PLASMA AND TISSUE PROTEINS PRODUCED BY NON-HEPATIC RAT ORGANS AS STUDIED WITH LYSINE-ε-C14*, ‡, §

GAMMA GLOBULINS THE CHIEF PLASMA PROTEIN FRACTION PRODUCED BY NON-HEPATIC TISSUES

By LEON L. MILLER, M.D., C. G. BLY,|| M.D., AND WILLIAM F. BALE, Ph.D.

(From the Departments of Radiation Biology and Pathology, The University of Rochester School of Medicine and Dentistry, Rochester, New York)

A recent report (1) presented direct evidence for the dominant role of the liver in the production of plasma proteins, as studied in the isolated perfused organ, and compared the results with those obtained in intact living animals.

This paper describes an attempt to assess the comparative role of the extra-hepatic tissues in plasma protein synthesis. For this purpose, ten "carcass"[1] perfusions have been carried out, using in general the same apparatus, lysine-ε-C14, and methods previously described (1). Here again comparisons are made with results obtained in intact animals. The data show that the extrahepatic tissues produce but very small amounts of plasma protein, entirely of the nature of globulins, despite the incorporation of large amounts of lysine-ε-C14 into tissue protein.

In order to define more clearly the plasma proteins produced by the non-hepatic tissues, plasma from eviscerated surviving rats injected with lysine-ε-C14 has been fractionated by the technique of preparative zone electrophoresis (2). The non-hepatic tissues of the eviscerated rat incorporate the labeled lysine chiefly into the plasma proteins with the mobility of the gamma globulins. The

[1] Carcass, as used in this paper, refers to the caudal 50 to 60 per cent of the rat, with or without infradiaphragmatic non-hepatic viscera.

beta and alpha globulin fractions contain only small but measurable activity, and the albumin fraction contains no significant radioactivity.

The theories on the origin of the plasma proteins have had a long and devious history. The generally indirect evidence indicating that the liver is probably the sole source of albumin, fibrinogen, and prothrombin, and the major source of most of the globulins has been reviewed (3, 4). The conclusions of these reviewers were based largely on clinical studies of the disappearance and depressed replacement rates of these plasma proteins from circulation in patients with specific liver injury, hepatectomies, Eck fistula, etc. This topic has been reviewed by Gutman (5). The confusion that still exists from the use of this approach has been surveyed in some detail (1) and is illustrated in the following paragraph, showing both extremes.

The profound (50 per cent) drop in albumin, and relative constancy of the globulin levels in living rats within 23 hours (without plasma volume data from which total amounts could be estimated) after abdominal evisceration suggest that either (a) the liver is necessary to produce albumin while extrahepatic tissues could produce most of the globulins, or (b) there is a (severe) preferential utilization of albumin by the peripheral tissues with cessation of removal of globulin from the blood (6). The immediate decline of all plasma proteins after hepatectomy in the dog has been ascribed to trauma, hemorrhage, and circulatory readjustments (7). Others (8) noted occasionally increased pseudoglobulins, together with absence of plasma protein regeneration after plasmapheresis, in hepatectomized dogs. The severe fall in plasma prothrombin and fibrinogen after hepatectomy (9, 10) may be as much related to surgical trauma and clotting as to the absence of the liver. Still others noted no change (electrophoretically) in the plasma protein patterns after hepatectomy except possibly a decrease in beta globulins (11). The tissue proteins of hepatectomized dogs showed higher specific activities than did those from sham-operated controls, and plasma proteins showed considerably lower activities, suggesting that the liver is essential to plasma protein synthesis but not to tissue protein production (4).

The suggestions, originating with Halliburton in 1887 (cited in reference 12) that some of the circulating proteins, particularly beta and gamma globulins, may be produced by lymphoid tissue, spleen, or reticuloendothelial (extrahepatic) phagocytes have been made by several other authors (6, 13–21) and reviewed by White and Gordon (12). These suggestions are based largely on the results of electrophoretic or immunological studies. The suggestion has been also made (12) that the lymphocyte might act as a protein storage cell or carrier, analogous to the fat cell. Tissue culture experiments (22) have indicated that immature lymphocytes and plasma cells may liberate antibody (beta and gamma globulin) proteins. The increased levels of these two proteins in the sera of rheumatoid arthritis have been associated with bone marrow plasmacytosis (23). The abnormally high plasma globulin levels occurring clinically in infections, such as hepatitis, lymphogranuloma venereum, kala-azar, granuloma inguinale, cirrhosis, multiple myeloma (5), and mesenchymal diseases (24), also suggest an origin of some globulins, normal or abnormal, from extrahepatic tissue such as bone marrow, plasma cells, leukocytes, or lymphoid tissue.

From the above sample of the literature, the only conclusion which may be safely drawn is that practically any cell in the body might contribute at least small amounts of protein to the circulating plasma, whether that protein be an unmodified intracellular protein or not.

Methods

The general methods and apparatus used, with certain changes as mentioned below, have been previously described (1). The perfusing pressure was kept at about 120 to 130 cm. of blood, equivalent to the normal rat blood pressure of 95 to 100 mm. mercury (25), with 3 to 5 cm. fluctuations related to the pump stroke volume.

Under ether or 50 per cent CO_2–50 per cent O_2 anesthesia, the peritoneal cavity of the carcass donor rat was opened from xiphoid nearly to symphysis. In the first carcass perfusion (RHQ 1)[2] the colon and other viscera and related vessels were resected, ties loosely placed about the aorta and vena cava, and the inflow cannula containing saline and heparin inserted and tied into the abdominal aorta just below the renal artery orifices. The outflow cannula, similarly prepared, was ligated into the inferior vena cava at the same level. The muscles, skin, and spine at the mid-lumbar level were quickly severed and the supine preparation suspended loosely above the flask on a sloping stainless steel platform. The outflow cannula was adjusted so that the rate of flow could be observed by drop-count, and calibrated by a graduated container. A saline-moistened gauze covering the preparation minimized drying and evaporation. This preparation thus included the muscle, bone and marrow, small lymph nodes, and skin of the caudal half of the animal, as well as the pooled red and white blood cells of the perfusate. In the last nine carcass perfusions (RHQ 2 through RHQ 10) the kidneys, ureters, pelvic urogenital viscera, bladder, and adrenals were also included in the preparation, and in the last eight, the testes also. This was effected by cannulating and sectioning the specimen at higher levels, and ligating the celiac axis branches where necessary.

Two of the preparations (RHQ 3 and RHQ 4) included subcutaneously implanted large growing Walker tumors. Since the C^{14}-labeled plasma proteins produced by them did not appear to differ in the gross from the other carcass preparations, they are included in the plasma protein results. Tissue protein synthesis by the tumor preparations will be discussed elsewhere.

In the last five experiments (RHQ 6 through RHQ 10) ties were loosely placed about the portal vein and inferior vena cava. The hepatic artery was ligated as distally as possible to allow for anomalous gastrointestinal and adrenal arteries, the thorax opened, and the aorta cannulated just above the diaphragm. Outflow cannulae were quickly placed in the portal vein and the vena cava, the liver removed, and the carcass sectioned just above the diaphragm and adjusted so that rates from both outflow cannulae could be individually measured. This type of preparation thus also included the gastrointestinal tract, spleen, pancreas, and mesenteric lymph nodes supported by the diaphragm and the clamped abdominal wall.

The total operative procedure usually lasted 30 to 35 minutes from skin incision to closing the perfusion apparatus. As soon as the inflow cannula was tied into the aorta, warm oxygenated blood could be introduced intermittently into the preparation from the perfusion apparatus while the operation was completed. This insured that the preparation was not without active blood flow for more than 5 to 8 minutes. The ooze from small sectioned muscle and skin vessels soon stopped by clotting and drying. The hind legs were remotely connected to the pump drive shaft and thus passively moved during the perfusion in an attempt to facilitate venous and lymphatic return from the large muscle masses.

