THE DOMINANT ROLE OF THE LIVER IN PLASMA PROTEIN SYNTHESIS

A Direct Study of the Isolated Perfused Rat Liver with the Aid of Lysine-ε-C^{14} * †

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This report presents direct experimental evidence of the dominant role of the liver in the biosynthesis of the plasma proteins. The qualitative and quantitative responses of the isolated intact liver perfused with homologous oxygenated blood are in accord with observations made in intact animals (18, 19, 31). The use of lysine-ε-C^{14} permits significant serial observations to be made in experiments of only 6 hours duration, and, with the perfusion procedure developed, affords a general method for the *in vitro* study of the factors affecting protein synthesis in mammalian tissue.

The impressive mass of clinical and experimental evidence summarized by Whipple and Madden (20) strongly supports the notion that the liver is the site of plasma protein formation. More recently, studies of proteins eluted from liver minces and slices (16, 28) by saline and serum media have been interpreted as being in accord with this view, although without adequate differentiation of the eluted proteins from the cellular proteins of the liver. Despite all this, experiments in rabbits and rats (11) have led some to minimize the liver's role in protein synthesis.

The data of the present report strongly support the view that perfusion of the intact liver with oxygenated blood permits the liver itself to synthesize plasma proteins, to separate them from its own tissue proteins at need, and to contribute them to the circulating plasma in a manner closely approximating the physiological as seen in intact normal animals.

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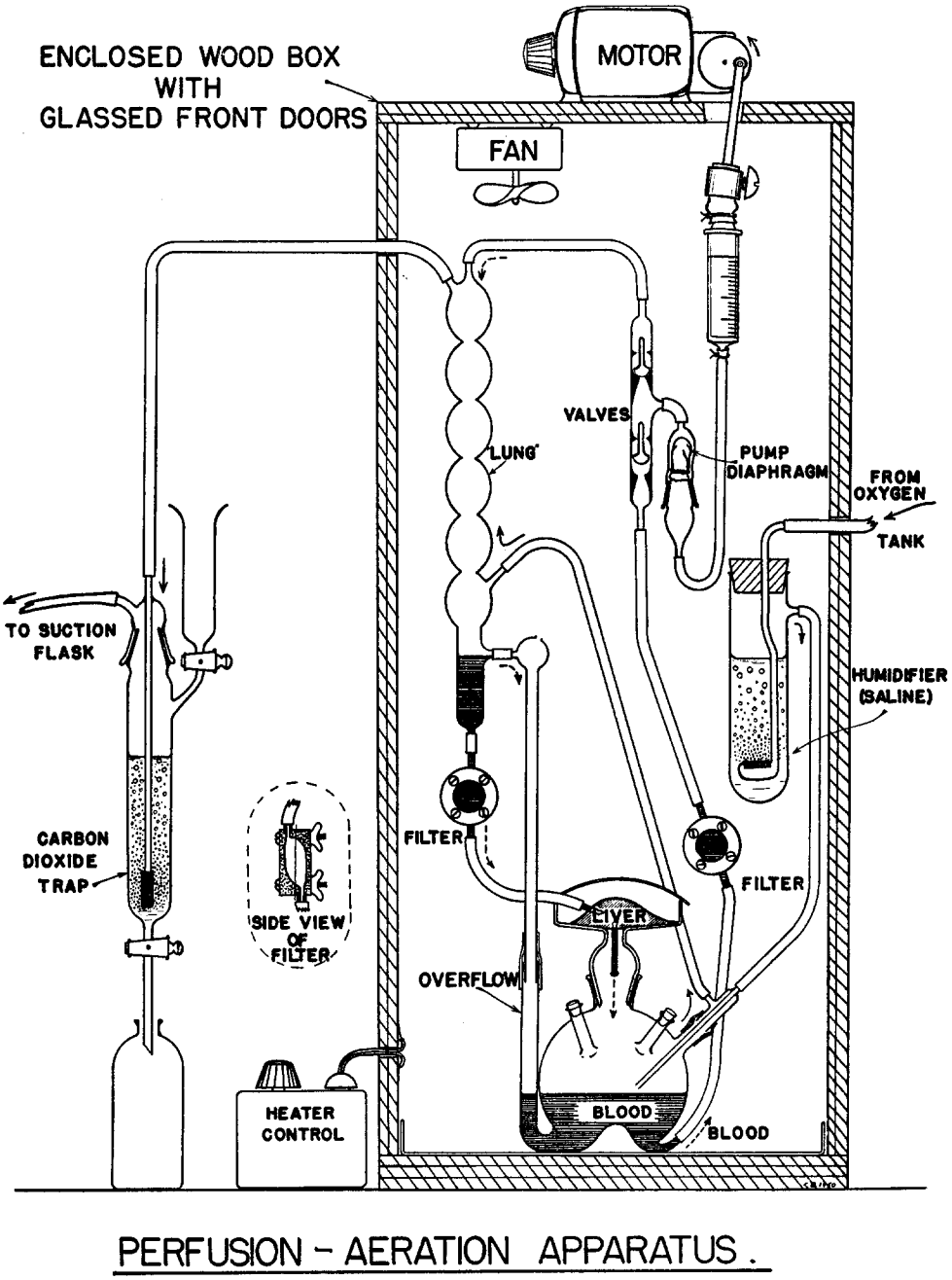

FIG. 1. For description see text.

Methods

Wistar strain adult albino rats (from 250 to 350 gm. weight) were maintained on a stock diet of commercial rat chow or checkers, and unless otherwise stated were deprived of food but allowed water for 16 to 18 hours before sacrifice as either blood or liver donors.

Under light ether anesthesia[1] blood donors were bled by cardiac puncture. Heparin in saline was used as the anticoagulant. Generally, from 130 to 180 ml. of pooled rat blood was obtained for each experiment, strained through a surgical cotton gauze sponge to remove small clots, and mixed with enough saline to total, with all the supplementary solutions used in an experiment, 135 to 240 ml. of diluted blood (final red blood cell hematocrit reading ranged from 25 to 40 volumes per cent).

The diluted blood was poured into the reservoir of the perfusion apparatus which is diagrammed in Fig. 1. This apparatus is similar in principle to many which have been described, yet deserves comment. The difficulties caused by clot formation in our early experiments were considerably minimized by the introduction of two simple filters. The filters, as shown in Fig. 1, consist of two discs of lucite machined to fit closely with an interposed disc of white silk between them. The silk used was 100 mesh per inch by 150 mesh per inch. The pump proper, of simple finger-stall design, can deliver from 25 to 200 ml. of blood per minute depending on the rate of the motor and the size of the syringe used to activate the finger-stall.

Oxygenation of the blood is effected primarily in the multibulbed tube. As in the experiments in which CO_2 collections have been made, the humidified air, or oxygen used for oxygenation, is first passed through the reservoir to sweep out any $C^{14}O_2$ which may enter the gas phase there, and then past the thin film of blood in the multibulbed "lung." The liberated CO_2 is collected by passage through 100 ml. of 40 per cent KOH, an aliquot of which is assayed for C^{14} activity. The values obtained for $C^{14}O_2$ activity are smaller than they should be to an extent dependent on CO_2 losses from the surface of the perfused liver (29).

The oxygenated blood is then accumulated in the small upper reservoir in which the level or perfusion pressure is maintained approximately constant (within 3 to 5 cm. of blood) by the overflow bypass back to the main reservoir flask. The blood flowing to the liver is thus maintained at a predetermined pressure of 20 to 25 cm. of blood. This is probably somewhat in excess of the normal portal venous pressure in the rat, if one accepts the values of Kunkel and Eisenmenger (17). The entire apparatus is maintained at a temperature of 38–40° by heat from a resistance wire strung on the walls of the box housing the apparatus, and carrying current adjustable by an autotransformer.

