PLASMA PROTEIN METABOLISM—NORMAL AND ASSOCIATED WITH SHOCK

Observations Using Protein Labeled by Heavy Nitrogen in Lysine*‡§

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The use of heavy nitrogen to label protein should enable the investigator to approach the study of protein exchange in the body with some prospect of success. These experiments are directed toward a better understanding of the behavior of the blood plasma proteins in health and disease. We hope this paper is but one in a series to be completed as emergencies and time permit.

Some of us for several years have been interested in the production and utilization of plasma proteins in the dog. Our understanding of the flow of plasma protein out of the circulation into the tissues and the reverse, we have described as a "dynamic equilibrium." We have shown that all nitrogen requirements of the dog can be supplied by dog plasma given by vein (1, 7, 14). The dog can be kept in positive nitrogen balance, weight balance, and in health for weeks while receiving carbohydrate, fat, minerals and accessories by mouth, and plasma protein in suitable amounts by vein. These plasma proteins under such conditions are utilized without significant nitrogen loss (8). No *hyperproteinemia* of significance is observed in spite of large injections of plasma. Moreover, these long continued plasma injections do not change significantly the albumin:globulin ratio. If albumin or any other plasma protein was particularly suited for tissue protein supply or maintenance, one would anticipate a change in the pattern of protein make-up of the plasma. Such changes have not been observed. The fact that no surplus of any plasma protein piles up in the blood plasma of these injected dogs would indicate that *all plasma proteins* can be utilized in the internal body protein metabolism under these conditions.

Schoenheimer, Rittenberg, and their collaborators (18) have found that on feeding to rats various amino acids labeled with deuterium or heavy nitrogen

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the plasma proteins show a greater metabolic activity than the proteins of any other tissue studied by them. In other experiments these workers (16) have demonstrated the extensive exchange of nitrogen that occurs between amino acids in the animal body.

It seemed possible that important evidence might be obtained on the *problem of shock* by experiments utilizing plasma whose protein constituents were labeled in as stable a manner as possible with an identifiable element. Since large quantities of heavy nitrogen were recently made available commercially this was the material chosen for the experiments. That the exchange reactions involving amino acids and plasma proteins might occur in the blood stream appears improbable. However, it seemed wise to use in these experiments an amino acid in which the uptake of nitrogen from the body pool of available nitrogen is at a minimum. Weissman and Schoenheimer (23) have presented evidence confirming earlier work in their laboratory that in the rat and the mouse the amino acid lysine does not take up nitrogen from other amino acids to any measurable extent. We have confirmed this finding in the dog in experiments to be published elsewhere. Lysine was therefore the amino acid selected for plasma protein labeling in the following experiments.

General Plan

The general outline of these experiments is as follows: To a dog selected as an eventual *donor of labeled plasma proteins* a complete allotment of the essential amino acids was fed or injected daily, the *lysine* being supplied by a product *synthesized with an excess of heavy nitrogen* in the epsilon amino position. During the period of labeled lysine feeding, blood was withdrawn periodically and the plasma separated and stored in a freezing cabinet for further experiments. The red blood cells removed from the donor were then replaced by equivalent amounts of washed red cells. The plasma protein level was kept low by suitable bleeding to insure maximum plasma protein production. As was expected, the concentration of labeled nitrogen in the lysine of this withdrawn plasma protein eventually reached a high enough level so that the amount could be measured with considerable accuracy even after a several-fold dilution with plasma protein of normal N^{15} content; that is, by injection into another dog.

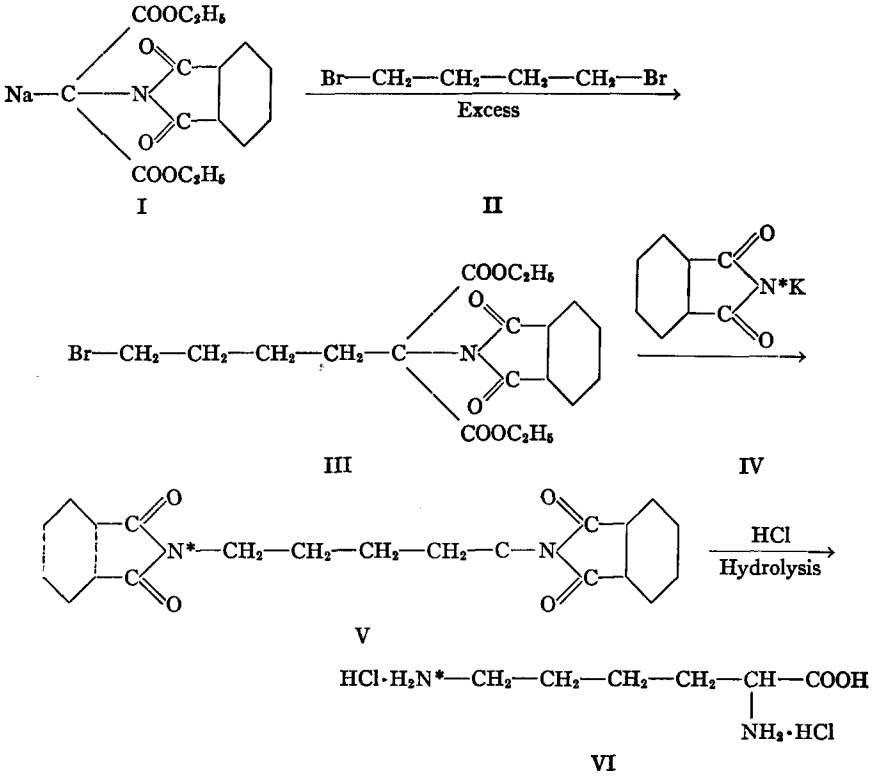
This *labeled plasma* was then used in several secondary experiments on which data are reported below. Portions of the blood of dogs, normal or otherwise, were withdrawn and replaced by blood whose plasma proteins were obtained from the labeled stores described above. Because of our interest in changes in plasma protein metabolism connected with *traumatic shock*, certain of these dogs were then subjected under anesthesia to experimental procedures designed to produce shock. In every instance blood samples were taken periodically and the heavy nitrogen content of lysine from the plasma proteins thereof determined.

A method has been developed for synthesizing lysine with heavy nitrogen in the epsilon amino position, and using this epsilon-tagged lysine, we have obtained evidence that nitrogen isolated from intact plasma proteins through deamination with nitrous acid is derived predominantly from the epsilon amino group of lysine. The use of epsilon-tagged lysine and the deamination procedure to be described make unnecessary the routine isolation of lysine from the plasma protein samples, and so greatly decrease the size of plasma samples needed and the time and effort required in preparing the samples for heavy nitrogen analysis.

The most striking result of these experiments is the rapidity with which this labeled plasma protein disappeared from the circulation and was replaced by unlabeled protein from the animals' own reserves. In 24 hours, some 50 per cent of the labeled protein administered had already been replaced in the circulation by protein containing normal lysine; that is, the normal abundance of heavy nitrogen.

Experimental Procedures

Preparation of Labeled Lysine.—The synthesis of lysine with N^{15} in the epsilon amino group was carried out according to the following reactions:



The ethyl sodium phthalimido malonate (I) was prepared according to the method of Dunn and Smart (3). One hundred sixty-four gm. (0.5 mole) of this material was stirred and heated at 150–160°C. with 650 gm. (3 moles) of tetramethylene bromide (II) for 2 hours. The excess of II was distilled off at reduced pressure, the residue taken up in 600 ml. of ether, filtered, and the ether removed from the filtrate under reduced pressure. The yield of syrupy product (III) was 206 gm. (93 per cent). Per cent Br calculated 18.2, found 17.8. Per cent N calculated 3.18, found 3.08.

