was injected into the liver donors via the penial vein immediately before the severance of the inferior vena cava. In addition to the heparin added to the blood at the time of its collection a further 1000 i.u. dissolved in 1 ml. of 0·9% NaCl was added to the perfusing blood soon after the introduction of the second liver. Carbon dioxide was collected and estimated for ^{14}C radioactivity exactly as described by Freeman, Gordon & Humphrey (1958).

Estimation of ^{14}C radioactivity of plasma free amino acids. Samples of plasma were mixed with equal volumes of 20% trichloroacetic acid. After centrifuging the free amino acids were isolated from the supernatants by means of columns of Zeo-Karb 225 (Gordon & Humphrey, 1960). Portions of the resulting solution were then analysed for amino acids by the ninhydrin method (Jacobs, 1956) and for ^{14}C radioactivity after combustion to CO_2 (Bradley, Holloway & McFarlane, 1954). To ensure the presence of sufficient CO_2 an accurately measured amount of the solution (approx. 0·1 ml.) was allowed to soak into a piece of filter paper which had previously been placed in the platinum boat.

Estimation of liver ^{14}C and ^{131}I radioactivities. At the end of each perfusion blood was removed from the liver by the passage of 30 ml. of 0·9% NaCl, which was slowly injected into the plastic tubing carrying the inflow cannula. Next, the remaining parts of the diaphragm and the vessels connected with the two cannulae were cut away and the liver was quickly rinsed and dried on filter paper. The whole liver was weighed and 3–4 g. cut from one of the main lobes, any part in which blood could still be seen being avoided. The sample thus obtained was then homogenized for approx. 1 min. with 10 vol. of 0·9% NaCl in a blender. After this treatment the homogenate was made 0·1 N with respect to NaOH by addition of N-NaOH. A sample of this solution was transferred to a platinum boat for estimation of its ^{14}C radioactivity.

Estimation of ^{131}I radioactivity. ^{131}I was measured by scintillation counting. S.D. was not more than ±3%.

Preparation of ^{14}C- and ^{131}I-labelled rat albumin. L-[^{14}C]-Leucine (90 μc) was added to the rat blood used for perfusion of a rat liver. After 5·5 hr. 14% of the total radioactivity had become incorporated into the plasma proteins. In order to obtain plasma protein of maximum specific

activity the liver was perfused with blood, the plasma of which had previously been separated and diluted with Hanks medium. For this purpose 72 ml. of heparinized rat blood was centrifuged for 12 min. at 1750*g*. The plasma was then removed, followed by the white-cell layer and approx. 20% of the red cells. Plasma (14 ml.) and 25 ml. of Hanks medium were added to the remaining 80% of the red cells. After thorough mixing the 62 ml. of blood containing 3·3 of plasma protein/ml. thus obtained was placed in the perfusion circuit and when the liver had been introduced, but before addition of the L-[^{14}C]leucine, 24 ml. of blood was removed. Finally 50 mg. of glucose was added.

The plasma obtained at the end of the perfusion was made to 20% by addition of 5 g. of NaCl/100 ml. of plasma. When this had completely dissolved the precipitate was centrifuged off and the supernatant solution plus carrier leucine was dialysed at 2° against several changes of water. After 3 days the solution was removed from the sac and centrifuged, and the supernatant was estimated for protein and for ^{14}C radioactivity. Plasma protein (470 mg.) was thus obtained with specific activity 64·8 μc/g. of protein C. Sufficient of this solution was pressure-dialysed against 5 mM-glycine buffer, pH 8·9, to give 131 mg. of plasma protein at a concentration of approx. 8·0%. The whole of this solution was iodinated at an average of 1 atom of I/molecule with 185 μc of ^{131}I by the ICl method of McFarlane (1958). After removal of free ^{131}I by means of De-Acidite FF the solution was again pressure-dialysed against the same buffer. After 1 night a volume of 1·1 ml. of clear solution was thus obtained. This was subjected to electrophoresis in starch gel as described below.

The [^{14}C]glycine-labelled albumin used in perfusion no. 52 was prepared as just described for [^{14}C]leucine-labelled albumin except that the initial labelling was done *in vivo* and as a preliminary to the starch-gel electrophoresis an albumin-rich fraction was prepared by electrophoresis on modified cellulose (Porath, 1954). Initially 25 μc of [U-^{14}C]glycine (U, uniformly labelled) was injected intravenously into a rat. The animal was exsanguinated 4 hr. later, and 4 ml. of the heparinized plasma was used for the first electrophoresis. The albumin thus obtained, after concentration by pressure dialysis, was iodinated and subjected in two lots to electrophoresis in starch gel.

Electrophoresis in starch gel. This was carried out in a horizontal slab of gel (0·9 cm. × 15 cm. × 25 cm.) containing 0·05 M-glycine buffer, pH 9·0. The electrode vessels contained borate buffer as described by Smithies (1955). A portion (0·6 ml.) of the concentrated plasma prepared as described above was allowed to soak into two strips of thick filter paper (Ford A4 paper) each 7 cm. long. After electrophoresis for 17 hr. at 2·5 v/cm. the albumin was visible as a faint brown band. To permit precise location of this band two vertical slices of gel, each approx. 1 mm. thick, were removed transversely to the brown band and stained with Amido Black. With these as guides the albumin band (1·2 cm. wide) was cut out and transferred to an elution apparatus (see Appendix).

The solution thus obtained from the gel was concentrated by pressure dialysis against water and centrifuged. Finally the supernatant was used for the liver perfusion described below.