In the intact animal experiments, in an attempt to at least partially by-pass the liver, the

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[2] Rat hind quarter.

substrate was slowly (for 2 minutes) injected intracardially through a No. 26 hypodermic needle, while the rat was anesthetized with ether.

Each of the perfused preparations was presented with the same substrate mixture added to 100 to 150 ml. of heparinized blood freshly drawn from 18-hour-fasted rats and diluted with saline and substrate; the initial hematocrit reading was 30 to 37 volumes per cent. The substrate mixture previously described in detail (1), consisted of 160 mg. of the essential amino acids (Merck, VujN), 167 mg. of the non-essential amino acids, 500 mg. of anhydrous dextrose, and 2.2 to 5.2 mg. (1.5 to 4.2 μc.) of DL-lysine-ϵ-C^{14} monohydrochloride[3] (so that the total L-lysine added ranged from 18.1 to 19.2 mg.). As soon as the perfusion was going smoothly the substrate was added into the reservoir, mixed thoroughly by swirling, and the experiment then reckoned as starting at this time.

Samples of the perfusing blood were withdrawn from the reservoir at 5 to 10 minutes, 30 minutes, and hourly thereafter, mixed with about 10 mg. of solid sodium oxalate in graduated centrifuge tubes and set aside without cooling until the close of the experiment. At the close of the experiment all tubes were centrifugalized simultaneously, hematocrit readings recorded, and the plasma separated. Aliquots of whole plasma were processed for independent measurements of C^{14} activities in the whole plasma, total plasma protein, albumin, globulin (dissolved from filter paper by warm saline), fibrin, and total serum protein fractions by methods previously described (1), and for plasma total protein, albumin, and non-protein nitrogen analyses using standard micro-Kjeldahl methods. One and two dimensional paper chromatograms were run with phenol and collidine on carrier-free, trichloracetic acid precipitated, ether-extracted aliquots of plasma in experiments RHQ 6 and RHQ 7.

Serial collections of "expired" carbon dioxide were made in 40 per cent potassium hydroxide, and aliquots analyzed for C^{14} activity in RHQ 7 and RHQ 8.

The feces and the urine from an experiment were approximately separated (in one experiment this was almost impossible because of diarrhea), urinary volumes measured, and the feces added to colon contents for analyses. A sample of terminal bladder urine was analyzed for C^{14} activity.

All tissues were accurately weighed and sampled, except skin, muscle, and bone and marrow. From dissections in the laboratory, the skin, muscle, and bone and marrow included in the carcass perfusion preparations amount to 13 to 14 per cent, 27 to 29 per cent, and 4.2 to 4.8 per cent of the intact animal, wet weight. The bone marrow alone was assumed to make up about 40 per cent of the weight of bone and marrow together, from data given in reference 27. Samples for assay of muscle and of skin were taken from back, flank, and leg. Initially bone marrow was separated from the bone of the femurs, tibias, and once, the fifth lumbar vertebral body; the specific radioactivities of marrow protein were the same from these three areas, and were practically unchanged if the bone ossein also was included.

The organs and tissue samples were dissected, frozen, and lyophilized. They were then ground in a mortar, crudely extracted with acetone at room temperature to remove most of the tissue fat, and again powdered in a mortar. Brief treatments of the tough fibrous tissue in muscle, skin, and portions of the gastrointestinal tract with added alkali (0.1 to 1.0 N NaOH), and reprecipitation by trichloracetic acid, all at room temperature and with inert carrier lysine, facilitated the process of obtaining representative aliquots. Duplicate samples were processed without the aid of alkali. They showed no difference in total weight or specific C^{14} activity, if the samples were reprecipitated from the sodium hydroxide solution by trichloracetic acid within a few hours and then allowed to stand for at least 24 hours. The trichloracetic acid–precipitated protein was then separated by centrifugation, resuspended and stirred

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[3] We are indebted to Dr. R. W. Helmkamp and Dr. Carl Claus for the synthesis of the DL-lysine-ϵ-C^{14} by methods previously described (26).

in fresh 6 per cent trichloracetic acid containing inert DL-lysine-HCl, allowed to stand a few hours and again centrifugalized. This washing procedure was repeated twice more, omitting the carrier lysine on the last wash. This crude tissue protein was then resuspended in acetone. Here, to be certain that no trichloracetic acid–protein complex remained dissolved in the acetone, an excess of acetone was added, or the trichloracetic acid was neutralized to pH 5–7 with a few drops of 1 N sodium hydroxide and allowed to stand 24 hours. The precipitate was filtered, washed on the filter with acetone, dried, and aliquots assayed for C^{14} activity and nitrogen. Calculations and radioactivity assays were carried out as previously described (1).

Serial blood glucose determinations were done by the method of Hagedorn and Jensen (28), modified so that the samples, after the heating and reduction of alkaline ferricyanide, were compared colorimetrically with similarly treated standards and blanks.

In order to study the plasma proteins produced by the eviscerated surviving rat, normal adult male Wistar strain rats were eviscerated by the two stage procedure of Ingle (29) with a constant intravenous infusion of glucose and insulin, in physiological saline as recommended by Ingle. This affords a surviving rat without its liver, gastrointestinal tract, pancreas, and spleen. In order to control the effects of the removal of the pancreas, spleen, and gastrointestinal tract, the evisceration procedure was carried out essentially as above except that the liver was allowed to remain supplied with blood by the hepatic artery. Shortly after closing the abdominal cavity, 1 ml. of a solution of DL-lysine-ε-C^{14} (6.76 mg. DL-lysine·HCl, 6.5 μc.) was injected into the femoral vein in about 5 minutes. 6 hours after the infusion of lysine the rats were exsanguinated by cardiac puncture. The heparinized plasma was then subjected to preparative electrophoresis by the technique of Kunkel and Slater (2), and the resultant fractions analyzed for protein and C^{14} activity as described previously (30).

Occasional histological sections from various organs of the experimental animals were compared with those from organs which had been allowed to remain in dead exsanguinated animals or in warm saline for the same length of time.

Sections of the tissues of the perfused carcasses were examined in each experiment by means of histologic methods used previously (1), and compared with fresh tissue and control tissues from rats which had lain dead for 6 hours. In the gross, the walls of the gastrointestinal tract at 6 hours were usually somewhat swollen and edematous around a lumen distended with bloody fluid, chyme, and feces; microscopically, most of the sections appeared normal except for only moderate edema and hyperemia of the muscle, submucosa, and mucosa, but there were in other focal areas considerable desquamation of mucosal cells, focal infiltration of small numbers of neutrophils into the mucosa, and occasional small fresh hemorrhages into the lumina in some sections. The *kidneys* generally appeared quite normal (with clear brush borders) except for diffuse slight hyperemia and occasional cloudy swelling; however, in occasional areas there were seen focal severe hydropic and vacuolar degeneration of the tubular epithelial cells, and focal perivascular edema and tiny hemorrhages; in only one section was there an area showing focal fresh hemoglobin casts in the tubules. The *spleen* appeared normal except for slight dilatation of the sinusoids and occasional hemolysis of the red cells. The *pancreas* sections were normal, without autolysis, but there were slight hyperemia and septal and

perivascular edema. The *bone marrow* was also normal but hyperemic with, occasional pale-staining areas suggesting autolysis. The *lymph nodes, testes* and *muscles* showed only mild hyperemia and edema.