Seventy-nine gm. (0.18 mole) of III was stirred and heated for 5 hours at 150–160°C. with 29.4 gm. (0.16 mole) of potassium phthalimide containing 65.5 atom per cent N^{15} excess. The product was extracted with three 200 ml. portions of hot absolute alcohol, filtered hot, and concentrated to a thick syrup (V) *in vacuo*. The syrup was refluxed for 5 hours with a mixture of 400 ml. each of concentrated hydrochloric acid, glacial acetic acid, and water, about 300 ml. of the mixture was distilled off, and refluxing then continued for 24 hours. The mixture was cooled, filtered, and concentrated *in vacuo* to about 100 to 150 ml. The residue was taken up with 120 ml. hot absolute alcohol and 1200 ml. of acetone added slowly. The epsilon-tagged *dl*-lysine dihydrochloride (VI) crystallized on standing in the refrigerator for 2 days. Yield 26.5 gm. (67 per cent). It was then taken up in 500 ml. of hot absolute alcohol, filtered, and recrystallized by addition of 700 ml. of ether. Yield 17.5 gm. (69 per cent). Per cent N calculated 12.8, found 13.0. The product contained 35.5 atom per cent N^{15} excess. Occasionally, conversion to the monohydrochloride (9) gives better yields and a better product than does a second crystallization as the dihydrochloride.

The tetramethylene bromide was very kindly prepared by the Departments of Chemistry of the University of Illinois and the University of Maryland. As much as 30-fold excess has been used in some preparations with apparently beneficial results. The potassium phthalimide was synthesized by Eastman Kodak Company using "heavy" ammonium nitrate.

Following the nomenclature of Schoenheimer and Rittenberg (17) the isotope concentration in a nitrogen sample is reported as *per cent N^{15} excess*, or the absolute concentration of heavy nitrogen minus the normal concentration of 0.37 per cent. In speaking of a substance other than pure nitrogen, the term *atom per cent N^{15} excess* is used to indicate the isotope concentration in the nitrogen atoms of the substance.

Method of Handling Plasma Samples

A. Free Amino Nitrogen Recovery.

In this procedure the non-protein nitrogen was removed, the intact plasma proteins subjected to deamination with nitrous acid, and the nitrogen gas evolved collected in a special sample tube for subsequent measurement in the mass spectrometer. The standard Van Slyke amino nitrogen apparatus (22) was found to be unsatisfactory for this purpose, for the various solutions retained a considerable amount of nitrogen from each sample and at the same time released an equivalent amount of nitrogen absorbed from previous samples or from air. Accordingly, a special deamination apparatus was designed which permitted degassing of the various solutions before the sample was released, facilitated the changing of the solutions and washing of the flasks between samples, and made it possible to carry out the deamination rapidly at an elevated temperature. The plasma samples were in general used soon after they were obtained, for old samples occasionally gave spuriously high enrichment values.

Two ml. of 12 per cent trichloracetic acid was added to 2 ml. of plasma, the mixture stirred well, allowed to stand 10 minutes, centrifuged, and the precipitate washed twice with about 4 ml. of 6 per cent trichloracetic acid. This procedure removes about 99 per cent of the labeled non-protein nitrogen. The amount of N.P.N. left with the protein was in general negligible in our experiments, but could be removed completely with about three times the amount of trichloracetic acid washing indicated above. The plasma proteins so obtained

were suspended in a small volume of water and transferred to the deamination apparatus shown in Fig. 1. Three ml. of acetic acid was added to the sample in the reaction flask (*R*). Ten ml. of 40 per cent sodium nitrite was placed in the tipping flask (*Ti*) and 50 ml. of a solution containing 20 gm. potassium hydroxide and 40 gm. of potassium permanganate per liter was placed in the absorption flask (*A*), followed by about 30 ml. of water. The flasks *R* and *Ti* were cooled by circulating ice water through the jackets, and the system was evacuated using a mercury diffusion pump. The stopcock between the *A* and the dry ice trap (*Tr*)

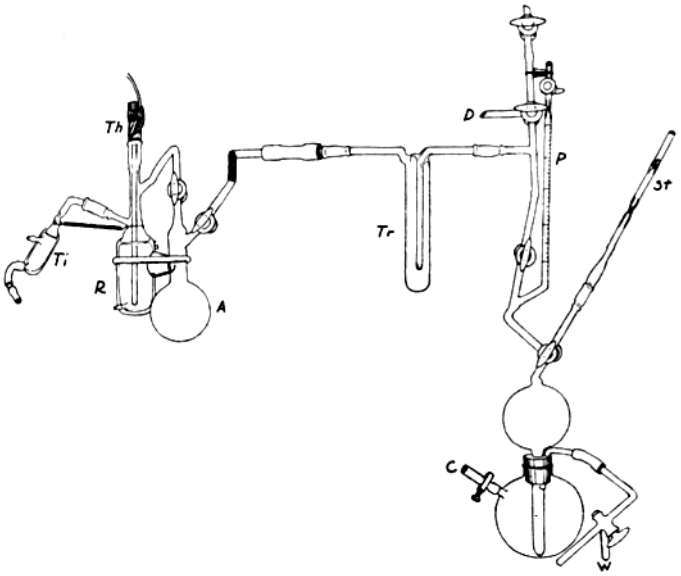


FIG. 1. Apparatus for deamination of proteins *in vacuo* at controlled temperatures. *Ti*, water-jacketed tipping flask for addition of sodium nitrite to reaction flask. *R*, water-jacketed reaction flask containing protein suspension and acetic acid. *Th*, thermocouple for measuring temperature of reaction mixture. *A*, absorption flask containing potassium permanganate and potassium hydroxide for removal of nitric oxides from gas sample. *Tr*, dry ice-acetone trap for removing water vapor. *D*, connection to mercury diffusion pump. *P*, pipette for measurement of sample volume. *St*, sample tube. *C*, tube to facilitate removal of mercury and cleaning solutions from Toeppler pump. *W*, connection to water aspirator for control of mercury in Toeppler pump.

was closed, the temperature in the reaction flask adjusted to 37°, and the sodium nitrite tipped in. The shaker was turned on for 5 minutes, the stopcock between *R* and *A* closed and the shaker again turned on for 3 minutes. The vacuum pumps were then closed off from the apparatus, the stopcock between *A* and *Tr* opened, and the sample pumped into the sample tube (*ST*) by means of the Toepler pump. The sample tube containing the nitrogen gas sample was then sealed off by a small flame, and set aside for use in the mass spectrometer. The cold trap and the absorption, reaction, and tipping flasks were washed thoroughly after each sample. Ordinarily eight or more samples can be loaded per day. The stationary part of the apparatus was cleaned every few weeks by removing the mercury through *C* and then filling the apparatus with nitric acid, cleaning solution, and several water rinses.