The rat furnishing the liver is placed under light surgical anesthesia and the peritoneal cavity opened. The gastrohepatic and gastroduodenal ligaments are divided, the latter between ties placed about the pancreaticoduodenal vessels. The common bile duct and portal lymphatics are isolated and divided. Ties are placed loosely about the portal vein. The inferior vena cava is ligated and divided between the liver and the right kidney. The portal vein is then rapidly cannulated and the thorax is opened for the cannulation of the superior vena cava. This vessel is then divided and the liver with the attached diaphragm is rapidly dissected out. The liver is then attached by the portal vein cannula to the oxygenated blood supply. The position of the liver is adjusted on the platform of the perfusion apparatus so that the hepatic vein outflow is readily estimated by counting the drops and measuring the volume of the vena cava outflow. The blood flow thus observed measured 1.0 to 3.0 ml. per gm. liver per minute. The total time of the operation is usually 25 to 35 minutes, and the liver is without an active blood flow for a period of 6 to 8 minutes. As soon as the perfusion is running

[1] In several experiments a mixture of 50 per cent oxygen and 50 per cent carbon dioxide was used as the anesthetic agent for both blood and liver donors, without any change in the results observed.

smoothly, the substrate mixture is added rapidly to the large reservoir and mixed throughly with the blood. The starting time of a perfusion is reckoned as the time at which the substrate is added.

Generally the amino acids essential for optimal growth in the rat and dog, hereafter referred to as the essential amino acids, were added in the form of an aqueous solution of the mixture Vuj-N by dissolving 3.20 gm. in distilled water to give 100 ml. of solution.[2] According to the manufacturer's assay, this Vuj-N mixture had the following composition: L-arginine hydrochloride 8.7 per cent, L-histidine hydrochloride monohydrate 4.35 per cent, L-isoleucine 8.50 per cent, L-leucine 19.0 per cent, L-lysine monohydrochloride 13.4 per cent, L-methionine 6.0 per cent, DL-methionine 1.3 per cent, L-phenylalanine 9.45 per cent, L-threonine 1.45 per cent, DL-threonine 8.05 per cent, DL-tryplophane 1.95 per cent, L-valine 6.60 per cent, and glycine 10.9 per cent.

Where the non-essential amino acids were used they were made up in a solution as follows: L-glutamic acid 1.02 gm., L-proline 0.32 gm., L-hydroxyproline 0.128 gm., DL-alanine 0.480 gm., DL-serine 0.488 gm., L-cysteine hydrochloride 0.112 gm., L-tyrosine 0.272 gm., DL-aspartic acid 0.520 gm., were dissolved in distilled water with the aid of 10 per cent sodium hydroxide and made up to 100 ml. (final pH 8.2).

The DL-lysine-ϵ-C^{14} was prepared as the monohydrochloride according to published methods (25).[3] The D- and L-lysine-ϵ-C^{14} were obtained through the preparation and resolution of DL-carbobenzoxylysine by the method of Bergmann as modified by Borsook (9) using papain and conversion of the L-carbobenzoxylysine to its anilide.[3] The L-lysine-ϵ-C^{14}·HCl so obtained had $(\alpha)_D^{25°} = +14.7°$ and the D-lysine-ϵ-C^{14}·HCl had $(\alpha)_D^{25°} = -14.1°$. The lysine was free of impurities detectable by paper partition chromatography and radioautographs of the resulting chromatograms.

The amounts of the various amino acids used are included in the data of the individual experiments. In some experiments extra glucose was added to the perfusing blood by mixing 2 or 3 ml. of 20 per cent glucose with the amino acid solution, and the mixture added together. The added substrates were thoroughly mixed with the blood in the reservoir flask by shaking the flask with a rotary motion.

Blood specimens were withdrawn from the reservoir flask at stated intervals, the initial sample usually being taken at 5 to 15 minutes, 30 minutes, 1, 2, 3, 4, 5, 6, and occasionally 7 hours after the start of an experiment. Approximately 10 mg. of solid potassium oxalate was added to each sample of blood to eliminate the slow formation of fibrin which otherwise occurred even with heparin present.

At the close of the experiment, 6 or 7 hours after the start, the liver was removed, cut into small pieces which were blotted on filter paper to remove excess blood, and weighed. Several small representative slices were taken in appropriate fixatives for histological examination, and the remainder was kept frozen until lyophilized. The dried liver was then easily powdered in a mortar and a representative sample taken for C^{14} assay. The rest of the finely powdered liver was then suspended in about 25 volumes of 6 per cent trichloracetic acid containing DL-lysine (10 to 20 mg.). After standing overnight the suspension of crude liver protein was centrifugalized, and the supernate discarded. The residual protein was resuspended with mechanical stirring in 6 per cent trichloracetic acid containing DL-lysine and allowed to stand for several hours; this process was again repeated twice (the last time without added DL-lysine), making a total of 4 washings with trichloracetic acid. The precipitated crude protein

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[2] We are indebted to Merck and Co., Rahway, New Jersey, for a generous supply of this material.

[3] We are indebted to Professor R. W. Helmkamp and Dr. Carl Claus for the preparation of the DL-lysine-ϵ-C^{14}, and to Dr. Morton Rothstein for the resolution.

was then suspended in acetone, centrifugalized, resuspended, filtered, and washed on the filter with acetone. After drying at 50–60°, aliquots of the resulting powder were taken for C^{14} assay and for determination of nitrogen content by the Kjeldahl method.

The blood samples were centrifugalized, hematocrit readings recorded, and the plasma separated. Aliquots of the whole plasma were taken for (*a*) C^{14} assay of the whole plasma; (*b*) for the precipitation of the total proteins with 20 volumes of trichloracetic acid after the addition of 10 to 20 mg. of DL-lysine; (*c*) for the precipitation of the albumin and globulin fractions by the method of Majoor (21). The total proteins and these fractions were repeatedly treated with DL-lysine and 6 per cent trichloracetic acid similarly to the liver. This procedure was considered efficacious for the complete removal of all non-protein C^{14} activity, particularly D-lysine-ϵ-C^{14} because almost all 5 or 10 minute plasma protein specimens had no measurable C^{14} activity; furthermore, in a control experiment in which rabbit blood containing labeled lysine and amino acids was circulated through the perfusion apparatus (without a liver) for 6 hours, the plasma proteins separated as described had *no* significant C^{14} activity; (*d*) for the chemical determination of total nitrogen, plasma protein, albumin, and globulin levels by the Kjeldahl method; (*e*) for the separation of fibrin. Aliquots of plasma were first mixed with about 20 mg. of DL-lysine, diluted with saline (10 to 15 volumes), and fibrin formed by adding beef thrombin[4] and allowing the mixtures to stand overnight. The resulting fibrin clots were wound out or spun down, excess serum protein expressed, and the clots washed twice with saline. The fibrin clot was dried after treatment with acetone, ground to a fine powder, and treated 4 times with 6 per cent trichloracetic acid and DL-lysine, then finally dried with acetone; this extensive latter treatment was found essential to remove active extraneous C^{14}, presumably adsorbed on the original fibrin.

In some experiments the residual serum protein was precipitated in the same manner described for the plasma proteins above and finally assayed for C^{14}. In two experiments serial fibrinogens were salted out (15) and found to possess the same specific activity as did the more easily prepared fibrin clot. The treatment of a considerable number of protein specimens with alkali (0.1 to 1.0 N NaOH) revealed no loss of C^{14} activity, in contrast to the findings of Winnick with glycine-1-C^{14} and homogenates (35).

In order to follow the ultimate disappearance of all the amino acids from the circulating blood, additional aliquots of the serial plasma specimens were first precipitated, in several experiments, with 6 per cent trichloracetic acid without the addition of DL-lysine, and the resulting protein-free solutions freed of the trichloracetic acid by ether extraction, and then examined for amino acids by one and two dimensional paper chromatography according to the method of Dent (13).

In some experiments "expired" carbon dioxide specimens were obtained serially at stated intervals by rapidly draining the 40 per cent potassium hydroxide solution from the CO_2 absorption trap (see Fig. 1), and refilling the trap with a measured volume (usually 100 or or 125 ml.) of fresh caustic solution. Aliquots of the trap contents were assayed for C^{14}.

Calculations of liver and plasma protein synthesized are arbitrarily based on the following assumptions: (1) Only the L-isomer of lysine is utilized for protein synthesis. (2) The C^{14} activity of both liver and plasma proteins is primarily present in the proteins as L-lysine. (These assumptions are based on failure of the liver to incorporate D-lysine into protein and the results of isolation of L-lysine as picrate from acid hydrolysates of the proteins with measurement of specific C^{14} activity). (3) The lysine content of these proteins from rats is approximately 7 per cent; (4) the incorporation of lysine occurs in protein only through synthesis of the entire protein molecule, and not by single amino acid shift.