Paper electrophoresis. A small volume of the [^{14}C]leucine and ^{131}I-labelled albumin solution obtained as described above was diluted with an equal volume of heparinized rat plasma and examined by paper electrophoresis (Jencks, Jetton & Durrum, 1955). The strip was then scanned for ^{131}I radioactivity with a slit 1·0 mm. in width. Twelve weeks later, scanning for ^{14}C was also carried out. A slit 6 mm. in width was required for this purpose. The results are shown in Fig. 1.

Extraction of plasma with organic solvent and estimation of such extracts for ^{14}C radioactivity. The plasma was extracted at low pH with 20% heptane in propan-2-ol (Dole, 1956). After addition of further heptane and water the organic solvent phase, which was water-clear, was removed, evaporated to dryness and estimated for ^{14}C radioactivity by solid counting. Two thicknesses of lens paper were present on the planchets to ensure even evaporation of the organic solvent used for transfer.

Calculations

The calculation of the rate of albumin catabolism based on the amount of ^{131}I radioactivity becoming soluble in 10% trichloroacetic acid has been carried out by the method given by Cohen & Gordon (1958). The calculation of the amount of ^{14}C radioactivity transformed into liver components has taken into account the amount of ^{14}C radioactivity still present in the organ after perfusion with 0·9% NaCl. This has been arrived at from the amount of ^{131}I radioactivity in a section of the liver chosen as being in excellent condition judged by freedom from traces of blood after perfusion with 0·9% NaCl. From the weight of this section, in relation to the total wet weight of the liver, and its total ^{131}I radioactivity, measured after homogenization as described above, and from the specific ^{131}I radioactivity of the final plasma, the volume of plasma still present in the whole liver was calculated (these amounts are given below). No correction for non-protein ^{131}I in the liver has been introduced because after perfusion with 30 ml. of 0·9% NaCl only 2% of the total ^{131}I radioactivity was found to be in this form. Since the specific ^{14}C radioactivity in the

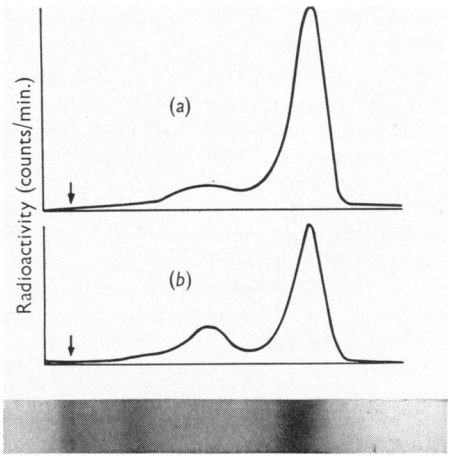


Fig. 1. Radioactivity tracings of doubly labelled (^{131}I and ^{14}C) albumin used for perfusion no. 61 after mixture with rat plasma and analysis by paper electrophoresis. (*a*) Scanning for ^{131}I with a 1 mm. slit. (*b*) ^{14}C radioactivity with a 6 mm. slit.

plasma was known, the total ^{14}C radioactivity present in the liver as unchanged albumin could be obtained. The rate of albumin catabolism is calculated from the rate of appearance of ^{14}C radioactivity in the liver, as plasma free amino acid radioactivity and as $^{14}CO_2$. As shown in Table 1, this total rate is divided by the initial ^{14}C-labelled plasma protein specific activity and the result multiplied by 0·50 and 0·42. These latter factors are required because albumin has been shown (Cohen, 1959) to account for 50% of the total protein present in rat plasma and to convert protein C into albumin as such.

RESULTS

Liver perfusions with ^{14}C-labelled whole plasma. Preliminary experiments with [^{14}C]glycine- and [^{14}C]lysine-labelled whole plasma gave 1·69 and 2·41% of the dose as CO_2 in 5 hr. with [^{14}C]glycine and 1·98% as CO_2 with [^{14}C]lysine.

Liver perfusions with ^{14}C- and ^{131}I-labelled albumin. Although the primary purpose of the initial livers which were perfused for 2 hr. before the start of the main part of each experiment was to remove modified doubly labelled albumin molecules, the amounts of $^{14}CO_2$ evolved in this part of the experiments were measured and is shown in Table 2. With [^{14}C]leucine-labelled albumin during the 4 hr. of perfusion of the second livers only 0·089–0·225% of the radioactivity originally added appeared as $^{14}CO_2$. With [^{14}C]-glycine-labelled albumin on the other hand 5–10 times more appeared as $^{14}CO_2$. Table 3 shows the rates of $^{14}CO_2$ formation and the rates of liberation of non-protein ^{131}I from the [^{14}C]leucine- and ^{131}I-labelled albumin. In order to make possible comparisons of these rates both have been converted into mg. of albumin catabolized/hr. In perfusion no. 60, 1 hr. after the introduction of the second liver the rate of catabolism, as measured both from the ^{131}I and from the ^{14}C labels, increased rapidly. In perfusion no. 61 this increase in rate did not take place.

Table 1 gives the amounts of ^{14}C radioactivity which remained in each liver after a final perfusion with 30 ml. of 0·9% NaCl (corrected for unchanged doubly labelled albumin), the final plasma free amino acid ^{14}C radioactivity and the $^{14}CO_2$ radioactivity resulting from the main part of each experiment, i.e. when each second liver was being perfused. The totals of these amounts are also shown calculated as rates of albumin catabolism (see Calculations section).