B. Preparation of Samples of Total Plasma Nitrogen.

Plasma samples were digested by the Kjeldahl procedure and the ammonia so obtained converted to nitrogen gas by means of sodium hypobromite essentially as described by Rittenberg and his coworkers (15). To 0.5 ml. of plasma was added 2 ml. of digestion mixture prepared as follows: Concentrated H_2SO_4, 1000 ml., was placed in a 2000 ml. beaker and warmed to 100°C. K_2SO_4 (C.P. Special), 300 gm., was added slowly with stirring, dissolving each portion before adding more. $CuSO_4$, 12.5 gm. in 50 ml. of hot water, and selenious acid, 10 gm., in 50 ml. of hot water were then added slowly, being careful to avoid sputtering, and the concentrated solution was kept in a warm place.

The mixture was digested for 3 hours after it had cleared, transferred to the distillation apparatus, concentrated alkali added, and the ammonia distilled into hydrochloric acid. The excess hydrochloric acid was back titrated with sodium hydroxide to a pH of 5.8, with a glass electrode. About 1 cc. of N/10 hydrochloric acid was then added and the volume of the solution reduced to about 5 ml. by boiling. Following the procedure of Rittenberg (15), this solution was treated with sodium hypobromite and the nitrogen gas pumped into a sample tube.

The reaction of nitrous acid with ammonia or lysine labeled with heavy nitrogen regularly gives nitrogen containing approximately *50 per cent* the N^{15} excess found after Kjeldahl digestion of the same sample and conversion of the ammonia to molecular nitrogen with NaOBr. This nitrogen obtained from the nitrous acid reaction generally contained negligible amounts of mass 30 ($N^{15}N^{15}$ and NO), in some cases as little as 0.5 per cent of the amount found by the Kjeldahl-NaOBr procedure for the same sample. This 50 per cent dilution of the N^{15} isotope enrichment by nitrogen obtained from the nitrous acid gives results upon deamination of intact protein which are about equal to those obtained for enrichment of the total nitrogen of lysine isolated from protein and determined by the Kjeldahl-NaOBr procedure, for in the latter case the *enriched epsilon* nitrogen is diluted by an equal quantity of *unenriched alpha* nitrogen.

Isotope Abundance Determination

Nitrogen isotope abundances were determined with a Nier type mass spectrometer (13). A description of certain modifications of this instrument and its operation will be published elsewhere. The mean error in nitrogen isotope 15 abundance measurements is 0.004 per cent for small abundances of 0.500 per cent or less.

The method of admitting samples into the mass spectrometer is shown in Fig. 2. The sample tube is introduced through the ground glass joint and the space about it is evacuated. The plunger is then lifted to break the capillary tip of the sample tube by passing current through the solenoid. The gas is then passed through the cold trap (liquid nitrogen) and enters the spectrometer through the capillary leak. The gas pressure is about 10 mm. Hg before entering the leak and 10^{-4} mm. Hg after passing through the leak.

Preparation of Labeled Plasma Proteins

All shock and sacrifice procedures were done under general anesthesia, using nembutal or ether. Plasmapheresis was done through an 18 gauge vena puncture needle in the jugular vein.

By daily feeding or injection of lysine tagged in the epsilon position with heavy nitrogen a dog (42-609) was built up as a *donor of labeled plasma*. Blood was removed from this animal by periodic plasmapheresis according to methods previously reviewed (10). Soon after removal the blood was centrifuged, and the plasma was then frozen and stored in this condition for subsequent usage. Clotting was prevented by the use of heparin.

It is conceivable that factors in the preparation of this labeled plasma may be pertinent in considering its ultimate fate. Data on this donor dog are therefore given in some detail. This apparently normal 10.45 kilo mongrel hound dog had been on a non-protein basal diet for 7 weeks. During the 10 days immediately preceding the experiment the dog was bled with the return of washed red cells. The plasma protein level dropped to 3.85 per cent. For 9 days before the labeled lysine administration was begun the dog was fed an amino acid mixture *complete except for lysine.* Then for 1 day the animal was given as a supplement to this amino acid mixture 3.6 grams *dl*-lysine dihydrochloride in which the epsilon amino nitrogen contained a nitrogen isotope 15 excess of 65.0 per cent. For 35 days thereafter 0.9 gm. of this labeled lysine was given as a daily supplement to the amino acid intake. The amino acids, including the labeled lysine, were administered intravenously for the first 7 days, orally

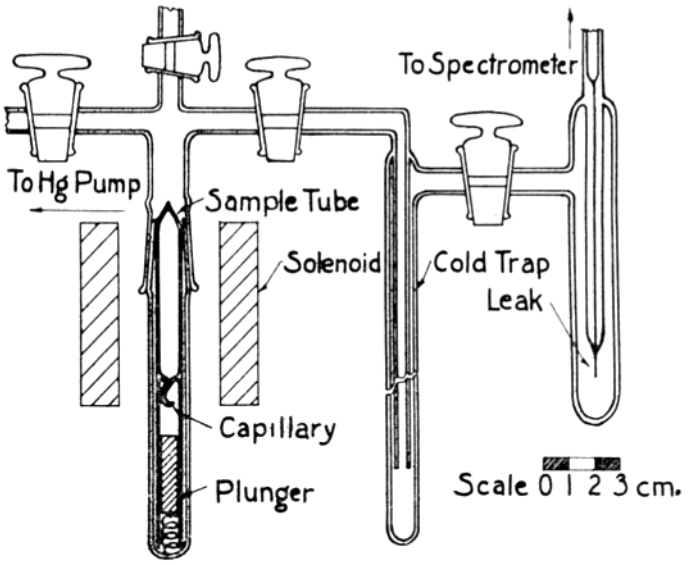


FIG. 2. Apparatus for introduction of gas samples into mass spectrometer.

for the next 9 days, and by various methods thereafter. Plasma collected during the 2nd and 4th days of this labeled lysine experiment was used for the labeled plasma experiment on dog *42-678.*

This donor dog (42-609) did not give the anticipated production of plasma protein and was therefore eventually sacrificed 64 days after the start of labeled lysine administration. The pertinent finding at autopsy was a suppurative pyelonephritis. It has been shown elsewhere (12) that a serious infection will limit or completely inhibit blood protein production in depleted dogs.

A single test was made of the remote possibility that plasma proteins might become labeled simply by contact with labeled lysine. Dog plasma was stirred for 2 hours at room temperature with 4 mg. of labeled lysine per ml. plasma. The plasma proteins contained no labeled nitrogen at the end of this time. A similar amount of labeled lysine injected intravenously into a dog resulted in the introduction of significant amounts of labeled nitrogen into the plasma proteins within 2 hours.

EXPERIMENTAL RESULTS

Labeled Plasma Exchange in Normal Dogs

Using the labeled plasma obtained as above an experiment was carried out to study the fate of transfused plasma in the normal dog.

The dog *42-678*, a mongrel hound that had recovered from *C. welchi* infection 6 weeks previously, was in good condition, eating the kennel diet before and during the experiment. Hematocrit 47.5 per cent. Total plasma protein 5.5 per cent. Weight 11.52 kilos. The experiment, performed February 9, 1943, consisted of a simple plasmapheresis done by removing 206 ml. of blood (containing 5.6 gm. of plasma protein and 103 ml. of packed red cells) and immediately replacing a volume of marked blood consisting of 142 ml. of the labeled plasma (containing 4.6 gm. plasma protein) together with 60 ml. washed red cells. The nitrogen obtained by deamination of the *intact donor plasma protein* showed an enrichment of 1.00 per cent N^{15} excess. The enrichment of *total plasma nitrogen* obtained by the Kjeldahl procedure was 0.24 per cent N^{15} excess.