It must be pointed out that the routine histological procedures would not be expected to reveal any very particular cellular damage within 6 hours and, while sections of the perfused carcass tissues in general looked no worse than either control group, neither did they (though supplied with oxygenated warm blood for the period) look any better than the tissues from the rat which had lain dead for 6 hours without any circulation. This reflects only on the present worthlessness of histologic criteria for short term (6 hours') evaluation of tissue viability. It is possible that special histochemical methods for testing the activity of enzymes, or even the sensitive methyl green–pyronin stain, might have revealed evidences of deterioration.

Preliminary Physiological Consideration

The authors are under no illusion that the "carcass" type of preparation functions as well as either the intact animal, the eviscerated surviving rat or the perfused liver. It is our feeling that such a preparation has a limited period of function and viability not greatly in excess of 4 or 5 hours. Given below is presumptive evidence of at least partial viability and continuing function in terms of blood flow, disappearance of substrate, glucose consumption, carbon dioxide production, and incorporation of labeled lysine into tissue and plasma proteins. No attempts were made to evaluate the effects due solely to denervation and spinal section, nor to remove any initial or subsequent vasoconstrictor substances from the perfusing blood. The large volume of perfusate used (120 to 182 ml.) probably served to supply limited amounts of certain circulating hormones as well as to dilute noxious products and to permit serial sampling.

At the perfusion pressures used, rates of flow varied widely from experiment to experiment. Maximum rates of from 4 to 32 ml. per minute were attained at from $\frac{1}{2}$ to $4\frac{1}{2}$ hours with a generally rapid decrease in rate at around 4 to 5 hours, down to 1 to 9 ml. per minute at 6 hours. Despite these large variations in rate, there were, as will be seen later, quite similar absorptions of amino acids from the perfusate and corresponding incorporation of lysine-ϵ-C^{14} into tissue and plasma proteins among the experiments.

The slowing of the rates of flow from about the 4th hour on was usually accompanied by the first appearances of detectable rigor together with the cessation of spontaneous gastrointestinal contractions. The use of 50 per cent CO_2–50 per cent O_2 instead of ether as the anesthesia for blood and carcass donor rats in two duplicate experiments produced no significant changes in rates of flow nor onset of rigor mortis.

Interstitial edema, as measured by increase in carcass weight, checked well

with the decrement in recovered perfusate volume, and ranged from 0 to 9 gm. in the first five experiments, appearing in the gross as retroperitoneal or muscle succulence. In RHQ 6 through RHQ 10, when the gastrointestinal tract was included, the accumulation of clear fluid within the bowel lumina was first noticeable at about 2 hours, blood tinged fluid appeared after 3 hours, and distention with dark red bloody fluid progressed from about the 4th hour on. Interstitial edema fluid also increased (to an average of 29 gm.). The extent of edema was probably not related to tissue invasion by the gastrointestinal bacteria since the addition of penicillin in large dosages (RHQ 9: 75,000 units, and RHQ 10: 100,000 units) was without significant effect. In spite of this the numbers of bacteria (practically all penicillin-sensitive *Staphylococcus albus* and less sensitive *Proteus vulgaris* and *Escherichia coli*) in the perfusate, serially measured on blood agar and in thioglycollate broth, were markedly reduced by the antibiotic although never completely eliminated, throughout the course of both experiments.

Bladder distention with urine and occasional micturition continued more and more slowly until the 5th and 6th hours. The total urine secreted in 6 hours was 1.1 to 2.5 ml.

Throughout the courses of all experiments the dark blackish-red color of the outflowing venous blood was in exceeding contrast to the bright red inflowing blood, indicating continuing oxygen consumption (as noted later, the oxidation of substrate and "expiration" of radioactive carbon dioxide did not begin to fall off until about the 4th hour).

<div align="center">EXPERIMENTAL RESULTS AND DISCUSSION</div>

A. Removal of the Amino Acid Substrate by Perfused Carcasses

In Fig. 1 are plotted curves showing the unabsorbed plasma non-protein radioactivity, which, as in the liver perfusion described in reference 1, is still at least 95 per cent in the form of lysine-ϵ-C^{14}, probably mostly D-lysine-ϵ-C^{14} at 6 hours.

Corrections have been made for plasma protein activity, for plasma and radioactivity removed in previous sampling, and for trapped plasma by assuming that the plasma resides between red cells (31) to the extent of 4 per cent of their volume on centrifugation.

The top group of two curves represents blank experiments in which the substrate mixtures were identical with those used in the other perfusions. However, no tissues were involved except the perfusate itself, including the red and white blood cells. In the preliminary blank run (rabbit blood) 86 per cent of the dose was still in the plasma after 6 hours' circulation in the apparatus. Correction for radioactivity retained by the blood cells would bring the total recovery dose to 100 per cent. Complete assays of all components in the second blank experiment (rat blood) resulted in 100 per cent recovery of the total radioactivity.

Evidence (1) indicates that within 6 hours, tissue such as the liver paren-
chyma can selectively remove most (about 82 per cent) of the natural L-lysine
isomer and some (about 7 per cent of which 1 to 2 per cent is possibly an L-
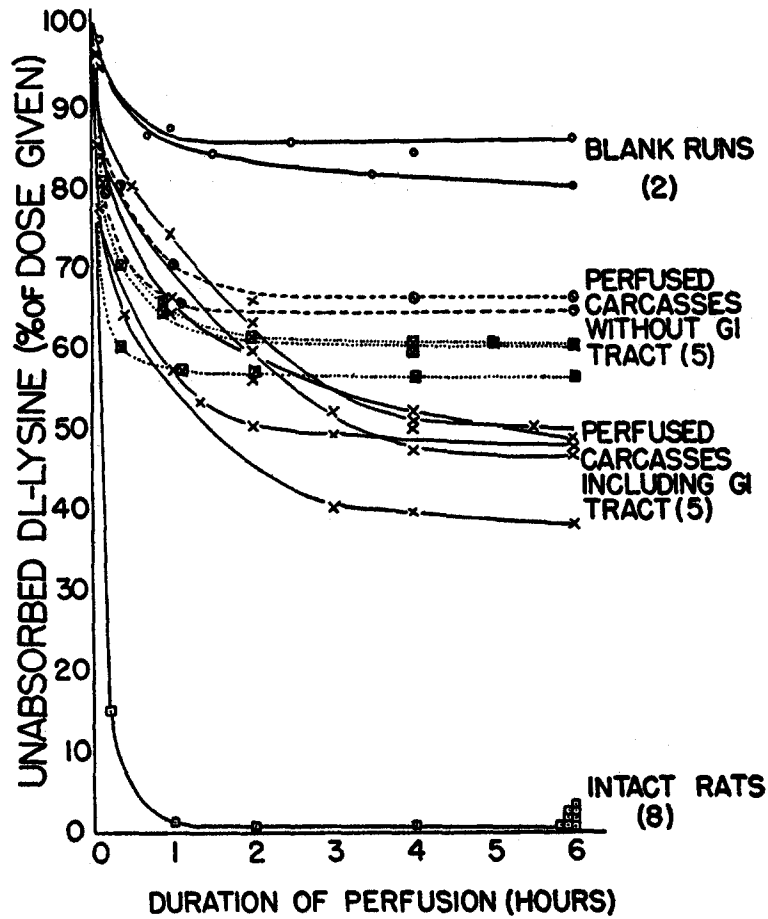lysine contaminant) D-lysine from the circulating plasma in a ratio of roughly

Fig. 1. Disappearance of non-protein radioactivity from the plasma. For description see
Results, section A.