Thus, in a typical experiment if 20 mg. of L-lysine was present in the amino acid mixture

[4] Parke-Davis Company.

added and had an arbitrary activity of 250 units, and if 2.0 gm. of liver protein was found to contain 4.0 units of radioactivity per gm., then $2(4/250) \times 20 \times 100/7 = 9.2$ mg. of liver protein made by the entire liver (2 gm. on dry "protein" basis).

The validity of some of the above assumptions will be discussed below.

Assays of C^{14} activity were carried out, using the apparatus designed and described by Bale (2). This apparatus makes use of the wet oxidation mixture of Van Slyke and Folch (33) and measures C^{14} activity of the CO_2 in an ionization chamber coupled with a dynamic vane electrometer and an Esterline-Angus automatic recorder.

On gross inspection after 6 to 9 hours of perfusion as above, the perfused livers (65 have been perfused to date) appeared normal and indistinguishable from freshly extirpated non-exsanguinated livers or from unperfused non-exsanguinated livers which had been kept in saline at 39°C. for 6 to 9 hours, except that some showed: (*a*) occasional peripheral areas (estimated at 0 to 2 per cent of the organ) in which contraction and wrinkling had resulted from drying exposure to the air beyond the saline-moistened protective gauze covering; (*b*) occasional scattered dark red petechiae, 1 to 3 mm. in diameter (totalling never more than 0 to 3 per cent of the surface area, and not occurring deeper in the liver); and (*c*) rarely a dark red apparently collapsed small proximal lobe (estimated at 3 to 7 per cent of the organ), the portal branch to which had possibly been occluded by the portal cannula tip. Edema was noted only in the one hypoxemia experiment, in which the blood oxygen content was maintained at 5 to 7 volumes per cent instead of the usual 10 to 15 volumes per cent (data to be published later).

For microscopic examination, Bouin's fixation and hematoxylin and eosin were used for general study, for fat formalin fixation and Sudan IV, and for glycogen formaldehyde-absolute alcohol fixation and the Bauer-Feulgen stain. Microscopically there was again no difference between sections of the two control types of liver and sections from the well perfused major portions of the experimental tissue. Since all animals had been fasted at least 18 hours before death, none of the livers contained more than barely visible glycogen, if any, compared with well fed glycogen control livers. Sections from the focal petechiae, the peripheral wedges, or the rare, poorly perfused small lobe showed congested, moderately widened sinusoids, narrowed hepatic cords, and occasional hemorrhage and bacteria; the hepatic cells except for a rare, cloudy hyaline necrotic-appearing cell, were still morphologically intact.

The limitations of morphology are recognized. The above evidence does not prove that any of the liver cells were still viable or physiologically active at the time of sectioning the organ, but indicates only that by gross and histological examination practically all the cells of the perfused liver appeared normal without detectable trauma to or damage of the cell membrane, nucleus, or some of the cytoplasmic constituents. (Conversely, some morphologically abnormal cells may still retain some functions.) The following studies show that even after several hours of perfusion, the liver maintains its functions of quantitatable amino acid clearance, bile secretion, plasma protein synthesis, CO_2 output, and best of all the ability to respond equally well in all of these to a second dose of substrate.

EXPERIMENTAL RESULTS

Fig. 2 presents the curves of disappearance of non-protein plasma C^{14} activity from the circulating plasma in terms of per cent of the calculated initial values (as 100 per cent). It will be noted that the presence of amino acids other than lysine does not significantly affect the rate of disappearance of the radioactivity since the curves are closely grouped together whether the DL-lysine is present alone or with substantial amounts of all the other amino acids. It will also be

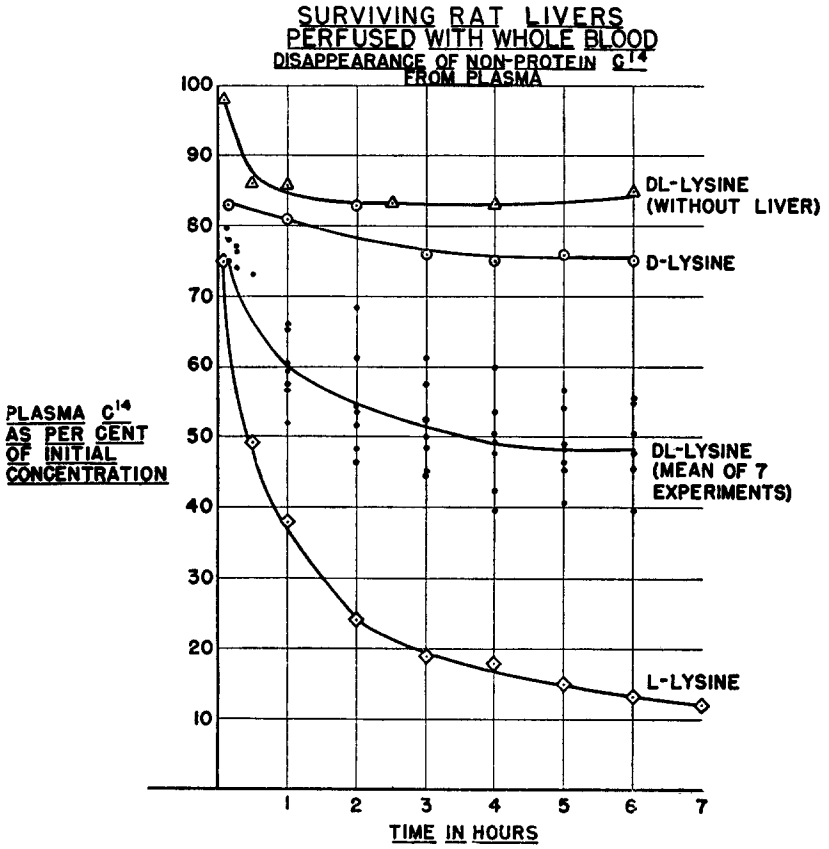

FIG. 2. The non-protein C^{14} activity of the plasma expressed as per cent of the initial concentration calculated on the assumption that the lysine C^{14} is distributed only throughout the plasma and does not enter the red cells.

DL-*Lysine without liver*, (RLP O), 2.09 mg. DL-lysine-ε-C^{14} (1.73 microcuries), 160 mg. Vuj-N mixture, 173 mg. non-essential amino acids, 500 mg. glucose added to 296 ml. of blood.

D-*Lysine*, (RLP 15), 24.6 mg. D-lysine-ε-C^{14} (3.47 microcuries), 160 mg. Vuj-N mixture (minus lysine), 167 mg. non-essential amino acids added to 207 ml. of blood.

DL-*Lysine*.—The seven experiments included here are: RLP 7, DL-lysine-ε-C^{14} 1.75 mg. (1.19 microcuries); RLP 6, 8, 9, 10, 11, 16, with DL-lysine-ε-C^{14} in amounts from 1.75 to 4.53 mg. (1.19 to 3.47 microcuries), Vuj-N mixture in amounts from 20 to 160 mg., and non-essential amino acids in experiments RLP 11 and 16 only, in amounts of 40 mg. and 160 mg. respectively, added to from 133 ml. to 175 ml. of blood.

L-lysine, (RLP 20), 24.6 mg. L-lysine-ε-C^{14} (3.47 microcuries), 338 mg. of Vuj-N mixture (minus lysine), and non-essential amino acids 374 mg. added to 218 ml. of blood.