By using [^{14}C]leucine-labelled albumin the amounts of radioactivity found in the livers at the end of the experiments after perfusion with 0·9% NaCl were 1·18 and 0·50% respectively of the totals initially added. These figures are subject to some inaccuracy since they have been obtained by subtraction of the amount of ^{14}C due to residual plasma as estimated by ^{131}I radioactivity (57 and 73% of total liver ^{14}C radioactivity in perfusions nos. 60 and 61 respectively) from the total ^{14}C radioactivity of the liver. The liver ^{14}C radioactivity

Table 1. (1) *Calculation of amount of albumin catabolized from radioactivity appearing as $^{14}CO_2$, as plasma free ^{14}C amino acids and as ^{14}C remaining in the liver after perfusion with 0·9% sodium chloride, and* (2) *amounts of albumin catabolized from ^{131}I becoming soluble in 10% trichloroacetic acid*

The blood used for the perfusion of each of these livers had circulated through a preliminary liver for 2 hr. after addition of the labelled albumin.

	Perfusion no.	60	61
(1)	Initial plasma protein ^{14}C (μc/g. of protein C)*	$4·37 \times 10^{-1}$	$5·46 \times 10^{-1}$
	Radioactivity in liver after perfusion with 30 ml. of 0·9% NaCl (μc)†	$5·73 \times 10^{-3}$	$3·05 \times 10^{-3}$
	Final plasma free amino acid radioactivity: total in perfusion circuit (μc)	$7·60 \times 10^{-4}$	$6·37 \times 10^{-4}$
	$^{14}CO_2$ evolved: total radioactivity (μc/liver)	$1·10 \times 10^{-3}$	$5·47 \times 10^{-4}$
	Liver ^{14}C + free amino acid ^{14}C + $^{14}CO_2$ (μc)	$7·59 \times 10^{-3}$	$4·23 \times 10^{-3}$
	Time perfused (hr.)	4·2	4·2
	Non-plasma-protein ^{14}C produced (μc/hr.)	$1·81 \times 10^{-3}$	$1·01 \times 10^{-3}$
	Non-plasma-protein ^{14}C produced, as albumin catabolized (mg./hr.)‡	4·93	2·21
	Non-plasma-protein ^{14}C produced, as albumin catabolized (mg./hr./300 g. rat)	4·77	2·41
(2)	Albumin catabolized calc. from ^{131}I becoming soluble in 10% trichloroacetic acid/300 g. rat (mg./hr.)	3·5	3·0

* [^{14}C]Leucine was incorporated into plasma protein by means of a liver perfusion.

† Corrected for ^{14}C-labelled albumin remaining after perfusion with 0·9% NaCl (see Calculations section).

‡ $\frac{\text{Non-plasma-protein } ^{14}C \text{ produced} \times 0·50}{\text{Initial plasma protein specific activity } (\mu c/\text{g. of protein})}$ (see Calculations section).

Table 2. *Results from perfusions nos.* 60 *and* 61

Perfusion no.	60		61	
	Liver 1	Liver 2	Liver 1	Liver 2
Liver donor wt. (g.)	350	310	367	275
Liver wt. after perfusion with 0·9% NaCl (g.)	—	10·2	11·2	7·1
Liver C at 3·31% of body wt. (mg.)	—	1025	1212	910
Initial haematocrit (%)*	—	33	—	36
Initial blood vol. (ml.)†	—	52·6	—	61·8
Initial plasma vol. (ml.)†	—	37·9	—	39·9
Initial plasma protein concn. (mg./ml.)*	—	72	—	68
Total ^{14}C radioactivity of labelled albumin added to perfusion (μc)	0·487	—	0·615	—
Total ^{131}I radioactivity of labelled albumin added to perfusion (μc)	11·6	—	19·2	—
Total radioactivity recovered as $^{14}CO_2$ (% of total in plasma)	0·082	0·225	0·093	0·089

* Sample taken 10 min. after introduction of liver 2.
† Calculated from ^{131}I specific activity of perfusing blood or plasma sampled 10 min. after introduction of liver 2.

Table 3. *Rate of catabolism of albumin calculated from rate of liberation of non-protein* ^{131}I *and from rate of formation of* $^{14}CO_2$ *only*

Perfusion no. ...	Albumin catabolized (mg./hr./liver)			
	60		61	
Time from addition of labelled albumin (hr.)	Liver 1	Liver 2	Liver 1	Liver 2
		Calc. from ^{131}I		
1	—	4·9	—	2·3
2	1·6*	2·0	1·9*	1·7
3	—	3·1	—	2·3
4	—	4·4	—	2·0
		Calc. from $^{14}CO_2$		
1·3	—	0·45	—	0·15
2·2	0·49*	—	0·60*	—
2·7	—	0·77	—	0·36
4·0	—	1·0	—	0·39

* Average rate for 2·2 hr.

estimated in this way nevertheless bore a fairly constant relationship, at 3·08 and 2·57, to the ^{14}C recovered as CO_2 plus plasma free amino acids.

In Table 4 are compared the rates of catabolism of [^{14}C]leucine-labelled albumin given by the first and second livers. These rates are derived both from the ^{131}I and the ^{14}C labels but in the latter case, for the first liver, in perfusion no. 60 the rate is based on the rate of evolution of $^{14}CO_2$ only. The factors used to obtain the total rates are shown in Table 4. Some of the reasons that may explain the observed differences in the rates of catabolism given by the two livers in each experiment are examined in the Discussion.