12:1. The D-lysine is not incorporated into protein or oxidized by the liver (1),
nor by the intact animal (32), but is selectively and rapidly excreted into the
urine by the kidneys if they are present and normal, and slowly into the bile
by the isolated perfused liver. Either DL-lysine or D-lysine can become attached
to the red blood cells to the extent of 6 to 10 per cent of the initial amount of
DL-lysine, probably by adsorption. However, 95 to 99 per cent of the L-lysine

seems readily available to whatever tissues are being perfused as evidenced by analyses of the red cells at the onset and close of a 6 hour experiment. Using D-lysine (1, 32), the red cells at 6 hours contained about 15 per cent in the liver perfusion, but only 0.9 to 1.1 per cent in intact animal experiments (rapidly excreted in urine). Thus, the red blood cells appear to retain significant amounts of lysine only when no competing tissue, excretion, or utilization is present.

The upper group of five curves shows the disappearance of 34 to 44 per cent of the initial DL-lysine-ϵ-C^{14} from the plasma in the first five perfusions of carcasses (without gastrointestinal tract), in which the tissue masses weighed 125 to 180 gm. For comparison, in two typical liver perfusions (8.1 and 9.1 gm. livers, one animal starved 7 days, the other fasted the standard 18 hours) using the same substrate mixture, 47.3 and 46.3 per cent of the labeled lysine were removed from the plasma, representing a greater amount of uptake by only one-fifteenth to one-twentieth as much wet weight of tissue as in the carcasses. The disappearance curves drawn from the results of these five carcass perfusions have the same general shapes as do those drawn from the liver for the first 3 hours, but show very little disappearance in the last 2 to 3 hours.

The lower group of five curves show that, although the rates of perfusion varied widely (over-all average rates of 25, 8, 22, 4 and 5 ml. per minute, respectively) fairly constant amounts (51 to 61 per cent of the radioactive lysine had disappeared from the plasma at 6 hours by these five perfusions of carcasses including the gastrointestinal tract, pancreas, and spleen. Since there was no increased excretion of lysine by the kidney and since the expiration of labeled CO_2 was minimal (0.4 per cent for perfused carcasses), the increased removal (average of 54 per cent compared to 42 per cent by the first three carcass perfusions) of radioactive lysine by these last five carcasses must be attributed to the gastrointestinal tract itself. However, a large portion of this increased disappearance from the blood in these five perfusions is spurious and related to hemorrhagic fluid within the bowel lumina and the interstitial edema fluid, which together contained an approximated average of 8.3 $\pm$ 3.7 per cent (4.0 to 13.6 per cent) of the DL-lysine dose.

The bottom curve and group of points in Fig. 1 show for comparison that by 6 hours 97.2 to 99.4 per cent of the non-protein DL-lysine-ϵ-C^{14} has been removed from the circulating plasma of ten intact rats. As reported in reference 32, 99.4 to 99.7 per cent of D-lysine disappears from the plasma in four intact rat experiments. It thus is obvious that the intact animal removes virtually all the labeled lysine from its blood in 6 hours, and that the perfused carcass, with no liver to oxidize the excess L-lysine to CO_2, is by no means able to remove the lysine as rapidly or as completely as the intact animal. The tissues of the carcass, however, are able to clear the perfusate of approximately half of the radioactivity, or about the same amount removed by a perfused rat liver (having only one-tenth as much tissue mass as the carcass).

To study the metabolism of the other substrate amino acids besides the labeled lysine, serial aliquots of the perfusate plasma were assayed for the Kjeldahl levels of non-protein nitrogen, and in two experiments (RHQ 8 and RHQ 10) for the colorimetric level of the amino acids as a group, and for the paper chromatographic distribution patterns and relative amounts of the individual amino acids.

The data for the disappearance of non-protein nitrogen are not presented. However, granting the non-specificity of non-protein nitrogen as a measure of amino acid–nitrogen, it is noteworthy that the levels rose to around 170 per cent of the base line value with the addition of the 330 mg. of substrate amino acids at 0 time, and then fell back towards 140 to 150 per cent of the base line value as the perfusions progressed. That the disappearance of excess non-protein nitrogen from the plasma is actually due to absorption of amino acids by the perfused liver or carcass is indicated by the fact that data obtained from the more specific ninhydrin reaction on serial specimens from several liver perfusions and two carcass perfusions (RHQ 8 and RHQ 10) by the quantitative colorimetric ninhydrin method yielded similar results.

In contrast to the perfused carcasses, the almost complete removal of the excess non-protein nitrogenous substrate by the perfused livers is at least as rapid as the removal of the labeled L-lysine-ϵ-C^{14} (1), and suggests that the fasted normal liver rapidly removes most of the excess available amino acid substrate in the given dose range.

On the other hand, the poor correlation in the carcass experiments between removals (from the perfusate plasma) of (a) the radioactive L-lysine-ϵ-C^{14}, i.e. 35 to 60 per cent of the DL-lysine corresponding by assumed analogy to the liver experiments (1) to 70 to 80 per cent of the L-lysine, and (b) the excess non-protein nitrogen, suggests that either the lysine-ϵ-C^{14} is absorbed preferentially or that relatively large (compared to the liver) amounts of free amino acids from the carcass tissues are in dynamic exchange with those in the perfusate, resulting in dilution of the labeled lysine with unlabeled lysine from the tissues simultaneously with removal of lysine from the perfusate.

One and two dimensional paper chromatograms of free plasma amino acids from the carcass perfusions showed by visual inspection that several of the individual amino acids decreased serially in quantity. As in the liver perfusion experiments (1), some amino acids such as glycine, serine and aspartic acid appeared to be largely removed. Others, such as glutamic acid, leucine, isoleucine, and valine, appeared to be about half removed. Some, such as lysine, arginine, and tyrosine, remained at the same or slightly increased concentrations. On the other hand, there appeared to be visibly increased amounts of alanine, proline, phenylalanine, and especially taurine and glutamine. It is to be emphasized that the spots were not eluted for objective quantitation or identification of the individual amino acids, their identities being based on locations.

A recent paper (33) has depicted the general increase of plasma α-amino

acid–nitrogen and the frequent increases in the concentrations of some individual amino acids in hepatectomized dogs. This increase is presumably due to the (physiological) release from the non-hepatic tissues and the accumulation of these amino acids in the plasma in the absence of the liver. No mention is made, however, of the hemoconcentration which is often seen in hepatectomized animals. In our carcass perfusions there often appeared a 10 to 20 per cent increase in hematocrit reading during the 6 hour experiment, accounting in part for the increased concentration of some amino acids. On the other hand, relatively large (3 to 5 times the normal plasma content) amounts of the amino acids had been added as substrate to these perfusates, so that one would expect most of the amino acid levels to decrease as they have in our perfusions, certainly by diffusion into the tissues and red blood cells, and possibly by other mechanisms.

B. The Removal of Blood Glucose by the Perfused Carcasses

The net total removals of glucose from the whole blood perfusates in three carcass perfusions (RHQ 7, RHQ 9, RHQ 10), the two liver perfusions, and one blank perfusion have been computed with correction for previous samplings. Small amounts of glucose (56 mg.) disappeared from the blank experiment, presumably as a result of utilization by the red and white blood cells. The standard 18-hour-fasted perfused liver, showed a very small (27 mg.) net absorption of glucose. The 18-hour-fasted carcass removed 564 mg. of glucose, more than twenty times that of the liver from a similarly fasted animal (the fasted carcass also weighs about twenty times as much as the liver). The "7 day" starved liver, completely depleted of glycogen showed almost five times as great an uptake (126 mg.) of glucose. The 7-day-fasted carcasses, (RHQ 7 and RHQ 10) showed net uptakes of 508 and 512 mg. of glucose respectively, four times that of the starved 8 gm. liver. The three carcasses showed only minimal (2 to 11 mg.) net uptake of glucose after the 4th hour probably indicating substrate depletion, (only 500 mg. of glucose was added to the blood of each perfusion). This is to be compared to the slow, almost linear, removal of glucose by the livers for the whole 6 hours. If one assumes that the gastrointestinal tract contents, edema fluid, and urine (none assayed for glucose) all contained glucose in the same concentration as the whole blood, then the amounts of glucose present in these fluids of known volume could be as high as 9 to 12 per cent of the total glucose which has disappeared from the perfusate in the three carcass experiments.