Wherever used the abbreviation RLP means rat liver perfusion.

noted that wherever DL-lysine is used, the residual plasma C^{14} activity approximates 50 per cent of the initial; whereas the use of D-lysine, the unnatural

isomer, is attended by minimal change in the course of the experiment. (The loss of plasma activity in this experiment can be largely accounted for by adsorption on red cells.) The use of the L-isomer, on the other hand, reveals that C^{14} activity is almost entirely removed from the plasma within 6 hours. This is in keeping with the known facts concerning the non-utilization of D-

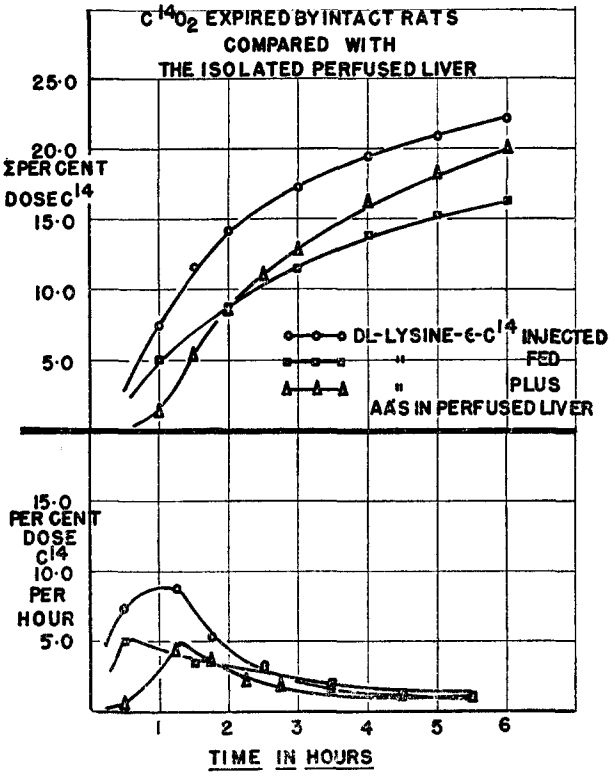

FIG. 3. *Intact rat*, fasted 18 hours, injected intracardially with 5.12 mg. DL-lysine-ε-C^{14} (4.22 microcuries).

Isolated rat liver, (RLP 23), perfused with a mixture of DL-lysine-ε-C^{14} 4.17 mg. (3.44 microcuries), Vuj-N mixture 180 mg., non-essential amino acids 187 mg. in 234 ml. of blood

Intact rat, feeding interrupted by intravenous injection of 4.78 mg. DL-lysine-ε-C^{14} (0.67 microcurie).

lysine as a substitute for L-lysine for nutritional purposes by higher mammals (3).

The positions of the curves in Fig. 2 have not been corrected for two known sources of error. The first is referable to the volume of plasma trapped among the red blood cells, and it is emphasized by the results of the control experiment in which the liver was omitted and blood was circulated in the perfusion

apparatus with a complete amino acid mixture including labeled lysine. The second lies in the adsorption of lysine cations by the negatively charged red blood cells. The first source of error has been measured by the isotope dilution

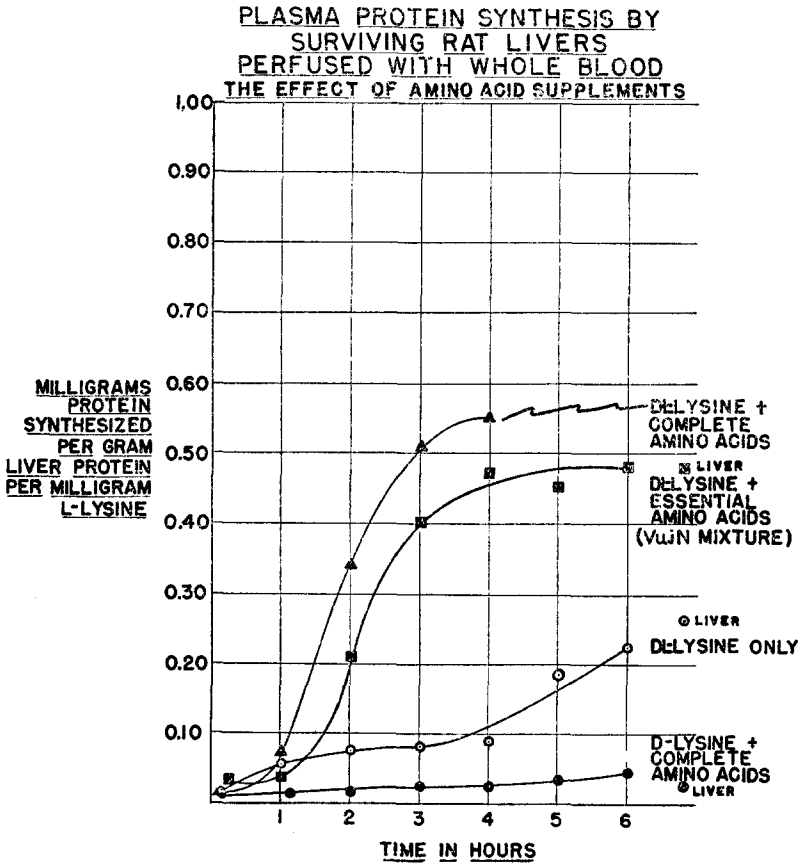

FIG. 4. From above down the curves represent the following experiments:

DL-*lysine plus complete amino acids.* This curve represents the first 4 hours of experiment RLP 19 as detailed in Fig. 7.

DL-*Lysine plus essential amino acids,* (RLP 8), DL-lysine-ε-C^{14} 1.75 mg. (1.19 microcuries), and Vuj-N mixture 20 mg.

DL-*Lysine only,* (RLP 7), DL-lysine-ε-C^{14} 1.74 mg. (1.19 microcuries).

D-*Lysine plus complete amino acids,* (RLP 15) corresponds to the experiment of Fig. 2.

technique and approximates 7 to 10 per cent of the volume of red blood cells centrifugalized under the conditions used in our experiments; the second source is in part subject to factors not controlled (such as red blood cell size, pH, temperature, etc.), but from calculations based on red cell and whole blood C^{14} samples it may range up to 8 per cent of the dose of DL-lysine, as

determined in two different perfusion experiments. The ultimate net fate of the C^{14} added to the perfusing blood has been quantitatively assessed in several experiments by totalling the measured activities of the "expired" $C^{14}O_2$, and the total C^{14} activity of the blood and liver. In this manner 80 to 85 per cent of the added isotope was accounted for. Probably the major loss of activity is associated with the escape of appreciable $C^{14}O_2$ through the liver capsule (29).

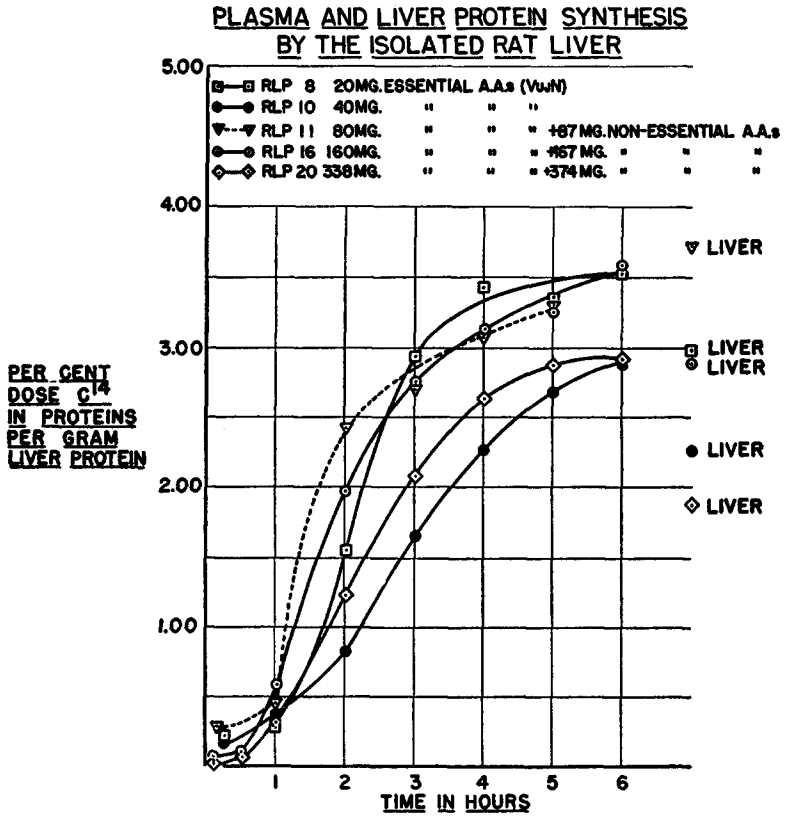

FIG. 5. Amino acid supplements are as indicated above. In RLP 16 glucose (500 mg.) was added with the amino acids.