Characterization of isolated albumin samples by electrophoresis on paper. As shown in Fig. 1 the [^{14}C]leucine-labelled albumin used in perfusion no. 61 contained a minor component with the mobility of an α-globulin. Scanning for ^{14}C and ^{131}I radioactivity showed this component to be relatively more strongly labelled with ^{14}C. Approximately 30% of the ^{14}C radioactivity was present as the minor component.

As shown in Fig. 2, the ^{131}I-labelled albumin which had been prepared by means of 1% trichloroacetic acid–96% ethanol (Charlwood, 1961) contained a considerable proportion of denatured molecules. Since carrier plasma was not present a considerable trail due to adsorption of albumin on the paper was found. Scanning for ^{131}I showed considerable radioactivity along the length of the trail. On the other hand, the albumin which has remained very near the origin was almost unlabelled.

^{14}C *Radioactivity in organic solvent extract of plasma.* [^{14}C]Leucine-labelled plasma which had

Table 4. *Comparison of rates of catabolism by first and second livers*

Results are given as mg. of albumin/hr./300 g. rat.

Perfusion no. ...	60		61	
	^{131}I	^{14}C	^{131}I	^{14}C
Liver 1	1·4	3·6*	1·6	4·1
Liver 2	3·5	4·8	3·0	2·4

* From $^{14}CO_2$ only, corrected assuming 11·8% of total as CO_2 as found in perfusion no. 61 (liver 1).

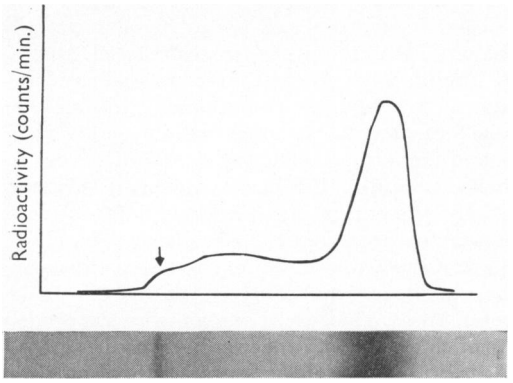


Fig. 2. Radioactivity tracing of rat ^{131}I-labelled albumin after analysis by paper electrophoresis. The plasma from which this albumin was prepared was taken from a preliminary liver perfusion. The albumin was prepared by means of 96% ethanol–1% trichloroacetic acid (Charlwood, 1961). A sample (1 mg.) was then iodinated with approx. 150 μc of ^{131}I and returned to the main bulk (590 mg. of albumin).

been prepared by means of a liver perfusion was extracted as described above (see Methods section): 0·66% of the total radioactivity was found to be thus extractable.

DISCUSSION

Conditions for agreement between results based on ^{131}I and ^{14}C labels. When a doubly labelled (^{14}C and ^{131}I) pure protein is catabolized both non-protein ^{131}I and ^{14}C in several forms different from that of the original protein will be produced. Assuming that the whole of the transformed ^{14}C can be measured, it should be possible to calculate the rate of catabolism of the original protein equally well from such measurements of ^{14}C and from those of the non-protein ^{131}I. The assumptions required for calculation of the catabolic rate of albumin from the rate of appearance of non-protein ^{131}I have been discussed by Cohen & Gordon (1958). With ^{131}I-labelled proteins and the isolated perfused rat liver, the observed rate of catabolism has been found to be greatly dependent on the presence or absence of traces of modified protein (Gordon, 1957). By using plasma proteins labelled only with ^{131}I the removal of almost all of such modified protein can be achieved by injection of the dose material into a living rat and by use of this animal's plasma after an interval of at least 48 hr. When a doubly labelled (^{14}C and ^{131}I) protein is used similar treatment would be required to ensure that the catabolic rate calculated from either of the labels corresponds with the catabolic rate characteristic of the fully native protein. Unfortunately in the present work the need for rather high ^{14}C specific activity has prevented the use of the screening procedure just mentioned. Instead the doubly labelled albumin was perfused for 2 hr. through an isolated rat liver which was then removed from the circuit before the start of the experiment proper. Although this procedure must certainly have decreased the proportion of denatured, or otherwise modified, doubly labelled albumin molecules, the catabolic rate deduced from the non-protein ^{131}I subsequently liberated by the second liver was still approximately three times that characteristic of native albumin (i.e. 35% of the rate of breakdown in the whole animal).

Consideration must therefore be given to the importance which should be attached to the observed agreement (Table 1) between the results obtained from the two labels when a slightly modified protein of the type actually used undergoes catabolism. Comparison of the rates of catabolism (Table 3) as measured by ^{131}I and by ^{14}C for the first and second livers shows higher values (faster catabolism as measured by ^{14}C) for the first liver. Possibly the simplest explanation for this finding is that the first liver is removing, and rapidly catabolizing, denatured albumin which carries less than the average amount of ^{131}I. By means of electrophoresis on paper, denatured albumin with just such properties was demonstrated to be present in a preparation of rat [^{131}I] albumin, made by the ethanol–trichloroacetic acid method (Charlwood, 1961). As can be seen in Fig. 2, the band of denatured albumin which remained at the origin was revealed by the staining technique but not by scanning for ^{131}I.