No glucose determinations were made in intact animal experiments.

C. Distribution and Recovery of Total Radioactivity

The distributions and recoveries of total radioactivity at the end of comparable 6 hour experiments using intact animals, carcass perfusions, and typical liver perfusions (together with one blank perfusion) have been computed from the analytical data.

Apart from the radioactivity in plasma, red cells, urine, and expired CO_2, the tissues in the intact animal were found to contain total radioactivity amounting to 50 per cent of the dose, and similarly the tissues in the perfused carcasses (with only three-fifths as much tissue mass) contained 20 to 35 per cent.

The activities present in the empty stomach, intestine, and colon of the carcass were all nearly the same as their respective counterparts in the intact rat. This fits well with the suggestion (from disappearance curves, Fig. 1) that in both types of preparations, the radioactive and other substrate was normally absorbed from the blood by the viscera within the first 1 to 3 hours; *i.e.*, before the development of hemorrhagic edema in the perfused carcasses.

The pancreases from perfused carcasses contained only 40 to 70 per cent as much radioactivity as did those from comparable intact animals. Pancreases from 7-day-starved intact rats or carcasses consistently contained more total activity than did those from 18-hour-fasted rats.

The smaller amounts (only 13 to 24 per cent of that in the intact animals) of radioactivity recovered in the combined kidneys and urine from the perfused carcasses, are only partially accounted for by ten to twenty times greater dilution of the radioactivity in the perfusate plasma. This depression of metabolic activity, and the previously mentioned oliguria, presumably were due to renal vasoconstriction, anoxia, or toxemia.

Usually 80 to 90 per cent of the radioactivity was accounted for, although in the blank perfusion complete recovery was attained. Possible losses may have occurred in various unassayed organs or because of non-representative sampling, which was especially difficult in muscle, skin, and bone marrow.

D. The Oxidation of Lysine-ε-C^{14} to $C^{14}O_2$ by the Perfused Carcass

As would be expected from the demonstration given in reference 1 that the perfused liver alone produced as much $C^{14}O_2$ as did the intact animal, the extra-hepatic tissues of the perfused carcasses produced very small but measurable amounts of $C^{14}O_2$ not exceeding a total of 0.4 per cent of the L-lysine dose. As in reference 1, the maximal rates of $C^{14}O_2$ expiration occurred at 1 to $1\frac{1}{2}$ hours for both the intact rats and the perfused livers. In the perfused carcass experiments, however, the peaks (0.04 per cent of the DL-lysine per hour, or 0.08 per cent of the L-lysine) did not occur till around the 4th hour. Since bacterial growth was suppressed by penicillin in both of the carcass experiments in which carbon dioxide was collected, this $C^{14}O_2$ production and the lagging peak are probably not related to bacterial oxidations.

E. Plasma Protein and Tissue Protein Synthesis by Non-Hepatic Tissues

Compared to the perfused livers, and to the intact animals, the perfused non-hepatic tissue incorporated considerable portions of the C^{14}-labeled lysine into the tissue proteins but only very small amounts into the plasma proteins.

The groups of experiments are too small for statistical manipulation. Hence, in comparisons of the perfused carcasses with the parenterally dosed intact animals, or of the perfused livers with the orally dosed intact rats certain inferences can only be suggested, not proved. Table I summarizes the averages

TABLE I

Protein Syntheses (Averages)

			Per-fusion blank experiment*	Substrate by parenteral or "non-hepatic" route					Substrate by oral or "hepatic" route			
				Perfused Carcasses*		Intact rats			Intact rats		Perfused livers*	
				Without GI tract, etc.	With GI tract, etc.							
Section	Item No.	Column	(a)	(b)	(c)	(d)	(e)	(f)	(g)	(h)	(i)	(j)
		Prior fasting (hrs.)	18	18	18	168	18	168	18	720‡	18	168
		No. of experiments	1	3	2	3	1	1	3	3	1	1
A		*Synthesis of Lysine into Protein* (Per cent of L-lysine-ϵ-C^{14})										
	1	Non-hepatic tissue protein	0.02	5.4	13.5	8.3	26.3	27.4	x	x		
	2	Liver protein only					5.8	4.2	3.7	5.0	4.0	4.8
	3	Plasma protein	0.03	0.38	0.56	0.32	2.7	2.1	4.3	3.8	4.6	6.2
	4	Total protein	0.05	5.8	14.1	8.6	34.8	33.7	x	x	8.6	11.0
B		*Tissue "efficiency"* (Per cent of L-lysine-ϵ-C^{14} synthesized into protein per gram of total wet tissue involved)										
	1	Non-hepatic tissue protein§	0.06	0.035	0.078	0.053	0.094	0.089	x	x		
	2	Liver protein only					0.021 (0.75)	0.014 (0.71)	0.014 (0.51)	0.029 (0.83)	0.44 (0.44)	0.60 (0.60)
	3	Plasma protein	0.09	0.0027	0.0038	0.0020	0.010 (0.36)	0.007 (0.35)	0.016 (0.58)	0.02 (0.69)	0.50 (0.50)	0.77 (0.77)
	4	Total protein	0.15	0.037	0.077	0.055	0.125	0.110	x	x	0.94	1.37

x, incomplete or no data available.
( ), expressed in terms of per cent/gm. liver weight rather than of whole animal weight.
* Substrate added directly to the perfusate.
‡ These rats had been chronically semifasted to constant bodyweight for 30 days, incidental to another study.

of tissue and plasma protein synthesis by the various groups of whole animals or perfusion experiments.

In all liver or carcass perfusion experiments, the complete substrate mixture was added directly to the perfusate. In order to compare the results of carcass perfusion with those obtained in the intact rat, data from two "parenterally dosed" intact rats, which had been given an incomplete substrate (4.1 mg. of

labeled lysine only) by intracardiac route, are included. It is assumed that both of these groups of experiments are comparable to the extent that the substrate would be made available through the arterial circulation primarily to the non-hepatic tissues, and only secondarily to the liver if present. As a means of comparing the perfused livers with intact animals, data have also been included from a group of six "orally dosed" intact rats from another study, in which a similar complete substrate mixture was given by stomach tube. It is assumed that in the intact animals the substrate would, in its initial circulation at least be made available primarily to the liver through the portal vein, and only secondarily to the non-hepatic or carcass tissues.

Because of the uncertainty as to the amounts of the free amino acids already present in the tissues and perfusate before adding the substrate or liberated by catabolism and because of the resultant uncertainty of calculations of actual amounts of protein synthesized, based on the dilution of the added lysine-ϵ-C^{14} by the non-labeled lysine already present in the system, the results are expressed as percentage of the administered L-lysine-ϵ-C^{14} which has been synthesized into protein.

The perfused carcasses in which the gastrointestinal tract, spleen, pancreas, and mesenteric nodes are included, (Table I A, columns c and d) are equivalent to only 58 to 60 per cent of the tissue mass of the intact rats, contain no liver, and incorporate 40 ± 11 per cent of labeled L-lysine into non-hepatic tissue protein.