Fig. 3 is presented to demonstrate the close similarity of the qualitative and quantitative pattern of $C^{14}O_2$ produced by the isolated perfused rat liver, and the intact rat when both are presented with somewhat varying amounts of labeled substrate. The quantitative values of $C^{14}O_2$ for the perfused liver are probably as much as 20 per cent low because of CO_2 lost through the liver capsule (29) as noted above. It is noteworthy that the maximum rate of plasma protein synthesis seen in the experiments of Figs. 4 to 7 coincides with the

interval in which the apparent rate of oxidation of lysine-ϵ-C^{14} is maximal; *i.e.*, between one-half and 2 hours after the addition of substrate.

Fig. 4 reveals that DL-lysine-ϵ-C^{14} alone leads to small incorporation of the C^{14} in the circulating plasma proteins. Admittedly, even the 18 hour fasted rat plasma and liver have available small amounts of other amino acids including the essentials as substrate. In striking contrast to this, the addition of the nine essential amino acids and glycine (Vuj-N mixture[5]) leads ultimately to a more than twofold incorporation of the C^{14} into the plasma proteins and into the gross liver proteins. What is more striking, is the comparatively more rapid response seen when at least the essential amino acids are made available. We take this to be a direct *in vitro* confirmation of previous observations concerning the role of the essential amino acids in plasma protein synthesis in the intact dog (18, 19) and rat (31).

In addition, Fig. 4 presents a comparison of the effectiveness of DL-lysine-ϵ-C^{14} and D-lysine-ϵ-C^{14} when they are supplemented identically with a mixture of all the essential and most of the non-essential amino acids. Not only is there very slight incorporation of the C^{14} into plasma proteins when D-lysine is used, but the presence of only slight activity[6] in the lysine isolated from the plasma and liver proteins makes clear the inability of the liver to utilize the unnatural isomer of lysine for plasma protein and liver protein synthesis. It can be concluded that the perfused liver in the *in vitro* system used not only discriminates between the natural and unnatural isomers of lysine, but also responds to the mixture of essential amino acids in a manner analogous to that of the intact animal.

It should be further noted from Fig. 4 that the curve of appearance of isotope in the plasma proteins has a lag period which becomes shorter with the addition of the non-essential amino acids. It is quite likely that this shortened lag period and increased rate of appearance of the isotope in the circulating proteins are a reflection of the ready availability in the experiments of Fig. 4 of the so-called non-essential amino acids. This is an impressive *in vitro* demonstration of the fact that the synthesis of blood and tissue protein is most rapid when *all* the amino acids are present and that the synthesis of the non-essential amino acids requires an appreciable period of time.

Figs. 5 and 6 present the results of experiments which were designed to assess the quantitative nature of the perfused liver's response to varying amounts of substrate, and also to examine further the effect of the addition of the amino acids non-essential for growth and plasma protein production.

All five curves in Fig. 5 show the lag phase noted in Fig. 4. It is also apparent

[5] Merck and Company.

[6] The lysine isolated as the picrate from the proteins of this experiment had a specific millimolar activity of 1.6 micromicrocuries. This is less than 2 per cent of the specific lysine activity found in comparable experiments with DL-lysine.

that there is roughly quantitative correlation between the amount of the essential amino acid substrate and the total cumulate incorporation of the C^{14} label in the plasma proteins. The correlation is equally good for the final content

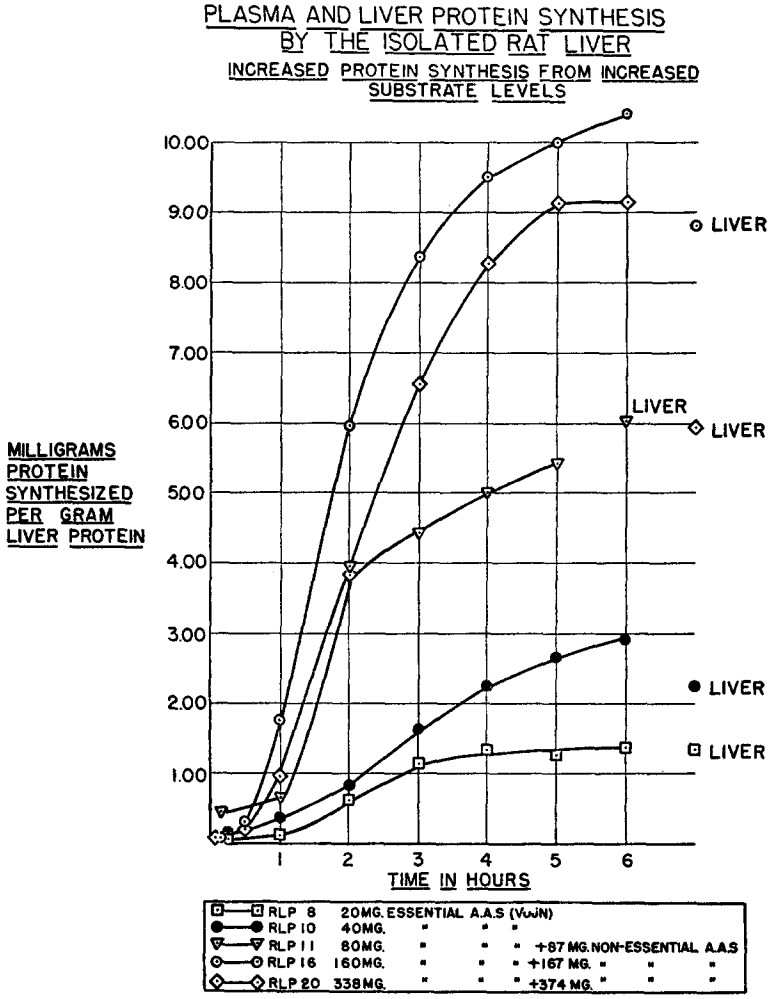

FIG. 6. Amino acid supplements are as indicated above. In RLP 16 glucose (500 mg.) was added with the amino acids.

of C^{14} in the liver proteins at the three lower levels used. The relatively low per cent of the labeled lysine incorporated in the liver protein of RLP 10 and RLP 20 is referable to the fact that no extra glucose was added to the circulating blood in these experiments. The effect of added glucose on protein synthesis varies with the kind and amount of amino acid substrate present and will be