It is well known that progressive degrees of denaturation, for instance by means of X-rays (Yalow & Berson, 1957), can convert albumin into a form which is strongly adsorbed by filter paper under the conditions used for paper electrophoresis. That the denatured material present in the sample of rat albumin prepared by ethanol–trichloroacetic acid became iodinated to a less than average extent is of some interest because denatured fractions in preparations of iodinated albumin have usually been detected on the basis of their radioactivity. Furthermore, according to P. H. Maurer (personal communication): 'Denatured albumin

iodinates more easily than does the native protein'. Evidently considerable further work would be required in order to characterize the materials removed by the first liver. For the second liver, however, in view of the much closer agreement between the rates of catabolism as calculated separately from the two labels it seems reasonable to assume that most of the denatured molecules originally present had been removed by the first liver. The preliminary perfusion thus led to the fairly close agreement between the rates of catabolism, as calculated separately from the two labels, which was actually observed with the second liver (Table 1). There seems no reason to assume that further removal of denatured material would be likely to introduce an opposite trend leading to non-agreement.

In perfusion no. 52 (Table 5), in which [^{14}C]-glycine- and ^{131}I-labelled rat albumin were used, the rate of catabolism calculated from the ^{131}I liberated was almost identical with the value obtained in the same way in perfusions nos. 61 and 62, in which leucine was the precursor [^{14}C]amino acid. On the other hand, in the [^{14}C]glycine experiment the rates of catabolism, observed for both livers, calculated from the ^{14}C results were much higher than when [^{14}C]leucine was used. This result would be difficult to explain if the doubly labelled albumin used could be assumed to have been essentially a homogeneous native protein both when [^{14}C]-glycine and when [^{14}C]leucine had served as the precursor amino acid. Since, however, examination of the [^{14}C]leucine-labelled albumin by paper electrophoresis revealed the presence of a second component it is almost certain that the [^{14}C]-glycine-labelled albumin must also have been heterogeneous. Furthermore, since glycine is known to be metabolized and then reincorporated into various non-protein substances the [^{14}C]-glycine-labelled albumin obtained by starch electrophoresis and used in perfusion no. 52 probably contained significant amounts of non-protein ^{14}C, in addition to ^{14}C-labelled plasma proteins other than albumin, capable of being catabolized relatively rapidly. As shown in Fig. 1, the mobility of the minor component present with the [^{14}C]leucine-labelled albumin was that of an α-globulin. Evidence that proteins of similar mobility isolated from rat plasma are rapidly catabolized has been given by Cohen (1958). In these circumstances little importance can be attributed to the non-correspondence in the rates of catabolism as measured by ^{14}C and ^{131}I with [^{14}C]glycine-labelled albumin. For this reason perfusion no. 52 is not reported in detail.

Actual rates of catabolism of native albumin and globulins by the perfused liver. It will be noticed that the rates of catabolism calculated from the ^{131}I liberated by the first liver were always lower than those given by the second. This may well have been due to the presence of soluble starch, derived from the starch gel, in solution with the labelled albumin. The large amount of this starch initially present in the blood used for perfusion presumably decreased the efficiency of the Kupffer cells in respect to their ability to remove denatured albumin (Benacerraf, Halpern, Stiffel, Cruchaud & Biozzi, 1955). This would explain why the observed rate of catabolism by the first liver, as measured by ^{131}I, is little above that characteristic of native rat albumin.

The present results with doubly labelled ([^{14}C]-leucine and ^{131}I) rat albumin support the previous claim of Cohen & Gordon (1958) that only 10–15% of all rat albumin is catabolized in the liver. In view of the contrary claim of Green & Miller (1960) that the perfused rat liver can catabolize ^{14}C-labelled plasma at a rate that 'is in good agreement with published estimates of the biological half-life of labelled plasma proteins', it becomes of great interest to determine whether a much larger proportion of the total catabolism of the globulins is carried out by the liver. Since recent experiments with ^{131}I-labelled γ-globulin (Cohen, Gordon & Matthews, 1962) suggest that only about one-third of this protein is catabolized in the liver it would seem to follow that if Green & Miller's claim is true much larger proportions of the other globulins must be catabolized by this organ.

The presence in whole plasma of ^{14}C-labelled non-protein components will doubtless have made some minor contribution to the high rate of $^{14}CO_2$ evolution of 0·13%/hr. from ^{14}C-labelled whole plasma reported by Green & Miller (1960). However, correction for the $^{14}CO_2$ evolved from such components can hardly be expected to decrease the rate of $^{14}CO_2$ production to one comparable with that now observed from [^{14}C]leucine-labelled isolated albumin (0·02–0·054%/hr.). As reported above, [^{14}C]leucine-labelled whole plasma was found to contain at least 0·66% of organic solvent-extractable ^{14}C radioactivity.

Table 5. *Perfusion no. 52 with doubly labelled ([^{14}C]glycine and ^{131}I) albumin*

The blood used for this perfusion had circulated through a liver for 2 hr. after addition of the ^{14}C-labelled albumin.