Data from the perfused livers (columns g to j, Table I A and I B) show a great similarity of perfused livers to livers in orally dosed intact animals, both in the quantity of new proteins synthesized and in the enhancing effect of starvation on liver protein synthesis.

The calculated "efficiency" (per cent of L-lysine-C^{14} incorporated into protein per gram of fresh tissue in the preparation) is a device introduced to correct for the difference in mass of the perfused carcasses, liver, or intact rats. With this as a criterion of the protein synthesis by the respective tissue groups (Table I B) it is found that gram for gram:—

(a) The perfused carcass tissues as a group (including gastrointestinal tract, spleen, pancreas, etc.) are about 70 ± 11 per cent as effective in tissue protein synthesis as are the non-hepatic tissues of the parenterally dosed intact rats. However, since the intact rats included livers, these tissue protein comparisons are correspondingly biased in favor of the intact rats.

(b) The perfused liver is about 7 ± 1 times as effective in its own tissue protein synthesis, after 18 hours of previous fasting of the donor animal, as are the non-hepatic carcass tissues (including the relatively inert bone and muscle) as a whole, and about 17 ± 4 times after 7 days of fasting. On the other hand, it is about 160 ± 35 times as effective in the synthesis and liberation of circulating plasma proteins after 18 hours' fasting as is the carcass, or about 440 ± 100

times after 7 days of fasting. If we combine the newly synthesized tissue and plasma proteins, the liver is still about 20 ± 5 times as effective after 18 hours' fasting, or about 40 ± 13 after 7 days' fasting.

The effects of fasting on plasma and tissue protein synthesis in the carcasses are noteworthy and are most consistent if the so called tissue "efficiencies" are studied (Table I B), although the experimental groups are too small to analyze statistically, and although the three periods of prolonged fasting, are not necessarily comparable to each other. The synthesis of non-hepatic tissue protein or non-hepatic plasma protein, by each gram of tissue involved as measured by the incorporation of L-lysine-ϵ-C^{14} is reduced by fasting.

Although it is believed that conclusions based on the summaries of percentage data as in Table I are the more defensible, calculations have been made of the amount of protein produced, based on the methods of calculation outlined in reference 1 and assuming among other things that the amounts of available free amino acid lysine present in the blood or tissues prior to the addition of the substrate or contributed by catabolism are negligible, and that only L-lysine and not D-lysine enters protein synthesis.

The calculations show that the tissues of the carcass vary in comparability with those of the intact rat in protein synthesis: the gastrointestinal tract and pancreas are about the same; the kidneys, lymph nodes, and testes are only 15 to 40 per cent as active; the remaining tissues range between 50 and 70 per cent as active; the per cent dose C^{14} incorporated into plasma protein of the perfused carcasses appear to be only about 15 per cent of that in the parenterally dosed intact rats and only 5 to 10 per cent of that in the perfused liver experiments or the orally dosed intact rats.

Fig 2 compares the uniformly very low and slowly released amounts of C^{14}-labeled plasma protein from the ten perfused carcasses, all at the bottom of the graph, with the large and rapid amounts produced by the perfused livers, one each from 18-hour-fasted and 7-day-fasted intact rats. The top curve represents composite data from eight intact rats which had been given the complete substrate orally or parenterally. The values in milligrams, calculated on basis of added substrate lysine only, are given with reservations and lose significance to the extent that the sizes, locations, and availabilities of various metabolic pools, particularly the lysine, are uncertain.

F. The Nature and Origin of the Plasma Proteins Produced by Non-Hepatic Tissues

Since the perfused carcasses do produce small but significant amounts of plasma proteins, the question arises as to what kinds of plasma proteins are produced. It has been shown in perfused rat livers, intact rats (1), and intact dogs (34) that globulin production exceeds albumin production which in turn exceeds fibrinogen production in the first several hours.

Serial independent determinations of the radioactivity present in the plasma albumin, globulin, fibrinogen, and total proteins, were done on specimens from 0 time through 6 hours. The time course of C^{14} incorporation into the plasma
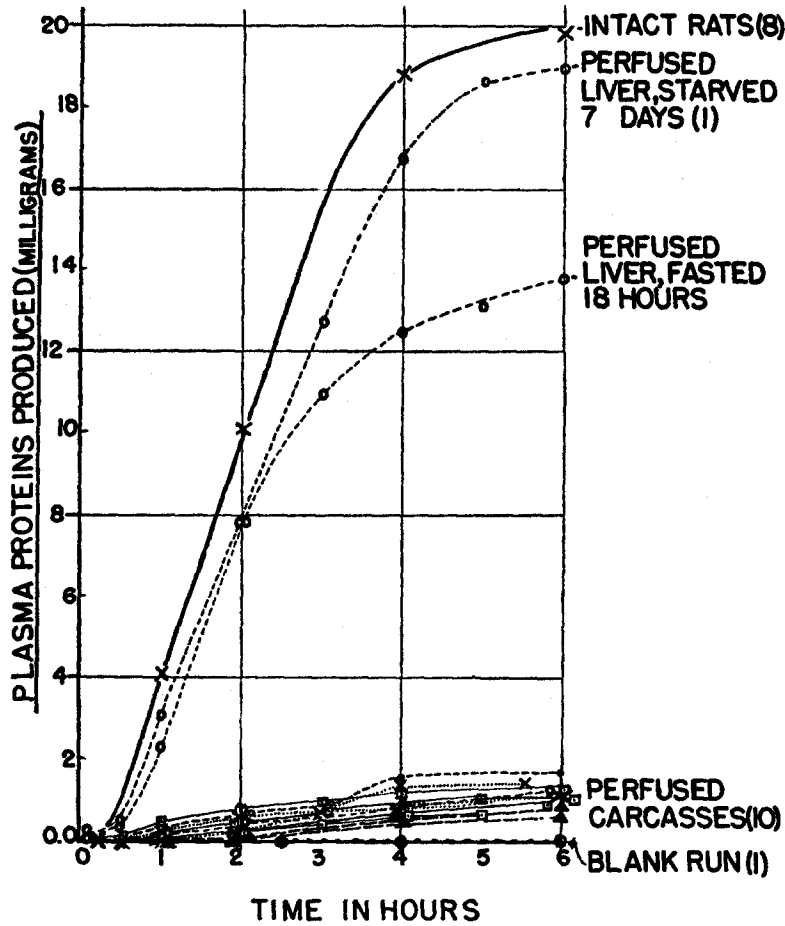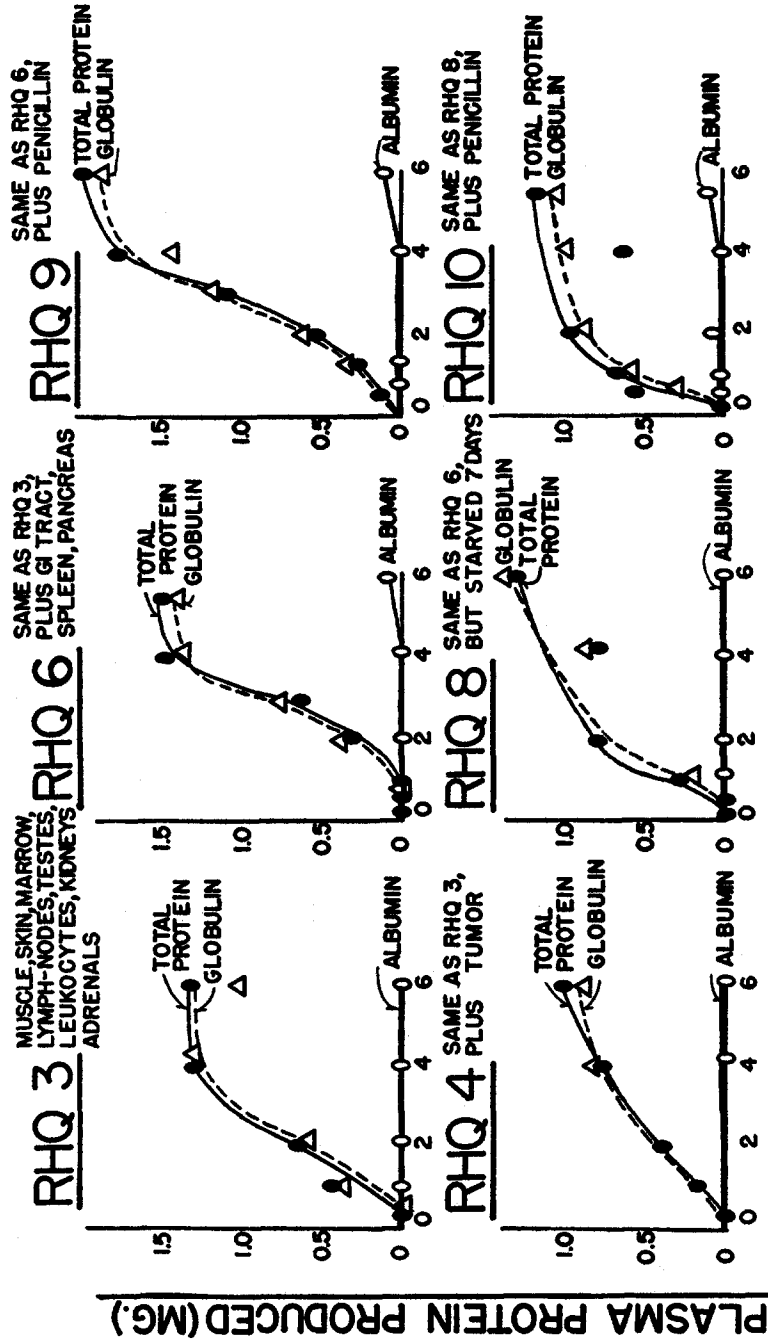