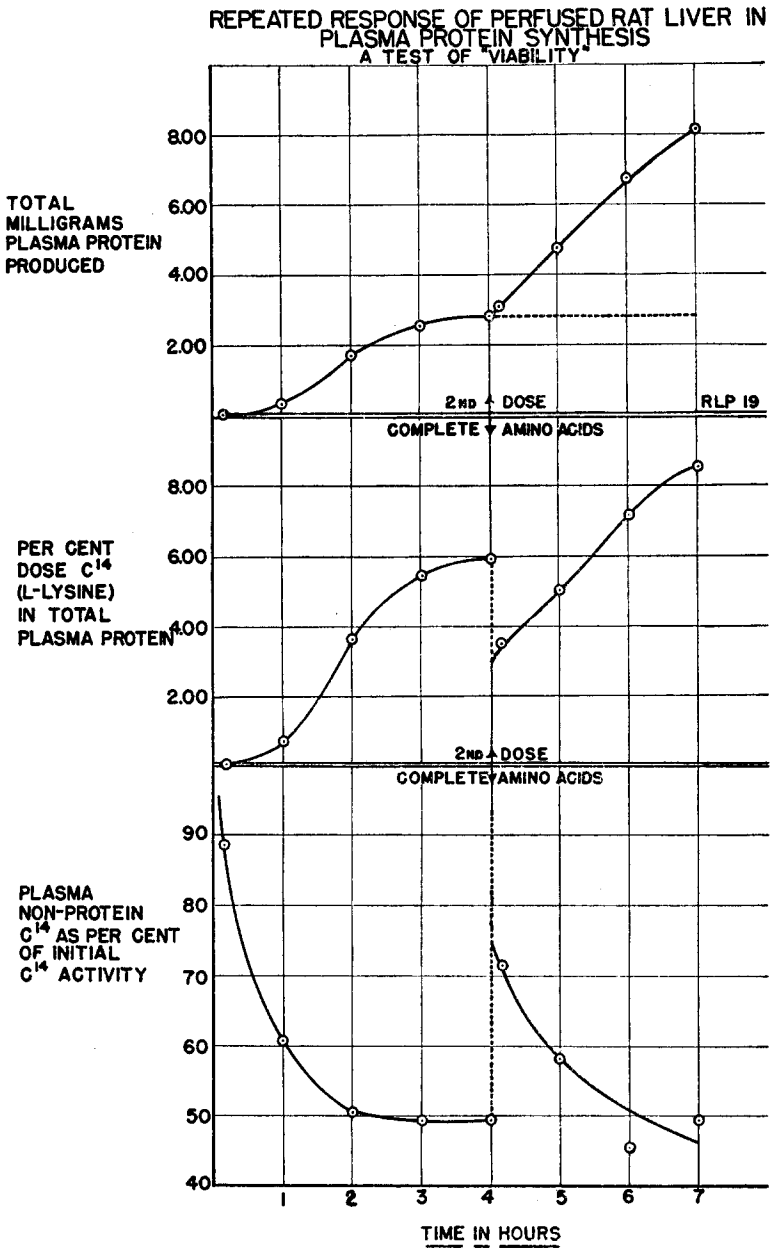

FIG. 7. Experiment RLP 19, DL-lysine-ϵ-C^{14} 2.08 mg. (1.69 microcuries) plus Vuj-N mixture 20 mg., and non-essential amino acids 20 mg. were added to 200 ml. of blood at the outset and this mixture was again added at 4 hours. The non-protein C^{14} values of the lowest graph are calculated on the basis of the computed 0 to 3 hour values taken as 100 per cent and 75 per cent respectively.

discussed in detail in a separate report dealing with the factors affecting protein synthesis. It should also be noted that the lag phase is shorter and the rate of C^{14} incorporation in the plasma proteins much more rapid and much greater quantitatively in the post-lag phase of the experiments in which the non-essential amino acids are included in the amino acid supplement. Taken by itself, Fig. 6 amply testifies to the broad quantitative capacity of the liver to convert amino acids to plasma and liver proteins with an efficiency roughly independent of the concentration of the substrate within the limits of these experiments (*i.e.* within 6 hours the liver will convert roughly the same percentage of whatever amount of substrate is available into protein).

Fig. 7 presents a typical experiment which shows that the final flat or declining phase of the C^{14} incorporation into plasma proteins is not a result of

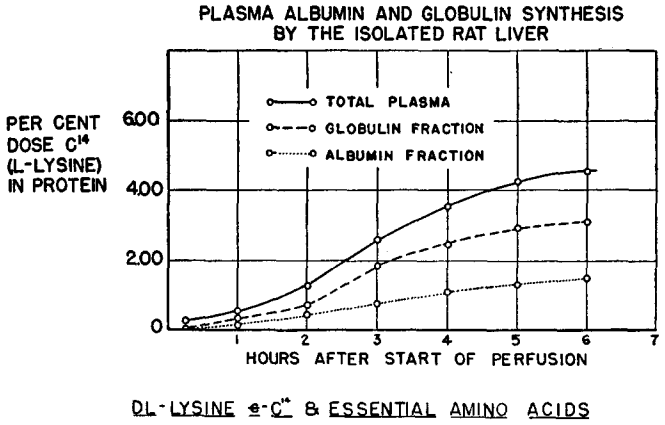

FIG. 8. Experiment RLP 10 with the supplements added as noted under Fig. 5.

weakening, exhaustion, or failure of the surviving livers used in our experiments, but is primarily related to the decrease and disappearance of essential substrates. This is attested by at least three different types of evidence. Firstly, in the level of circulating non-protein radioactivity (which is primarily unused D- and L-lysine with only the latter being utilizable), the curves flatten out inversely as the protein incorporation curves. Secondly, serial chromatograms of the amino acids in the non-protein filtrates reveal a *progressive* disappearance of virtually all the amino acids except hydroxyproline; the final 7 hour chromatogram reveals small amounts of glutamic and aspartic acids and lysine (probably the D-isomer) and considerable valine and the leucines. Thirdly, and most directly, is the prompt and quantitatively equal or greater response of the isolated liver to the exhibition of an additional complete supplement of the amino acids (Fig. 7). The obvious absence of a lag phase in the second portion of the curve of C^{14} incorporation provokes speculation and suggests

the presence of *labile* plasma protein precursors or intermediates in the liver which are still present in the liver of the experiment in Fig. 7 (RLP 19) at the time when the second dose of amino acid substrate was administered; *viz.*, 4 hours after the first dose.

The Nature of the Protein Fractions into Which C^{14} Is Incorporated by the Liver. —Blood plasma protein albumin and globulin fractions were obtained by the method of Majoor (21). The results of a typical experiment (RLP 10) are

TABLE I

Greater Extent of Incorporation of Lysine-ε-C^{14} into Plasma Globulin Fraction

Experiment No.	Amino acid supplement	C^{14} albumin/globulin ratio* Hrs. after start						
		1	2	3	4	5	6	7
RLP 7	DL-Lysine only		0.32	0.36	0.42	0.25	0.27	
RLP 8	Essential, 20 mg.	0.40	0.36	0.36	0.42	0.46	0.75	
RLP 10	Essential, 40 mg.	0.54	0.47	0.41	0.47	0.42	0.49	
RLP 11	Essential, 80 mg. Non-essential, 87	1.0	0.46	0.45	0.44	0.40		
RLP 16	Essential, 160 mg. Non-essential, 167		0.56		0.55	0.57	0.57	
RLP 19	Essential, 20 mg. Non-essential, 20	0.93	0.78	0.53	0.53	0.54	0.57	0.57
RLP 20	Essential, 338 mg. Non-essential, 374	1.1	0.84	0.78	0.75	0.71	0.65	0.52

* This ratio is obtained by dividing the total C^{14} activity of the plasma albumin fraction in a given volume of plasma by the total C^{14} in the globulin fraction of the same volume of plasma.

plotted in terms of per cent dose C^{14} incorporated in the protein fractions and are shown in Fig. 8.

Although only the essential amino acids (and glycine) were used in this experiment (RLP 10), the qualitative results are similar in all 35 experiments studied to date regardless of substrate, dose level, essential amino acids, non-essential amino acids, or glucose. The results bear a striking resemblance to those in the normal intact dog (24) and in intact rats. It is clear that C^{14} enters both the albumin and globulin fractions. Here the C^{14} enters the globulin fraction more rapidly than the albumin fraction; in fact, the final C^{14} A/G ratio (experiments in Table I) varies between 0.27 and 0.75 (in the intact dog and

rat experiments the C^{14} A/G ratio varied between 0.42 and 0.66). Furthermore, as indicated in Table I, there is no significant change in the final C^{14} A/G ratio in the experiments in which considerably larger amounts of the complete amino acid mixture were used.

The relatively greater rate of incorporation of C^{14} in the globulin fraction as contrasted with the albumin fraction is seen in Fig. 9, in which are plotted

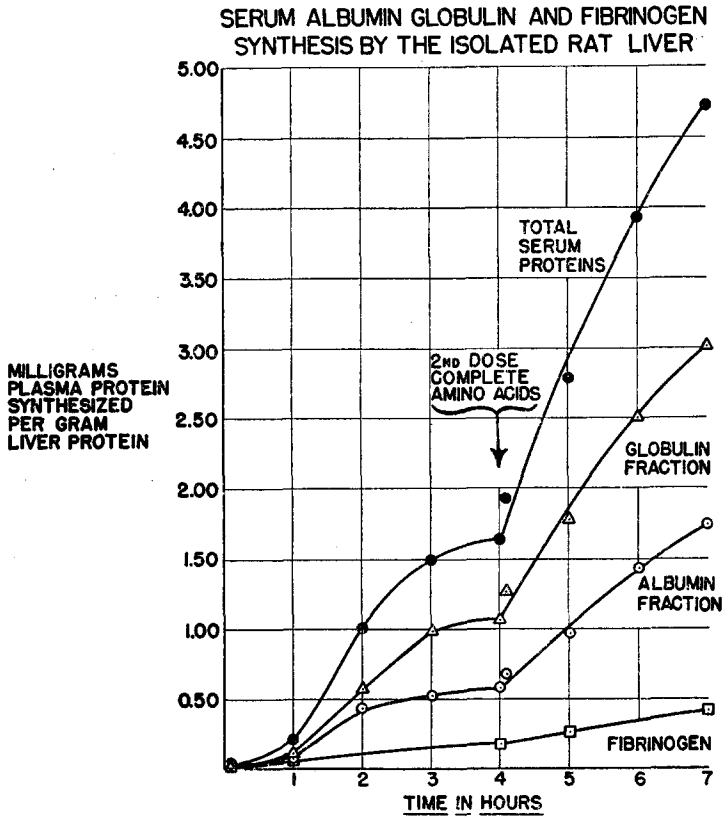

FIG. 9. Experiment RLP 19 with the supplements added as noted under Fig. 7.