Table 1 gives data collected over the course of this experiment. The percentage of heavy nitrogen found in the plasma samples decreased steadily, more rapidly in the first few hours after the injection of labeled plasma, but with measurable speed over the entire 6 days of the experiment.

Column 4, giving the per cent of circulating plasma lysine consisting of donor plasma lysine, is calculated on the assumption that the free amino nitrogen collected and measured is derived entirely from lysine epsilon amino nitrogen. However, the interpretation of the data is not seriously affected if one assumed that we are here isolating some representative fraction reproducibly from the different plasma samples. It is of interest that the figures shown here are very close to those found if the N^{15} excess obtained in the total nitrogen of plasma samples is calculated in terms of the per cent of circulating plasma nitrogen consisting of donor plasma nitrogen.

One must note that an element of uncertainty is introduced into the interpretation of these data because an appreciable portion of the total plasma nitrogen of the dog has been removed by the necessary sampling for this analysis. It is not easy to correct the enrichments found for this periodic sampling. An attempt has been made to do so on the following assumptions: (1) That the plasma volume remained constant over the course of the experiment or, more accurately, that it was quickly restored to its original value following the removal of each sample; (2) that the plasma proteins removed by sampling were replaced by untagged plasma proteins derived from reserve stores of protein within the dog. The milligrams of nitrogen removed in any plasma sample divided by the milligrams of nitrogen circulating gives the correction factor to be applied to subsequent measurements due to the withdrawal of that plasma sample. One plus the sum of the correction factors for all previous samples gives the correction factor by which the percentages in column 4 are multiplied to give the corrected values shown in column 4*a*.

There are several factors that conspire to make these "corrected" *data* give *too high* enrichments. First, it is very unlikely that the animal is able to replace completely the removed plasma proteins with new plasma proteins from reserve stores during the intervals between frequent samples. Second, as the labeled nitrogen disappears from the blood stream it is probably, at least to an appreciable extent, introduced into the labile protein from which the new plasma proteins are made. Therefore, the protein components coming

TABLE 1

Decline in Labeled Plasma Nitrogen Concentrations in Normal Dog

Dog 42-678

1	2	3	4	4a	5	6	7
Time after injection of labeled plasma protein	Plasma removed in sampling	N^{15} excess found on deamination of intact protein	Circulating plasma lysine, per cent consisting of donor plasma lysine		N^{15} excess in total plasma nitrogen	Circulating plasma nitrogen, per cent consisting of donor plasma nitrogen	N^{15} excess in plasma non-protein nitrogen
			Found	Corrected for sampling			
	ml.	*per cent*			*per cent*		*per cent*
4 min.	4.3	0.184	18.3	18.3	0.043	18.1	0.025
15 min.	4.6	0.175	17.4	17.6	0.036	15.1	0.027
45 min.	4.9	0.157	15.6	15.9	0.033	13.9	0.014
90 min.	4.8	0.155	15.4	15.8	0.028	11.8	—
3 hrs.	5.1	0.137	13.6	14.1	0.029	12.2	0.010
6 hrs.	4.7	0.126	12.5	13.1	0.028	11.8	0.007
9 hrs.	5.3	0.112	11.1	11.8	0.025	10.5	0.005
17 hrs.	5.0	0.101	10.1	10.8	0.020	8.4	0.003
24 hrs.	4.5	0.093	9.3	10.1	0.021	8.8	0.020
48 hrs.	5.0	0.069	6.9	7.5	0.012	5.0	0.005
72 hrs.	6.0	0.066	6.6	7.3	0.015	6.3	0.001
96 hrs.	7.0	0.056	5.6	6.3	0.012	5.0	0.005
144 hrs.	5.6	0.048	4.8	5.4	0.011	4.6	0.000

into the circulation as a result of the plasma withdrawal stimulus may be appreciably enriched instead of containing no excess heavy nitrogen as the method of correction assumes. As a result, the true curve to be expected if no samples had been taken would be expected to lie somewhere between the uncorrected values and the values found on correcting for sampling with the above method. These *"corrected" values* then simply *set an upper limit* on the possible effects of blood withdrawal.

Fortunately it has been possible in most instances to keep the blood withdrawals small and the two sets of data do not deviate widely. This has been possible largely because the method of deamination employed did not require

nearly the amount of protein that would have been necessary for isolating amino acids directly from each plasma sample, the proteins from 1 to 2 ml. of plasma furnishing sufficient nitrogen for a single isotope analysis.

From Table 1 and the value given earlier as the isotope concentration of the non-protein nitrogen of the donor plasma, it is evident that the labeled nitrogen present in the non-protein nitrogen of the injected blood plasma disappeared very rapidly from the circulation of the recipient dog. The concentration of protein in the circulating plasma of the recipient immediately after plasma exchange was 5.2 per cent; after 1 day, 5.6 per cent; after 6 days, 5.2 per cent.

Dog 42-1071.—Normal hound dog. Kennel diet before and during experiment. Weight 7.65 kilos. Plasma volume (dye) 340 ml. Red blood cell volume 330 ml. Red cell hematocrit 48.2 per cent. On the day of the experiment 200 ml. of blood was withdrawn from this dog, apparently bringing it near to shock. The blood withdrawn contained 98 ml. plasma which analyzed as 6.1 per cent protein. The dog was then immediately transfused with 330 ml. of blood freshly drawn from the donor dog (42-609) of the preceding experiment. This transfused blood contained 188 ml. plasma with 4.1 per cent protein and on deamination of the plasma proteins an N^{15} excess of 1.48 per cent was found. The total plasma nitrogen contained 0.36 per cent N^{15} excess and the plasma non-protein nitrogen 0.23 per cent N^{15} excess. At the time of this experiment the donor dog (42-609) was not receiving labeled lysine. It had been discontinued 10 days previously. During these 10 days the N^{15} excess found on deamination of the plasma proteins of the donor dog had fallen from 2.0 per cent to 1.5 per cent.

The recipient dog (42-1071) was followed with periodic blood sampling for 3 days, then sacrificed by bleeding from the carotid artery under ether anesthesia. *Table 2* shows data collected over the course of this experiment. Table headings and methods of calculation are the same as for Table 1. A fibrin clot which formed in part of the last sample was removed and washed. The total nitrogen of this clot contained 0.037 per cent N^{15} excess, or approximately the same as that found in the total plasma nitrogen. The concentration of protein in the circulating plasma of dog 42-1071 was initially 6.1 per cent; immediately after plasma exchange, 5.6 per cent; after 1 day, 5.8 per cent; after 2 days, 5.5 per cent; and on sacrifice, 5.5 per cent.

Chart A (solid line) shows the per cent of donor plasma lysine remaining in circulation in the plasma protein, based upon the assumption that the free amino nitrogen isolated from the intact protein is derived from the epsilon amino lysine nitrogen. It is obtained from the data of Tables 1 and 2, by dividing all of the values of column 4 by the first value and multiplying by 100. The assumption is also inherent that at the time of the first sample no tagged plasma has been lost from the circulation and that mixing of the donor plasma and recipient plasma is complete at this time. The same data are also presented here (dotted line) using the values of column 4*a* corrected for sampling as source data for this calculation.