FIG. 2. Plasma protein synthesis. Comparison between rat liver and carcass, perfusing with same substrates. For description see Results, section E.

protein fractions is depicted in Fig. 3, expressed as plasma protein produced. With our salt fractionation methods (35-37,9) the albumin/globulin ratios (amount in albumin fraction divided by amount in globulin fraction) for protein-nitrogen were 0.90 ± 0.03 for all 18-hour-fasted animals, but for protein C^{14} were from 0.50 to 0.70 for the orally dosed intact rats, the parenterally dosed rats, and the perfused livers, and 0.00 in five out of the ten perfused carcasses

FIG. 3. Non-hepatic plasma proteins. For description see Results, section F.

at the end of 6 hour experiments. The 0.00 values in the carcass experiments are the more significant because assays were run on a volume of sample four times that usually used. In the other five carcass perfusions, the C^{14} activities of the albumins and fibrinogens were not determined in two experiments, and the C^{14}-albumin/globulin ratios in the other three stayed at 0.00 until the last samples at 6 hours in which the C^{14}-albumin/globulin ratio rose to 0.11, 0.13, and 0.15. Since the measured levels of radioactivity in these albumin samples were very low, they are of questionable significance.

No significant radioactivity was found in the serial purified fibrin proteins from these carcass perfusions, even with larger samples. In brief then, the perfused carcass can apparently produce 10 to 20 per cent as much plasma globulin as the perfused liver or intact rat in 6 hours with the substrate used, but practically no plasma albumin or fibrinogen.

The conclusions concerning the nature of the plasma proteins produced by the perfused carcasses have been amply verified by the examination of C^{14}-labeled plasma protein fractions using the preparative electrophoretic technique of Kunkel and Slater (2). These experiments reveal that the eviscerated surviving rat produces in large amount only plasma proteins with the mobility of gamma globulins. In distinct and complementary contrast the isolated perfused liver produces all of the plasma proteins except those with the mobility of the gamma globulins (30). Fig. 4 presents the results of a typical experiment of this kind. It is apparent that the proteins of the gamma globulin fraction contain most of the incorporated C^{14}. Furthermore the beta globulin and alpha globulin fractions contain comparatively very small but detectable C^{14} activity. The albumin is notably devoid of C^{14} activity. It is the specific activity curve which best portrays the quantitatively minor extent to which the normal rats' non-hepatic tissues synthesize plasma proteins other than the gamma globulins. The observed quantitative variation in the specific activity of the gamma globulins with varying mobility is compatible with the demonstrated non-homogeneity of the gamma globulins (38).

Because of the probable overlap of the fastest migrating gamma globulins and the beta globulins, a more precise evaluation of the extent of production of beta globulins by normal non-hepatic tissues is not possible on the basis of the data at hand.

By way of controlling the factors of shock incident to operative trauma, and the absence of the gastrointestinal tract, the pancreas, the spleen, and the lymph nodes of the mesenteric bed, eviscerated surviving rats were prepared in which the liver was allowed to remain supplied by the hepatic artery. Plasma obtained from blood removed 6 hours after the intravenous infusion of DL-lysine-ϵ-C^{14} was separated electrophoretically and the resulting qualitative and quantitative electrophoretic distribution of C^{14}-labeled plasma protein was essentially similar to that from the intact rat as described in the preceding paper (30).

Although the eviscerated surviving rat used in our studies was surgically deprived of the gastrointestinal tract and spleen it appears unlikely that the inclusion of these organs in a preparation such as the surviving hepatectomized rat would significantly alter our conclusion; viz., that the non-hepatic tissues are concerned primarily with the elaboration of the gamma globulins. This
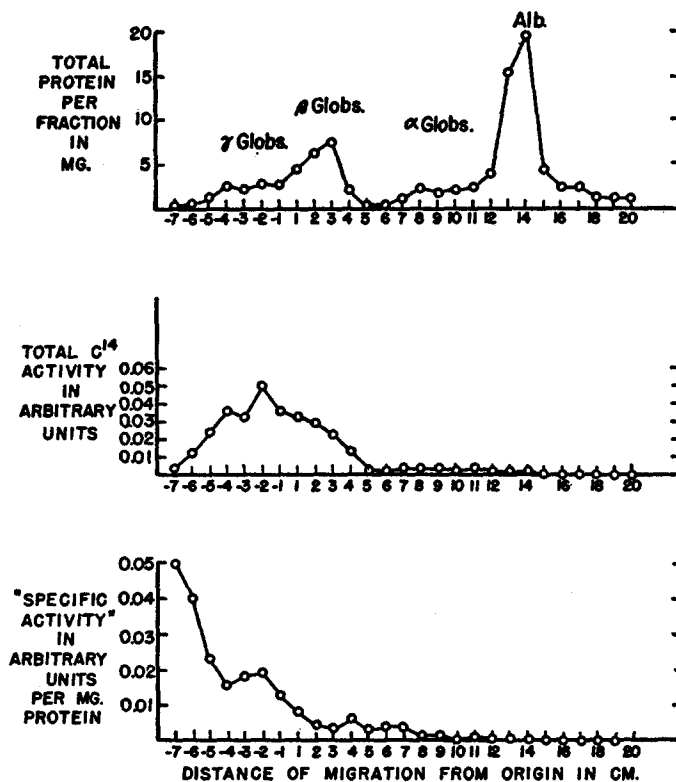

Fig. 4. Electrophoretic separation of plasma protein fractions of plasma from eviscerated surviving rat injected with DL-lysine-ϵ-C^{14}. Zone electrophoresis of plasma obtained from blood of surviving eviscerated rat 6 hours after intravenous injection of DL-lysine-ϵ-C^{14} (3.4 mg., 3.7 μc.). Protein content of fractions determined by the method described in reference 3 in the accompanying paper.

view is affirmed by the above described results of our isolated "carcass" perfusion studies in which the inclusion of all the non-hepatic abdominal viscera led to no greater incorporation of C^{14}-labeled lysine into plasma globulins than was observed in their absence.