Time perfused (hr.)	4·1
Albumin catabolized calc. from ^{131}I becoming soluble in 10% trichloroacetic acid/300 g. rat (mg./hr.)	2·3
Non-plasma-protein ^{14}C produced (mg. of albumin catabolized/hr./300 g. rat)	15·6
$^{14}CO_2$ evolved (albumin catabolized, % of total albumin catabolized)	20·0

Difficulties involved in simultaneous measurement of catabolic rates of more than one plasma protein with either ^{131}I *or* ^{14}C. Attempts to estimate the proportion of whole plasma normally broken down in the liver are complicated by the existence in plasma of proteins of widely different catabolic rates. Unless synthesis is occurring the average rate of catabolism of such a mixture will decrease as the more rapidly catabolized components are used up. Although with ^{131}I as the label synthesis need not be considered, and although for the short times, and with the relatively large-sized pools of plasma, employed for liver-perfusion experiments the changes in rate of catabolism during the perfusion may not be very large, two serious difficulties still remain. The first of these is that before use iodinated proteins must be freed from modified components by passage through a living animal. During this preliminary stage, with mixtures of proteins of very different catabolic rates, the proportions present would be seriously modified. Thus iodinated plasma prepared for a liver perfusion by this means would contain greatly reduced concentrations of the more rapidly catabolized components. Secondly, the interpretation of the results of such experiments would require knowledge of the relative degree of iodination of each component. Thus even for the perfused liver with complex mixtures such as plasma, the average catabolic rate cannot be directly measured by means of the ^{131}I label. When ^{131}I-labelled whole plasma is catabolized *in vivo* similar difficulties prevent calculation of the average catabolic rate of the plasma proteins. Since the alternative, the injection of ^{14}C-labelled whole plasma into the whole animal, involves complications due to reutilization of the label, no direct means is at present available for accurately measuring the average rate of catabolism of a mixture of proteins either in a perfusion system or *in vivo*. In these circumstances the comparison made by Green & Miller (1960) of the catabolic rate of ^{14}C-labelled whole plasma in the rat liver with the rates *in vivo* of isolated proteins (Cohen, 1958; Oeff, 1954) hardly seems valid.

Thus the alternative of measuring and comparing the constant catabolic rates of individual proteins in the liver perfusion system and *in vivo* seems to be necessary. Since preliminary studies on the screening periods necessary for the more rapidly catabolized proteins will also be required, such investigations will take considerable time. The present experiments do, however, suggest that the data thus obtainable will be interpretable directly as evidence of the catabolic rates of each protein. Ultimately sufficient information may thus be accumulated for an accurate estimate of the share of the liver in the total rate of catabolism of the plasma proteins *in vivo* to emerge.

SUMMARY

1. Rat albumin doubly labelled with ^{14}C and ^{131}I has been prepared with [^{14}C]glycine or [^{14}C]-leucine. The former was incorporated into plasma by injection into a living rat. The biosynthesis with [^{14}C]leucine was by means of a liver perfusion. After removal of fibrin the plasmas were labelled with ^{131}I and subjected to preparative electrophoresis in starch gel.

2. The rate of catabolism of this doubly labelled albumin was estimated during perfusion through isolated rat livers both from the rate of formation of non-protein ^{131}I and from the amount of $^{14}CO_2$, liver ^{14}C and plasma free amino acid ^{14}C finally present.

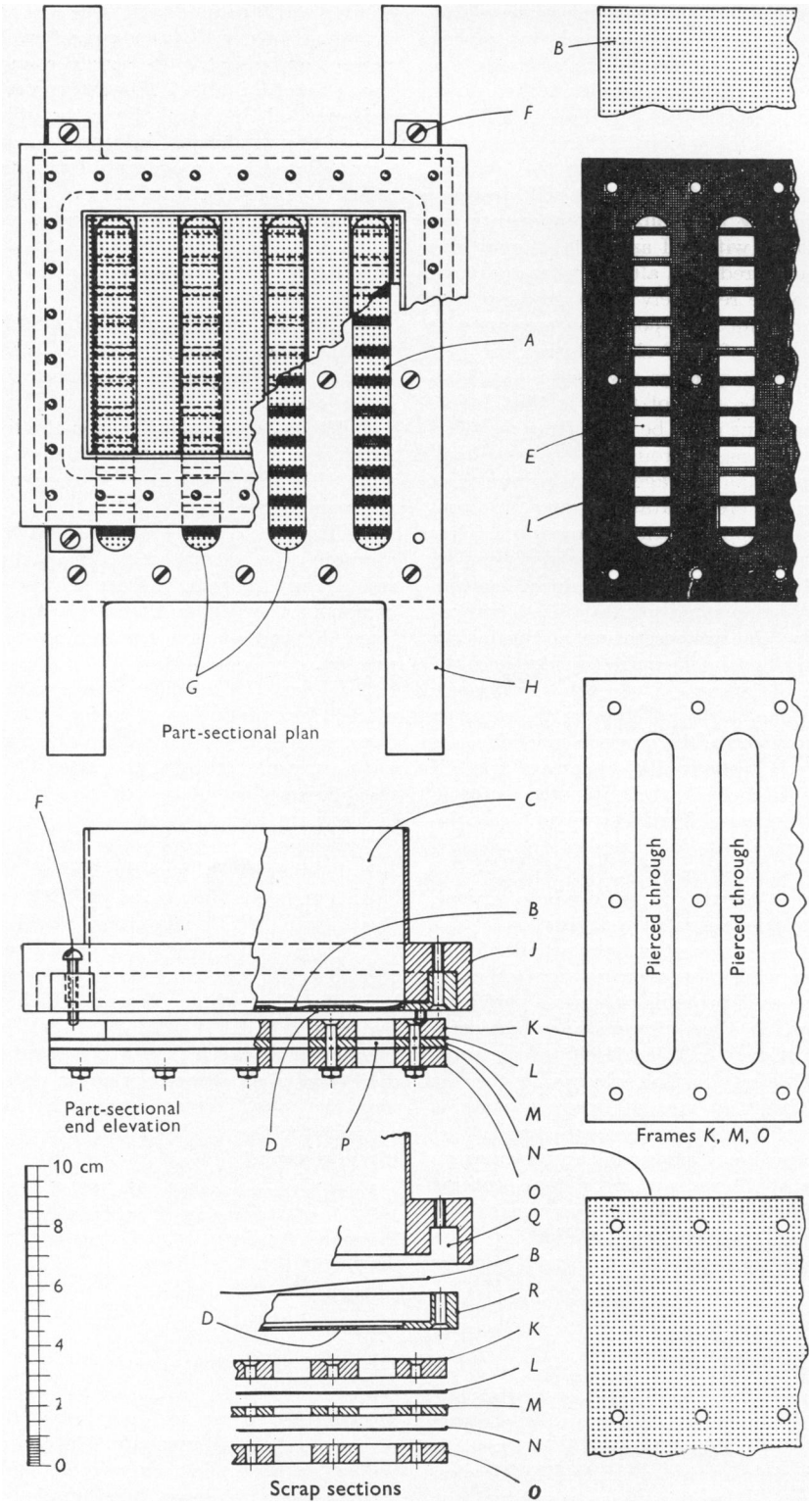
3. When [^{14}C]leucine was the precursor and after 2 hr. preliminary perfusion through a rat liver, which served to reduce the amount of non-native protein present, the rates of catabolism of the albumin calculated on the basis of the two labels were found to be in fair agreement. With [^{14}C]glycine as precursor the rate of catabolism as calculated from ^{14}C was the higher.