TABLE 2

Decline in Labeled Plasma Nitrogen Concentrations in Normal Dog

Dog 42-1071

1	2	3	4	4a	5	6	7
Time after injection of labeled plasma protein	Plasma removed in sampling	N^{15} excess found on deamination of intact protein	Circulating plasma lysine, per cent consisting of donor plasma lysine		N^{15} excess in total plasma nitrogen	Circulating plasma nitrogen, per cent consisting of donor plasma nitrogen	N^{15} excess in plasma non-protein nitrogen
			Found	Corrected for sampling			
	ml.	*per cent*			*per cent*		*per cent*
4 min.	5.4	0.484	32.8	32.8	0.132	36.2	0.020
15 min.	5.7	0.447	30.3	30.8	0.128	35.2	0.009
45 min.	5.9	0.380	25.8	26.7	0.111	30.5	0.026
90 min.	5.7	0.379	25.6	26.9	0.111	30.5	0.006
2.5 hrs.	6.0	—	—	—	0.110	30.2	0.008
6 hrs.	4.7	0.325	22.0	23.8	0.095	26.1	0.010
9 hrs.	5.8	0.293	19.9	21.8	0.098	26.9	0.019
18 hrs.	7.2	0.260	17.6	19.6	0.077	21.2	0.002
24 hrs.	4.7	0.245	16.6	18.7	0.069	19.0	0.000
48 hrs.	8.0	0.177	12.0	13.8	0.054	14.8	0.003
67.5 hrs.	6.9	0.157	10.6	12.6	0.043	11.8	−0.005

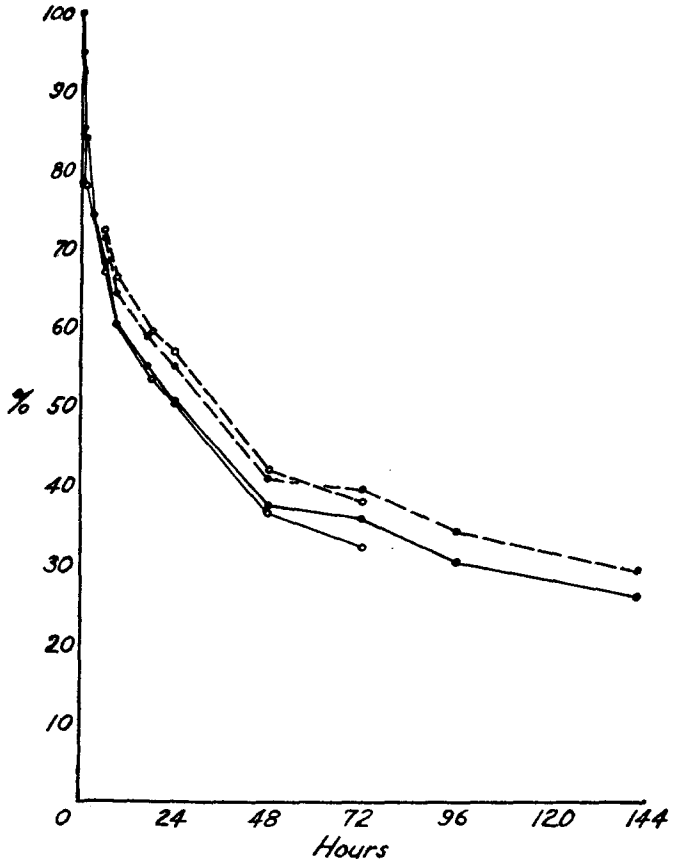


CHART A. Percentage of donor plasma protein lysine remaining in the circulation of the normal dog. Solid circles, data for dog 42-678. Open circles, data for dog 42-1071. Solid lines, data uncorrected. Dotted lines, data with maximal corrections for sampling.

Labeled Plasma Exchange in Dogs Subjected to Shock

1. Intestinal Trauma.

Dog 42-835, a normal hound dog, weighing 15.45 kilos was given nembutal solution by vein A plasma volume was done and 30 minutes after giving the nembutal *intestinal trauma* was induced for 13 minutes using the methods developed by Mahoney *et al.* (11). At the conclusion of trauma 216 ml. of labeled plasma was injected intravenously. This labeled plasma, containing 8.85 gm. of protein was obtained by pooling plasma samples which had been preserved by freezing after their withdrawal from donor dog 42-609. The pooled plasma protein on deamination gave an N^{15} excess of 1.90 per cent, while the total plasma nitrogen and the non-protein each contained 0.38 per cent N^{15} excess.

TABLE 3

Decline in Labeled Plasma Nitrogen Concentrations during Shock from Intestinal Trauma
Dog 42-835

1	2	3	4	5	6	7
Time after injection of labeled plasma protein	Plasma removed in sampling	N^{15} excess found on deamination of intact protein	Circulating plasma lysine, per cent consisting of donor plasma lysine	N^{15} excess in total plasma nitrogen	Circulating plasma nitrogen, per cent consisting of donor plasma nitrogen	N^{15} excess in plasma non-protein nitrogen
	ml.	*per cent*		*per cent*		*per cent*
3 min.	6.2	0.426	22.4	0.092	24.2	0.025
15 min.	6.4	0.419	22.0	0.078	20.5	0.014
30 min.	4.8	0.392	20.6	0.076	19.9	0.018
60 min.	5.9	0.359	18.9	0.076	19.9	0.020
90 min.	4.9	0.326	17.1	0.081	21.2	0.033
2 hrs.	5.0	0.329	17.2	0.070	18.4	0.023
3 hrs.	4.4	0.321	16.9	0.064	16.8	0.018
4 hrs.	5.8	0.323	17.0	0.068	17.8	0.016
5 hrs.	5.6	0.304	16.0	0.060	15.7	0.015
6 hrs.	5.4	0.303	15.9	0.062	16.3	0.012

This animal (42-835) was apparently in shock as indicated by low blood pressure before the time of labeled plasma injection, recovered for about 20 minutes, and then relapsed into a steady state of shock for the remaining 5½ hours before sacrifice. Immediately after injection of plasma the protein level was 4.9 per cent. The plasma volume before trauma was 860 ml. and protein level 5.1 per cent. Two hours after trauma the plasma volume was 470 ml. and the protein level 4.6 per cent. The protein level before death was 4.7 per cent. The volume of exudate lost into the lumen and from the surface of the traumatized bowel during the experiment was 250 ml.; it contained 12 gm. of protein.

About 20 per cent of the N^{15} excess injected in the lysine fraction of the donor plasma was recovered by the deamination technique from the *traumatized tissue and the exudate from it,* about equal quantities of N^{15} from each.

Table 3 gives data collected over the course of this experiment. In general, the same methods of calculation have been followed and the same headings

used as in Tables 1 and 2, except that no corrections for sampling have been made since it does not seem possible in view of plasma volume shrinkage during shock to estimate with any assurance the changes in plasma volume during the experiment nor to assume restoration of plasma proteins lost by sampling.

2. Leg Trauma.

Dog 43-54, a mongrel weighing 13.2 kilos, had been *splenectomized* 1 month previously. In this experiment, done in collaboration with Drs. Morton, Mahoney, and Howland, the dog was anesthetized with morphine and nembutal, then about one-third of its blood was rapidly withdrawn and immediately replaced with an equivalent amount of tagged donor plasma and washed red cells according to the data below. Ten minutes later, after a preliminary sample of arterial blood had been withdrawn, the *Blalock clamp* (2) was applied as high as possible on the upper thigh of the *left hind leg*. This clamp was removed $5\frac{1}{4}$ hours later. Within a few minutes the dog went into shock and died $2\frac{1}{2}$ hours after removal of the clamp, several hours before death was expected. Death usually occurs about 10 hours after removal of the clamp in such experiments and such decreased plasma volumes are characteristic of the later stages. The premature exitus here was probably due to the removal of more blood in sampling than is ordinarily removed in shock experiments of this type.