The question as to exactly which tissue is the origin of the plasma protein produced by the perfused carcass cannot be answered here. The fact that the perfused carcasses including the gastrointestinal tract, pancreas, mesenteric lymph nodes, and spleen all synthesize a total of two to three times as much

tissue protein but little if any more plasma protein than those carcasses without these tissues, is strong evidence that these four tissues are not primarily or uniquely responsible for the plasma protein synthesis by non-hepatic tissues. The inclusion of the adrenals and kidneys and genito-urinary accessory apparatus in the last seven carcasses perfused and testes in the last eight, resulted in no marked measurable change in the amount of plasma protein produced. Bacterial protein synthesis was shown to be insignificant by the two penicillin experiments in which bacterial growth in the perfusate was almost completely suppressed without any effect on protein synthesis by any organ or tissue. This leaves only the skin, muscle, and bone marrow; the first two of these had only about 20 to 25 per cent as high a protein metabolic activity as the bone marrow, although much larger masses of tissue. This may implicate the white cell mother areas, such as the bone marrow and lymph nodes and disseminated follicles in the non-hepatic synthesis of plasma protein. Since lymphatic areas such as the spleen and mesenteric nodes, however, did not appear to be quantitatively important as discussed above, one looks more to the bone marrow than to the lymphatic system. We have already speculated concerning the cells of origin of the gamma globulins (30) and voiced the opinion that the radiosensitive white cell areas are of prime importance in the elaboration of the antibody gamma globulins. This conclusion is thoroughly explored in two recent reviews (39, 40).

SUMMARY

The non-hepatic tissues in a perfused "carcass" (caudal half of the rat) maintain some physiological functions for as long as 5 to 6 hours of perfusion, including good clearance of lysine-ϵ-C^{14} and glucose from the perfusate, and synthesis of both tissue and plasma proteins.

The perfused "carcass" tissues incorporate only small amounts of lysine-ϵ-C^{14} into the plasma proteins to an extent not markedly affected by the presence of the gastrointestinal tract, pancreas, spleen, or kidneys. This activity is found only in the globulin fraction obtained by sodium sulfate fractionation. No significant activity was detected in the plasma fibrinogen or albumin fractions.

C^{14}-labeled plasma proteins obtained from the eviscerated surviving rat 6 hours after intravenous lysine-ϵ-C^{14} have been separated by zone electrophoresis. The gamma globulins contain most of the C^{14} activity, with small but measurable activity in the beta and alpha globulins, and no activity in the albumin fraction.

BIBLIOGRAPHY

1. Miller, L. L., Bly, C. G., Watson, M. L., and Bale, W. F., *J. Exp. Med.*, 1951, **94**, 431.
2. Kunkel, H. G., and Slater, R. J., *Proc. Soc. Exp. Biol. and Med.*, 1952, **80**, 42.
3. Madden, S. C., and Whipple, G. H., *Physiol. Rev.*, 1940, **20**, 194.

4. Tarver, H., and Reinhardt, W. O., *J. Biol. Chem.*, 1947, **167**, 395.
5. Gutman, A. B., *Advances Protein Chem.*, 1948, **4**, 157 and 221.
6. Roberts, S., and White, A., *J. Biol. Chem.*, 1949, **180**, 505.
7. Munro, F. L., Hart, E. R., Munro, M. P., and Walkling, A. A., *Am. J. Physiol.*, 1945, **145**, 206.
8. Berryman, G. H., Bollman, J. L., and Mann, F. C., *Am. J. Physiol.*, 1943, **139**, 556.
9. Mann, F. D., Shonyo, E. S., and Mann, F. C., *Am. J. Physiol.*, 1951, **164**, 111.
10. Ratnoff, O. D., and Conley, C. L., *Bull. Johns Hopkins Hosp.*, 1951, **88**, 414.
11. Lewis, L. A., Page, I. H., and Reinhard, J. J., Jr., *Am. J. Physiol.*, 1949, **159**, 73.
12. White, A., and Gordon, E. S., Symposium on Steroid Hormones, Madison, University of Wisconsin Press, 1950, 195.
13. Roberts, S., and White, A., *J. Biol. Chem.*, 1949, **178**, 151.
14. Taliaferro, W. H., and Taliaferro, L. G., *J. Infect. Dis.*, 1950, **87**, 37.
15. Sabin, F. R., *J. Exp. Med.*, 1939, **70**, 67.
16. Treffers, H. P., *Advances Protein Chem.*, 1944, **1**, 80.
17. Cannon, P. R., *Advances Protein Chem.*, 1945, **2**, 140.
18. Roberts, S., Adams, E., and White, A., *J. Immunol.*, 1949, **62**, 155.
19. Cheng, K. K., *J. Path. and Bact.*, 1949, **61**, 23.
20. Flink, E. B., Hastings, A. B., and Lowry, J. K., *Am. J. Physiol.*, 1950, **163**, 598.
21. White, A., and Dougherty, T. F., *Ann. New York Acad. Sc.*, 1946, **46**, 859.
22. Keuning, F. J., and Van Der Slikke, L. B., *J. Lab. and Clin. Med.*, 1950, **36**, 167.
23. Hayhoe, F. G. J., and Smith, D. R., *J. Clin. Path.*, 1951, **4**, 47.
24. Leinwand, I., *J. Lab. and Clin. Med.*, 1951, **37**, 532.
25. Farris, E. J., and Griffith, J. Q., Jr., The Rat in Laboratory Investigation, Philadelphia, J. B. Lippincott Company, 2nd edition, 1949.
26. Olynyk, P., Camp, D. B., Griffith, A. M., Woislowski, S., and Helmkamp, R. W., *J. Org. Chem.*, 1948, **13**, 465.
27. Fairman, E., and Corner, G. W., *Anat. Rec.*, 1934, **60**, 1.
28. Hagedorn, H. C., and Jensen, B. N., *Biochem. Z.*, 1923, **135**, 46; **137**, 92.
29. Ingle, D. J., *Exp. Med. and Surg.*, 1949, **7**, 34.
30. Miller, L. L., and Bale, W. F., *J. Exp. Med.*, 1954, **99**, 125.
31. Gregersen, M. I., and Hall, V. E., *Ann. Rev. Physiol.*, 1951, **13**, 405.
32. Rothstein, M., Bly, C. G., and Miller, L. L., data to be published.
33. Flock, E. V., Mann, F. C., and Bollman, J. L., *J. Biol. Chem.*, 1951, **192**, 293.
34. Miller, L. L., Bale, W. F., Yuile, C. L., Masters, R. E., Tishkoff, G. H., and Whipple, G. H., *J. Exp. Med.*, 1949, **90**, 297.
35. Majoor, C. L. H., *J. Biol. Chem.*, 1947, **169**, 583.
36. Milne, J., *J. Biol. Chem.*, 1947, **169**, 595.
37. Levin, B., Oberholzer, V. G., and Whitehead, T. P., *J. Clin. Path.*, 1950, **3**, 260.
38. Kirkwood, J. G., Cann, J. R., and Brown, R. A., *J. Biol. Chem.*, 1950, **185**, 663.
39. McMaster, P. D., The Nature and Significance of the Antibody Response, New York, Columbia University Press, 1953, 13.
40. Ehrich, W. E., Blood Cells and Plasma Proteins, New York, Academic Press, Inc., 1953, 187.