4. The rate of formation of $^{14}CO_2$ by the isolated liver from [^{14}C]leucine-labelled albumin (0·02–0·054 %/hr.) observed in the present work has been considered in relation to the $^{14}CO_2$ rate from [^{14}C]-lysine-labelled whole plasma, which was reported by Green & Miller (1960) to be 0·13 %/hr. The rate of catabolism deduced from the present findings with doubly labelled albumin is not in accord with their view that the liver can catabolize fast enough to account for the whole of the turnover rate *in vivo* of the plasma.

I am glad to acknowledge the skilled technical assistance of Mr Louis Assemakis. I am most grateful to Dr J. H. Humphrey for discussion and criticism of many aspects of the present study.

APPENDIX

The apparatus shown in Figs. 3 and 4 (Letters *A–R* in text appear in Fig. 3, and *S–V* in Fig. 4) is the same in principle as that described by Gordon (1960) for recovery of proteins from starch gel but it is simpler to use and is enlarged to handle four pieces of gel simultaneously. The compartments for buffer solution, in which the eluted proteins accumulate (*P*), have been made rather longer



For legend see opposite page

than each piece of gel to facilitate control of the meniscus level during the run. Tests with [^{131}I]-albumin in alternate pieces of gel have shown absence of transfer from compartment to compartment so that elution from pieces of gel containing separate bands can be carried out simultaneously without mutual contamination.

The following constructional and operational details may be noted. The current is applied to the upper surfaces of the pieces of gel by means of a hanging cellophan membrane (*B*) filled with 0·02M-phosphate buffer, pH 8·3. This membrane, which forms the bottom of an open-topped Perspex box (*C*), is partially supported by three wide Perspex bars (*D*). The four operative areas thus formed are positioned to coincide with the upper surfaces of the pieces of gel. The degree of contact with the pieces of gel can be adjusted by means of screws (*F*), which raise or lower the box (*C*) in relation to the gel holder. The thickness of the pieces of gel must be sufficient for there to be air gaps under each Perspex bar (*D*) without at the same time any loss of adequate contact between gel and membrane. The whole apparatus is filled with 0·02M-phosphate buffer, pH 8·3, to the levels S^1, S^2 and S^3 shown in Fig. 4, and the air bubbles trapped under the bottom frame are removed. The buffer level in vessel (*T*), which must not be allowed to fall below the bottom of the cell, is determined by the exact position of sucker U^1. During passage of the current (200 mA for four pieces of gel), overheating and pH changes are prevented by means of the pumping system, which circulates buffer continuously between the cathode and anode vessels. Since, however, small gas bubbles are carried under the cell by the stream of buffer if the anode is placed in vessel (*T*), the use of a separate anode vessel (*V*) connected by a cloth bridge (*W*) has been found to be convenient. With albumin, passage of 200 mA for 3 hr. has been found to give 95% recovery.

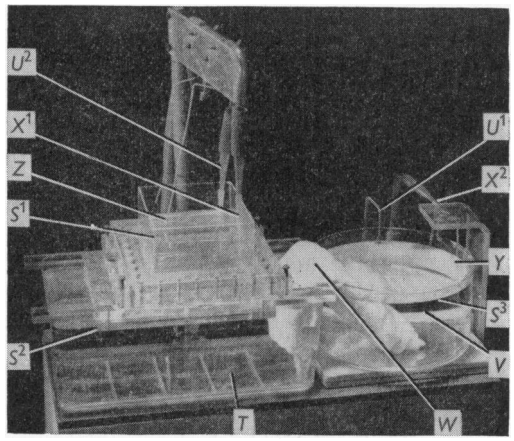


Fig. 4. General view of elution apparatus. Levels of the buffer in the anode and cathode vessels are controlled by means of suckers U^1 and U^2 and delivery tubes X^1 and X^2. Buffer circulation is brought about by a peristaltic pump with separate tubes for the flow from anode to cathode vessels and vice versa. S^1, S^2 and S^3, Buffer levels in cathode vessel (open-topped box), vessel *T* and anode vessel respectively: *T*, Perspex box on which anode cell rests; U^1, U^2, suckers which maintain levels in anode and cathode vessels; *V*, anode vessel; *W*, cloth bridge; X^1, X^2, delivery tubes from buffer circulation pump; *Y*, anode; *Z*, cathode.