Under nembutal anesthesia, the initial plasma volume measurement (Evans' blue dye) was 610 ml., plasma protein level 5.4 per cent. The bleeding of 425 ml. from the carotid artery contained 226 ml. of plasma and 12.2 gm. of plasma protein. A mixture of 225 ml. of washed red cells and 235 ml. of plasma obtained from dog 42-609 was injected immediately following the bleeding. This plasma had been preserved in a frozen condition for 5 months. It contained 10.5 gm. of protein which on deamination showed an N^{15} excess of 2.60 per cent. The total plasma nitrogen contained 0.57 per cent N^{15} excess. Immediately after exchange the plasma protein level was 4.8 per cent and after 5 hours clamp application just before its removal was 5.2 per cent. A second plasma volume measurement made 5 minutes after removal of the clamp showed a circulating volume of 570 ml. and protein level of 5.5 per cent. As is characteristic in this type of experiment, after removal of the clamp the leg gradually became greatly distended. Just before death and plasma protein level was 5.1 per cent.

Table 4 gives data collected over the course of this experiment, calculated in the same manner as in Table 1. Since the change in plasma volume was relatively small up to the time of clamp removal, corrections for sampling up to this time have been included as of some possible significance.

3. Leg Trauma.

Dog 40-149, a normal mongrel, weighing 11.8 kilos had been in the animal house several months. Anesthesia was induced by morphine and nembutal, more nembutal being administered as indicated through the course of the experiment. Four hundred ml. of blood containing 210 ml. of plasma and 15 gm. of plasma protein was withdrawn from the carotid artery. One hundred thirty ml. of washed red blood cells suspended in 15 ml. of Locke's solution and 242 ml. of labeled plasma were immediately introduced. This plasma contained 13 gm. of plasma protein which on deamination showed an N^{15} excess of 0.61 per cent. The total plasma nitrogen contained 0.18 per cent N^{15} excess. Plasma protein level was 6.3 per cent before exchange and 5.6 per cent immediately after exchange.

The labeled plasma used in this experiment was derived from a different donor dog (42-1364) and had been frozen for a few days. This donor animal had been bled about one-fourth of its blood volume and 4 hours later given a subcutaneous injection of the essential amino acids

including 2 gm. of tagged *dl*-lysine monohydrochloride. Large samples of blood were withdrawn 20 and 40 hours later. The plasma obtained from these pooled samples was the donor material used.

Twenty-five minutes after the administration of donor plasma the *Blalock clamp* was applied to the *right hind quarter.* Blood samples were taken periodically for N^{15} measurement and plasma volume determination. The clamp was removed 5½ hours after it was applied. The animal died about 14 hours after the clamp was removed. During the first 6 hours normal blood was injected to replace that withdrawn for samples. A preliminary plasma volume based upon the dilution of the tagged plasma injected gave a plasma volume of 520 ml. The first plasma volume determination by the dye method made just before the clamp was removed was 360 ml., plasma protein level 5.8 per cent. Plasma protein level

TABLE 4

Decline in Labeled Plasma Nitrogen Concentrations during Shock from Leg Trauma

Dog 43-54

1	2	3	4	4a	5	6
Time after injection of labeled plasma protein	Plasma removed in sampling	N^{15} excess found on deamination of intact protein	Circulating plasma lysine, per cent consisting of donor plasma lysine		N^{15} excess in total plasma nitrogen	Circulating plasma nitrogen per cent consisting of donor lasma nitrogen
			Found	Corrected for sampling		
	ml.	*per cent*			*per cent*	
8 min.	3.4	0.771	29.6	29.6	0.184	32.4
15 min.	Clamp	Applied				
30 min.	4.8	0.751	28.9	29.1	0.173	30.4
55 min.	5.4	0.751	28.9	29.3	0.171	30.1
1.5 hrs.	5.0	0.723	27.8	28.4	0.170	29.9
2.5 hrs.	4.5	0.704	27.1	27.9	0.169	29.8
3.5 hrs.	5.4	0.669	25.7	26.6	0.147	25.9
5.5 hrs.	5.1	0.632	24.3	25.4	0.147	25.9
5.6 hrs.	Clamp	Removed				
6.3 hrs.	5.4	0.619	23.8		0.150	26.4
7.5 hrs.	7.7	0.596	22.9		0.147	25.9

was 6.9 per cent 30 minutes after removal. A second determination by the dye method 4.4 hours after the clamp was removed was 240 ml., with plasma protein level 7.4 per cent. Eleven hours after removal the plasma protein level was 7.8 per cent.

Table 5 shows data collected over the course of this experiment. Except for the columns of corrected values, 4*a* and 4*b*, the data are in general calculated in the same way as in the previous tables. In the preceding two shock dogs corrections for sampling were impossible in most cases because of insufficient plasma volume data. In this experiment, however, the plasma volume at any time could be estimated with reasonable accuracy by drawing a smooth curve through the three experimentally determined volumes plotted against time, and these estimated volumes have been used in calculating the corrected values shown in column 4*a*. Although it is possible to calculate sampling corrections

for this animal, such corrections are open to objection, inasmuch as the plasma volume was decreasing to a greater extent than was accounted for by the plasma samples withdrawn. Obviously the body tissues during this shock state could not supply materials for plasma to replace that withdrawn as samples. Accordingly, where the samples were not replaced by injections of ordinary blood, the values in column 4*a* are even greater over-corrections than those given in the preceding tables. Therefore, another column has been added, column 4*b*, in which the assumption is made that the dog cannot restore from tissues

TABLE 5

Decline in Labeled Plasma Nitrogen Concentrations during Shock from Leg Trauma
Dog 40-149

1	2	3	4	4*a*	4*b*	5	6	7
Time after injection of labeled plasma protein	Plasma removed in sampling	N^{15} excess found on deamination of intact protein	Circulating plasma lysine, per cent consisting of donor plasma lysine			N^{15} excess in total plasma nitrogen	Circulating plasma nitrogen, per cent consisting of donor plasma nitrogen	N^{15} excess in plasma non-protein nitrogen
			Found	Corrected for sampling	Corrected for injections			
	ml.	*per cent*				*per cent*		*per cent*
8 min.	10.8	0.264	44.0	44.0	44.0	0.048	41.7	0.014
25 min.	Clamp	Applied						
40 min.	5.3	0.255	42.4	43.2	42.4	0.045	39.1	0.009
1.5 hrs.	15.0	0.235	39.1	40.2	40.7	0.036	31.3	−0.005
5.3 hrs.	11.2	0.192	32.0	33.6	34.6	0.035	30.5	0.001
5.6 hrs.	Clamp	Removed						
6.1 hrs.	10.0	0.182	30.3	33.1	34.1	0.033	28.7	0.011
7.4 hrs.	4.0	0.168	28.0	31.4	32.0	0.039	33.9	0.007
10.0 hrs.	4.2	0.165	27.4	31.1	31.4	0.037	32.2	0.006
10.4 hrs.	2.6	0.162	27.0	31.2	30.9	—	—	0.002
13.0 hrs.	10.7	0.149	24.8	28.9	28.4	0.032	27.8	−0.002
14.6 hrs.	3.6	0.145	24.1	29.3	27.6	0.024	20.9	0.004
16.6 hrs.	3.1	0.132	22.0	27.2	25.2	0.033	28.7	0.004

the protein removed in sampling, and the data are corrected only for the ordinary, that is, unlabeled blood injected during the first 6 hours of the experiment. The method of making this correction is very similar to that used for column 4*a*. The milligrams of ordinary plasma nitrogen injected divided by the estimated number of milligrams of plasma nitrogen circulating at the time of the injection gives the correction factor to be applied for each injection. One plus the sum of all such previous correction factors gives the number by which the percentages shown in column 4 are multiplied to obtain the corrected values in column 4*b*. This is a straightforward type of correction, the accuracy of which is limited chiefly by the accuracy of the estimated plasma volumes. The

early values are larger than those of column 4*a* because the amounts of plasma nitrogen injected were slightly greater than the amounts withdrawn in samples.