the results of the protein fractionation of Experiment RLP 19. Here the response of the liver to the second dose of the complete amino acid mixture is seen to result in the continued incorporation of C^{14} in the albumin and globulin fractions without significant change in the C^{14} A/G ratio. Shown here, too, is the incorporation of the C^{14} in the fibrin fraction. The incorporation of the isotope in the fibrinogen (*i.e.* fibrin) of this experiment is typical of the findings in ten experiments in which the fibrin was isolated. In these experiments the total C^{14} in the fibrin approximated 5 to 10 per cent of the total in the plasma

proteins; since the fibrinogen approximates 3 to 4 per cent of the total plasma proteins on a chemical basis, the fibrinogen has incorporated about twice as much activity on a gram basis as the plasma proteins in general. This may simply be a reflection of a higher lysine content of fibrinogen, or a greater rate of formation, or both. In brief, Fig. 9 provides graphic and direct testimony of the liver's ability to synthesize not only fibrinogen but also serum albumin and globulin.

The isolation of the lysine as lysine picrate from the plasma and liver proteins of six experiments indicates that at least 95 per cent of the protein C^{14} activity is indeed present as lysine. The most important controls of this experiment are described in a forthcoming paper (5) in which perfusion of rat hindquarters and viscera shows considerable incorporation of the isotope in the tissue proteins, small but significant incorporation of the isotope in the serum globulin fraction, and none in the albumin or fibrinogen fraction.

DISCUSSION

The experiments presented in this report would appear to leave little doubt as to the dominant role of the liver in plasma protein synthesis, notably the synthesis of practically all albumin and fibrinogen and probably more than 80 per cent of the globulin fractions. However, many questions concerning the identity of the components of the globulin fraction remain to be answered. In particular it is desirable to precisely assess the hepatic contribution, if any, to the biosynthesis of antibody γ-globulin. This is desirable in the light of a recent report (12) which, on the basis of demonstrated localization of antigenic material in the liver, assumes that the liver is involved in antibody formation. The liver is well known to be the site of localization of intravenously injected foreign material, especially when such material is colloidal in nature. The isolated perfused liver from an actively immunized animal can perhaps be used as a test system to study the role of the liver in antibody formation.

The demonstration (5) that the isolated non-hepatic tissues rapidly remove amino acids from the circulating blood, primarily for tissue protein synthesis but also for the elaboration of a fraction of the plasma globulin, is in harmony with other observations made on hepatectomized dogs (4, 32). Furthermore, they afford a basis for a working hypothesis as concerns the hypoalbuminemia and inverted A/G ratio noted in advanced cirrhosis with clinical signs of portal vein obstruction. Normally the liver must be traversed before amino acids can reach the systemic circulation, and to the extent that they circumvent the liver as a consequence of portal obstruction they become available to the non-hepatic organs in larger measure. Since these latter are capable of synthesizing some serum globulin but not albumin, it is conceivable that a larger portion of absorbed amino acids are metabolized by the non-hepatic tissues with a corresponding disproportionate synthesis of serum globulin. This is not to minimize

the factor of impaired functional ability of the cirrhotic liver *per se*, which reduces the ability of the organ to metabolize substrate becoming available to it.

The mechanism of the biosynthesis of proteins has long fascinated biochemists, but the technical difficulties of isolating and characterizing proteins have prevented progress in the attack on this problem. The advent of isotope techniques has led to renewed interest, and the results of the liver perfusion studies just described above invite comparison with experiments using other techniques.

The use of homogenates (38, 36), tissue minces (28), and tissue slices (1, 6, 7, 14 22–26, 37) as *in vitro* protein synthetic systems is fraught with the as yet unsurmounted difficulty of separating and differentiating (or proving identical) isotopically labelled extracellular proteins (such as the plasma proteins) from the complex mixture of scrambled enzymes, intracellular proteins, or protein-like substances from which they may have arisen and which may also be isotopically labelled and show other similarities.

It is often difficult to believe that some of the reported isotopically labelled coagula are any more related to native proteins than the "plastein" (10) once described. The observation of Winnick using homogenates (35) that C^{14}-labelled glycine may be incorporated in and remain with a trichloracetic acid-insoluble protein coagulum only to become readily diffusible after mild alkaline treatment emphasizes the fact that the products of *in vitro* protein synthetic systems must be more clearly separated and adequately characterized. As previously mentioned, the specific activities of the protein fractions obtained from our perfusion studies were unaltered by repeated or prolonged treatment with 0.1 N or 1.0 N NaOH at room temperature. Controls on simple physical adsorption, entrapment in interstices, or the formation of loose bonds with the free or superficial carboxyl amino or other groups, may be possible, using labelled unnatural amino acids; *e.g.*, D-lysine in the work of this paper and that of reference 8.

Because of frequently possible serological cross-reactions for somewhat similar homologous intra- and extracellular proteins (30), the use of specific immune serum does not insure separation or identification of plasma proteins from an incubated liver homogenate. Two studies (26), using liver slices from unfasted chickens and C^{14}-labelled protein products, have shown a net synthesis of C^{14}-labelled chicken serum albumin. With no evidence that the anti-chicken–albumin serum (used in a quantitative precipitin reaction to identify the newly labelled protein) had been tested to control the real possibilities of cross-reactions with liver cytoplasmic or nuclear constituents, it becomes impossible to ascribe the occurrence of an albumin-like protein in the aqueous buffer medium solely to a net synthesis of serum (extracellular) albumin by the liver cells. Although the most radioactive (highest specific activity) protein fraction obtained from the incubated medium was closely associated with that of added carrier chicken serum albumin during electrophoresis and behaved similarly to chicken serum albumin on ultracentrifugation, it appears unusual that the "serum albumin" fraction had a specific activity some 47 times that of the globulin components (C^{14} A/G ratio of 47). Unless experiments with the intact chicken make plain

that this species differs to this extreme from the dog, rat, and rabbit with their C^{14} A/G ratios of 0.3 to 2.0 in both intact animals and isolated perfused livers (24, 5, this paper, and unpublished rabbit experiments in this laboratory), it becomes very difficult to reconcile such an enormous C^{14} A/G ratio with true plasma protein synthesis.

Moreover, in both of the studies now under consideration, as well as in a third (1) in which with the aid of added crystalline ribonuclease in 100-fold amount as inert carrier crystalline ribonuclease containing radioactivity was isolated from incubated beef pancreas slices, C^{14}-bicarbonate was the only precursor of the labelled carbon in the isolated protein. Furthermore, none of the above or similar attempts to demonstrate protein synthesis with *in vitro* homogenate or slice systems has shown either the necessity of having at least the essential amino acids present or any quantitative dependence of resultant protein product upon available amino acid substrate, two conditions which characterize the results in both intact animals and experiments with the isolated perfused liver. It is no longer enough to assume that the scrambled homogenate or slice system contains "within itself" all necessary substrate.

The use of aqueous media, buffered or otherwise, in liver slice or homogenate experiments does not exclude the strong possibility of literally washing out intracellular proteins from the mechanically or chemically (anoxia, wastes) damaged cells; many published curves of protein synthesis or incorporation by homogenates or slices strikingly resemble the expected curve of a purely physical diffusion or washing-out process.