Fig. 3. Diagram of elution cell. The cell is constructed entirely from Perspex except for the two cellophan membranes, the sheet of 0·13 mm. thick polythene (which forms the bridges on which the pieces of gel rest) and the brass screws. Upper surfaces of the intermediate and bottom frames *M* and *O* are lightly greased with petroleum jelly before assembly to prevent possible liquid interchange between the compartments. The slot in frame *J* is sufficiently deep for contact with *R* to be on the inner edge of the frame rather than at the bottom of the slot. The cellophan membrane *B* should not be so large that its edges fold into the slot. After assembly the heads of the screws in *K* are covered with a layer of petroleum jelly. *A*, Gel compartments; *B*, cellophan membrane, which makes contact with upper surfaces of pieces of gel; *C*, open box; *D*, supporting bars; *E*, holes in polythene sheet; *F*, adjusting screws; *G*, open ends of compartments which contain solution into which protein is eluted; *H*, bars on which cell rests; *J*, frame forming upper part of the base of the open box (the frame is fabricated from sheet Perspex; details of fabrication are not shown); *K*, frame for holding pieces of gel; *L*, polythene sheet cut to form bridges; *M*, intermediate frame, similar to *K*; *N*, cellophan membrane; *O*, bottom frame, similar to *K*; *P*, compartment which contains buffer solution into which protein is eluted; *Q*, slot in frame into which base of open box is screwed, thus sealing edges of cellophan membrane; *R*, base of open box with support bars for membrane.

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The Lipid Composition of Phosphorylating 'Digitonin Particles' and Water- and Saline-extracted Mitochondria from Rat Liver

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Cooper & Lehninger (1956*a*) described the preparation of fragments of the mitochondrial membrane, by treatment with digitonin, that still retained the capacity for oxidative phosphorylation (see also Devlin & Lehninger, 1956; Cooper & Lehninger, 1956*b*). Devlin & Lehninger (1958) stated that 29 % of the digitonin particles was lipid and 95 % of this was phospholipid of undetermined nature. Remmert & Lehninger (1959) (see also Hulsmann, Elliott & Rudney, 1958; Elliott, Hulsman & Slater, 1959) have suggested that lipid factors may be concerned in the control of phosphorylation, and Pressman & Lardy (1956) have shown that oleic acid is a potent uncoupler of oxidative phosphorylation. Green (1959) has shown that lipid factors are concerned in the oxidation of succinate by electron-transport particles prepared from ox heart. The composition of the lipids from rat-liver mitochondria has been studied by Getz & Bartley (1961), Macfarlane, Gray & Wheeldon (1960), Paoletti & Grossi (1960), Clément, Clément & Le Breton (1956), Clément, Haimovici & Le Breton (1955), Collins & Shotlander (1961) and Getz, Bartley, Stirpe, Notton & Renshaw (1962), but no work has so far been published on the detailed lipid composition of digitonin particles prepared according to Devlin & Lehninger (1958). Among other methods for fractionating mitochondria are water extraction (Claude, 1946; Watson & Siekevitz, 1956) and saline extraction (Dallam, 1958). The present paper describes the fatty acid pattern of lipids extracted from digitonin particles and the insoluble mitochondrial residues remaining after extraction of these organelles with water or with saline.

EXPERIMENTAL

Chemicals. Digitonin was AnalaR grade (British Drug Houses Ltd.); it was used without further crystallization. All solvents were of analytical grade except ethanol, which was purchased as azeotropic.

Animals. White rats of the Wistar strain bred in the laboratory were used. They were fed on a standard diet of rat cubes supplied by Oxo Ltd. (Medical Department), Southwark Bridge Road, London, S.E. 1. The fatty acid analysis of the diet is given by Getz & Bartley (1961).

Preparation of the digitonin particles. The procedure described by Devlin & Lehninger (1958) was followed.

Extraction and fractionation of the lipids of digitonin particles. Digitonin particles (0·36 g. dry weight) were extracted with chloroform–methanol (2:1, v/v) according to Getz & Bartley (1961). The lipid extract was evaporated to dryness under nitrogen at 60° and chloroform was added to the residue. The mixture was warmed to 50° and filtered. The residue was extracted with warm chloroform several times (final vol. 80 ml.). The remaining precipitate and the filter paper were extracted with warm chloroform–methanol (2:1, v/v) (final vol. 50 ml.). Samples of each extract were estimated for their ester content. The chloroform extract contained 164 μmoles of ester and the subsequent chloroform–methanol extract 9·5 μmoles. After combining the two extracts they were evaporated to dryness and the solids were dissolved in chloroform. The extract (171 μmoles of lipid ester) was applied to an 18 g. silicic acid column (Hirsch & Ahrens, 1958) that had been exhaustively washed with chloroform. Neutral lipids were eluted from the column by 1 l. of chloroform. Tests on the last 16 ml. of chloroform passing through the column showed it to be free from lipid esters. The chloroform eluent containing the neutral lipids was free from phosphorus. Subsequent elution of the phospholipids was with the solvent sequence shown in Table 1. A fraction collector was used to collect 10 ml. samples of the eluent. These were analysed for phosphate by wet-ashing a 0·5 ml. sample and