Chart B presents the data of column 4 of Tables 3 and 4, and of column 4*b* of Table 5 for comparison with the data of the early portion of Chart A. *All*

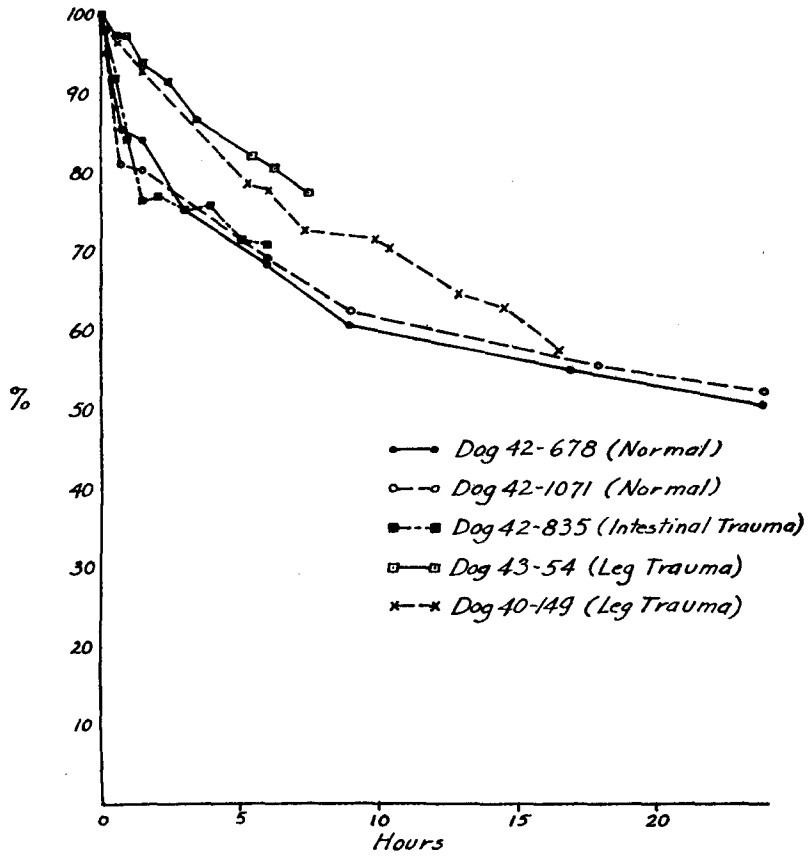


CHART B. Decline in labeled plasma protein lysine nitrogen concentration in normal and shocked dogs.

these curves are constructed from measurement of the *proportions of labeled* (donor) plasma protein lysine nitrogen *to unlabeled* (recipient) plasma protein lysine nitrogen *per unit volume* expressed as percentages of the initial measurement. The *curves for the normal dogs* also correspond to the percentage of labeled plasma remaining in the circulation insofar as the total circulating mass of protein remains constant. The *curves for the shock dogs* do not reflect the percentage of labeled plasma protein remaining in the circulation because

of the shrinkage of total circulating plasma protein (plasma volume × plasma protein level).

DISCUSSION

In the experiments on normal dogs (Tables 1 and 2 and Chart A) there is at first a rather rapid disappearance of the labeled plasma from the circulation, then a slower disappearance as time goes on. It is not a logarithmic curve and therefore no strict half-life can be deduced. The labeled nitrogen concentration had fallen to 50 per cent (uncorrected) in approximately 26 hours, but the further fall to 25 per cent required 5 days.

One might interpret the curves as having two phases. The presence of a highly *mobile fraction of tissue proteins* exchanging constantly with the plasma proteins would tend to cause the curve of disappearance of labeled plasma proteins to assume an initial steep phase. The plasma would at first be exchanging with an unlabeled fraction of the tissue proteins, but as this latter fraction built up a higher isotope concentration, the apparent rate of loss of plasma protein would be slowed down due to the return of labeled protein from this mobile tissue protein pool. At the same time but more apparently when the highly mobile fraction of the tissue proteins had attained an isotope concentration approximating that of the plasma the labeled plasma protein would disappear at a slower rate consistent with the slower phase of Chart A. This rate would be a combination rate including the rate of synthesis of new protein for replacing the loss through metabolic degradation and the rate of exchange with the less mobile tissue proteins. On this basis, the form of the curve of disappearance for an individual animal might be influenced by the nutritional status and food intake of the animal with respect to protein.

It is difficult to say whether there is any real difference between the curves for our normal dogs and those in shock (Charts A and B). We are inclined to believe that the close similarity between the two normal dogs may be fortuitous and that all the curves may fall within a normal range. It should be mentioned again that the curves for the shocked dogs cannot be properly called disappearance curves since they do not reflect the massive losses of plasma protein from the circulation but only the relative amounts of labeled and unlabeled protein in circulation. If the shocked animals had been able to mobilize quickly large quantities of protein from the circulation, one might have expected a considerably increased rate of dilution of the labeled plasma in circulation to occur in the dog with intestinal trauma. Similarly one might have expected sharp changes in the rate of dilution during the development of shock following removal of the clamp from the dogs with leg trauma. Indeed, if even a normal rate of exchange between plasma proteins and tissue protein had continued during shock, the influx of this normal amount of unlabeled protein into the greatly decreased amount of circulating plasma might have been ex-

pected to increase the rate of dilution. From this viewpoint we might interpret the lack of evidence for any such marked change in slope of the shock dogs (Chart B) as evidence that the rate of mobilization of tissue protein into plasma protein is greatly reduced in shock. It is of interest that the rate of sodium exchange across the capillary membrane has been shown to be greatly reduced in shock (5).

Experiments that are similar to those reported here have been described by Fine and Seligman (4). They report very briefly experiments in which plasma containing cystine labeled with radioactive sulfur was transfused into dogs. A curve is shown covering the first 14 hours of one experiment. Two hours after the administration of labeled plasma, 93 per cent of the radioactivity was still circulating; after 6½ hours, 90 per cent; and after 14 hours, 70 per cent. The data for two dogs are summarized by stating that 90 per cent of the radioactive protein was circulating 5 hours after injection; 70 per cent, 15 hours after injection; and 45 per cent, 48 hours after injection. They do not give data on the amount of labeled plasma protein injected. The amount of plasma removed for sampling and measurement was presumably small. The 48 hour value is quite similar to that found in our experiments with normal dogs (Chart A).