Thus to us it seems much better to leave the cell membrane, the exchange surfaces, and the bile, lymphatic, and vascular channels and flows intact—untraumatized by handling, slicing, compression or rupture, and uncompromised by relying upon diffusion through a passive slice (however thin) to supply oxygen and substrate and to remove products and wastes. We believe that in the isolated perfused liver with minimal handling and disruption of blood flow, the cells remain physiologically as well as anatomically intact and are able to perform for us the bioseparation of the extracellular products from the intracellular contents of enzymes, substrates, or precursors.

The conclusion that the isolated perfused liver affords a method for studying protein synthesis *in vitro* under conditions simulating those in the intact animal draws strong support from the following considerations:

(*a*) The perfused liver discriminates between the D- and L-isomers of lysine with a virtual failure of response to the unnatural isomer (Experiment RLP 15), in spite of otherwise optimal conditions for protein synthesis.

(*b*) The perfused liver *repeats its action quantitatively* when a second dose of C^{14}-labeled substrate is given, as concerns (*a*) the removal of amino acids, (*b*) the oxidation of amino acids to CO_2, and (*c*) the synthesis of the plasma proteins even after already having been perfused for 4 hours. This is in distinct contrast to the failure of a liver homogenate to respond to a second dose of substrate at any time in spite of the addition of numerous potentially involved factors (6, 7), or indeed after about 1 hour to respond to anything.

(*c*) Amino acids are removed continuously from the perfusing blood as noted in serial chromatograms of the non-protein fraction of the plasma. The ability to clear amino acids from the blood has been recognized as a normal physiological function of the liver. This disappearance of amino acids, corroborated by quantitative colorimetric ninhydrin determinations done on the non-protein fraction, closely parallels the disappearance of non-protein radioactivity in perfused blood and in the intact animal.

(*d*) Bile secretion totalling 0.7 to 1.5 ml. in 6 hours has been observed to occur continuously throughout six rat liver experiments in which the common bile duct was cannulated incidentally to the perfusion. Shortly after the start of perfusion this bile contains visibly increased pigment presumably owing to slight hemolysis in the perfusate, samples of which contained much less hemoglobin from hemolysis than in those experiments in which the bile duct had not been cannulated, but tied off.

(*e*) The essential similarity of the quantitative response of the perfused liver to that of the intact animal in (*a*) the per cent of C^{14}-labeled amino acid converted to CO_2,—which has varied between 19 per cent and 30 per cent of L-lysine-ϵ-C^{14} in both types of experiments,— (*b*) the percent of L-lysine-ϵ-C^{14} converted to liver protein,—which under optimal conditions has varied between 2 per cent and 8 per cent of the total dose in the dog (30) and rat (unpublished observations),—and (*c*) the per cent of L-lysine-ϵ-C^{14} converted to plasma proteins with similar C^{14} A/G ratios in both dog (30) and rat (unpublished observations). Although not figuring amongst the data presented here, the results from eight rabbit experiments are in harmony with these conclusions.

The potential protein synthetic capacity of the isolated liver can be estimated from the following simple considerations:—The isolated rat liver synthesizes within 6 hours a total of about 20 mg. of plasma proteins and an equal amount of liver protein from a complete mixture of amino acids totalling 327 mg. Assuming that a rat of 300 gm. weight eats a daily diet of which 20 per cent is a complete amino acid mixture, then such a rat consuming 15 gm. of food a day would consume the equivalent of some 3000 mg. of amino acids. Assuming that both plasma and liver proteins are synthesized to the same extent as in the isolated liver receiving 327 mg. of amino acids, then $\left[\frac{20(3000)}{327}\right] = 183$ mg. each of plasma and liver proteins would be produced per 24 hours.

With the liver of such a rat containing about 2000 mg. of protein, and the circulating plasma about [300 (8.0/100) (5.1/100) (60)] = 740 mg. of protein, it is at once apparent that the liver as studied above is fully capable of producing enough liver protein and plasma protein to permit a daily turnover of about 9 per cent of the liver protein and about 25 per cent of the total circulating plasma protein. These estimates are well within or in excess of the turnover rates arrived at by isotopic studies in intact animals. Taken together

with similar calculations made in experiments on the isolated hindquarters of the carcass with and without the non-hepatic viscera (5), one may surmise that the liver is capable of synthesizing all the plasma albumin fraction, all the fibrinogen, and at least 80 per cent of the total serum globulin fraction.

APPENDIX TO DISCUSSION

Comments Regarding the Effect of Anoxia upon the Viability of Hepatic Epithelium

The desirability of objectively defining early hepatic cellular damage or death is implicit in much that has been written about the effects of anoxia on hepatic morphology and function. Govan (*Brit. J. Exp. Path.*, 1950, **31,** 485) believed he could see the first microscopic signs of liver cell damage at one-half hour after experimental fat emboli reached the portal circulation, with fatty vacuolation at 2 hours and definite necrosis in 4 to 8 hours; he of course had the advantage of comparing small focal damaged areas with large surrounding normal areas, and he measured no function. Popper and his group have elaborated on histologic criteria of human liver cell damage and death (*Arch. Path.*, 1948, **46,** 132; *J. Lab. and Clin. Med.*, 1948, **33,** 435; *Am. J. Med.*, 1949, **6,** 278), but the only time-clue offered is that the "perisinusoidal spaces of Disse" do not appear in liver biopsies unless at least 10 minutes to 1 hour of agonal suffocation has preceded the biopsy. Markowitz (*Physiol. Rev.*, 1951, **31,** 188) has reviewed the literature on the interruption of the hepatic arterial supply in dogs and rabbits in which death of the liver and the animal is a matter of days unless prevented with antibiotics. Similar studies in rats have apparently not been made.

Physiologically speaking, attempts to define cell damage or death are equally lacking in precise criteria. Many homogenate systems in which there may not be a single intact cell, continue to "respire" at a decreasing rate for over an hour; liver slices, even though most of the cells may be leached or dead (Opie, *J. Exp. Med.*, 1948, **87,** 428) also continue to show decreasing enzyme activity for as long as 4 to 5 hours (Anfinsen, *J. Biol. Chem.*, 1949, **179,** 1001), though only the superficial peripheral cells are able to produce glycogen (Deane, *J. Cell. and Comp. Physiol.*, 1947, **30,** 255). Using a perfusion technique very similar to ours, Brauer (*Am. J. Med.*, 1951, **11,** 237) has observed continuous bile flow for 26 hours.

Clinically, Hay (*Surgery*, 1951, **29,** 826) has shown that survival of shock cases can be greatly improved by increasing the arterial blood flow to the liver, while Kaufman (*New England J. Med.*, 1950, **242,** 90) has shown that diagnostic anoxia (breathing 10 per cent oxygen) may transform a borderline function test into a positive test of liver dysfunction. Engstrand (*Acta chir. scand.*, suppl. 146, 1949) has compiled a few data and a monumental review on variations of blood and oxygen supply to the liver, including the finding by Tanturi and Ivy (*Am. J. Physiol.*, 1938, **121,** 61) that after 30 minutes of total ischemia (occlusion of portal vein and hepatic artery) bile secretion returned from zero to normal within 40 minutes. They stress the importance of the intrahepatic vascular pressure and note that in shock, decreased liver function (bile and BSP[7] excretion falls, blood amino acid nitrogen rises) is associated with the hypotension and hypoxia.

[7] Bromsulfonphthalein.

We are thus left with a conclusion that is more inferential than definite, namely that the isolated liver perfused with whole blood very closely resembles in performance the liver in the intact animal and to that extent may be regarded functionally if not philosophically as a living organ.

SUMMARY AND CONCLUSIONS

A direct study of the isolated rat liver perfused with oxygenated blood containing amino acids and lysine-ϵ-C^{14} has yielded facts indicating that the liver synthesizes practically all the plasma fibrinogen, the albumin fraction, and probably more than 80 per cent of the plasma globulin fraction.

The response of the isolated perfused liver in protein synthesis is qualitatively and quantitatively analogous to that of the intact animal, notably in (*a*) the ability to discriminate between natural L-lysine and D-lysine, (*b*) the per cent of isotopic amino acid converted to CO_2, (*c*) the per cent utilized in liver and plasma protein synthesis.

The results obtained with the perfused liver are compared and contrasted with those reported for tissue homogenates, minces, and slices.

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