Fine and Seligman also used plasma protein labeled *in vitro* with radioactive bromine. The labeling procedure caused partial denaturation of the protein so that its rate of disappearance varied according to the bromine content, and accordingly the material could not be employed in studying the normal metabolism of plasma proteins. The ease of preparing such labeled protein and the relative accuracy with which its movements in the body could be followed, however, made it quite useful in comparing its rate of disappearance in shock animals and normal controls under nearly identical conditions. They found no significant differences between the rate of loss in normal animals and those in untreated hemorrhagic shock.

It is of some interest to compare the rate of disappearance of labeled plasma proteins with that of a transferred homologous antibody. Antibodies are in general considered to be very similar chemically and physically to the ordinary serum protein gamma globulin, with slightly different groups or arrangements which cause them to react against specific antigens. The metabolism of an actively produced antibody in the circulation of a rabbit or rat which had received antigen some time previously could not be differentiated by use of isotopes from the metabolism of the other serum proteins (19, 20). However, when an antibody produced by one rabbit was injected into another, it failed to take up labeled nitrogen from the body of the recipient and gradually disappeared from the circulation, indicating that the recipient was able to carry out its degradation, but was unable to re-synthesize it (16). If the catabolism of such an antibody, in the absence of specific antigen, is carried out in the

same manner as that of the closely related ordinary serum protein, the antibody would be a very nearly perfect labeled protein.

Thus, it may be significant that the rate of loss of injected antibody in the rabbit is quite similar to that reported here for labeled plasma proteins in the dog. According to the data of Heidelberger *et al.* (6), and assuming that 100 per cent of the passive antibody was circulating 20 minutes after the injection, at 22½ hours after the injection 62 per cent of the antibody was left in circulation, at 48 hours, 44 per cent remained, and after 168 hours, 17 per cent still remained. This last figure would probably have been higher except that a large blood sample was taken earlier and made up by a transfusion of ordinary blood.

The non-logarithmic nature of the antibody disappearance curve could hardly be due to incomplete mixing since 20 minutes elapsed between the injection and the first sample. It is probably not due to having a composite curve of differing rates because the antibody is presumably a homogeneous protein. The recipient is unable to re-synthesize the antibody, thus eliminating that complication. Partial injury of the protein during transfusion is possible, but seems improbable as pointed out in the discussion of our two normal dogs. The antibody probably has a better chance than other plasma protein of being treated as a foreign substance by the recipient, but since it is a homogeneous protein such treatment would not account for the slower than logarithmic decrease in its rate of disappearance. Indeed, if it were to be treated as a foreign protein, it might be expected that as the body mechanisms were gradually mobilized against the antibody, its rate of disappearance might actually increase. In this connection it is of further interest that even heterologous antibodies (21) show curves of disappearance similar to those of homologous antibodies and labeled plasma proteins, at least during the first few days. It thus seems probable that a major proportion of the early relatively rapid loss of passive antibody from the circulation is due to the escape of the antibody protein molecules into the tissues, presumably with the return of an equivalent amount of protein to the blood. After the antibody labeled concentration of the extravascular mobile protein reaches that of the plasma the further and slower decline of antibody protein in the plasma is probably related to its rate of degradation within the body.

The data for antibody disappearance as well the experiments of Chart A might be interpreted to point to an active tissue mass of protein about equal in size in the normal animal to the mass of plasma protein, in that the rapid dilution phase appears to end near the 50 per cent level. It is of further interest that the labile portion of plasma protein producing material in the normal dog is approximately equal to the normal mass of circulating plasma protein as measured by chronic plasmapheresis (10).

SUMMARY

Labeled plasma proteins are produced by administering to dogs the amino acid lysine synthesized with heavy nitrogen. Such labeled proteins are apparently indistinguishable biologically from proteins of normal isotope concentration.

Labeled plasma proteins, as plasma, injected into normal dogs pass out of the blood stream at an initially rapid but constantly decreasing non-logarithmic rate. This outflow is balanced by a simultaneous inflow of plasma proteins from the tissues. Fifty per cent of the labeled protein is out of the blood stream in about 24 hours; 75 per cent in about 6 days.

Shock due to trauma of intestine or leg shows a dilution curve of labeled plasma protein not unlike that of the normal dog. If anything, dilution appears a little less rapid in shock. Since the usual shrinkage of plasma volume and plasma protein mass is present in these shocked dogs, these data are compatible with a decreased inflow of protein into the plasma during shock.

Methods are described which are suitable for the use of heavy nitrogen incorporated in the epsilon group of lysine and its subsequent analysis in body fluids.

These data may indicate that the plasma proteins are normally in constant and rapid exchange with a mobile pool of body protein.

BIBLIOGRAPHY

1. Daft, F. S., Robscheit-Robbins, F. S., and Whipple, G. H., *J. Biol. Chem.*, 1938, **123,** 87.
2. Duncan, G. W., and Blalock, A., *Ann. Surg.*, 1942, **115,** 684.
3. Dunn, M. S., and Smart, B. W., *J. Biol. Chem.*, 1930, **89,** 41.
4. Fine, I., and Seligman, A. M., *J. Clin. Inv.*, 1943, **22,** 285.
5. Gellhorn, A., Merrell, M., and Rankin, R., *Am. J. Physiol.*, 1944, **142,** 407.
6. Heidelberger, M., Treffers, H. P., Schoenheimer, R., Ratner, S., and Rittenberg, D., *J. Biol. Chem.*, 1942, **144,** 555.
7. Holman, R. L., Mahoney, E. B., and Whipple, G. H., *J. Exp. Med.*, 1934, **59,** 269.
8. Howland, J. W., and Hawkins, W. B., *J. Biol. Chem.*, 1938, **123,** 99.
9. Johnson, J. R., Organic synthesis, New York, John Wiley & Sons, 1939, **19,** 62.
10. Madden, S. C., and Whipple, G. H., *Physiol. Rev.*, 1940, **20,** 194.
11. Mahoney, E. B., Kingsley, H. D., and Howland, J. W., *Ann. Surg.*, 1941, **113,** 969.
12. NcNaught, J. B., Scott, V. C., Woods, F. M., and Whipple, G. H., *J. Exp. Med.*, 1936, **63,** 277.
13. Nier, A. O., *Rev. Scient. Instr.*, 1940, **11,** 212.
14. Pommerenke, W. T., Slavin, H. B., Kariher, D. H., and Whipple, G. H., *J. Exp. Med.*, 1935, **61,** 283.
15. Rittenberg, D., Keaton, A. S., Rosebury, F., and Schoenheimer, R., *J. Biol. Chem.*, 1939, **127,** 291.
16. Schoenheimer, R., The dynamic state of body constituents, Cambridge, Harvard University Press, 1942.

17. Schoenheimer, R., and Rittenberg, D., *J. Biol. Chem.*, 1939, **127,** 285.
18. Schoenheimer, R., and Rittenberg, D., *Physiol. Rev.*, 1940, **20,** 218.
19. Schoenheimer, R., Ratner, S., Rittenberg, D., and Heidelberger, M., *J. Biol. Chem.*, 1942, **144,** 541.
20. Schoenheimer, R., Ratner, S., Rittenberg, D., and Heidelberger, M., *J. Biol. Chem.*, 1942, **144,** 545.
21. Topley, W. W. C., and Wilson, G. S., Principles of bacteriology and immunity, Baltimore, William Wood & Co., 2nd edition, 1936, 883.
22. Van Slyke, D. D., *J. Biol. Chem.*, 1915, **22,** 281.
23. Weissman, N., and Schoenheimer, R., *J. Biol. Chem.*, 1941, **140,** 779.