CIRCULATION OF PLASMA PROTEINS: THEIR TRANSPORT TO LYMPH*

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As part of a study of protein turnover in diabetes (1), S^{35}-labeled serum proteins were introduced intravenously into dogs, and the disappearance of the labeled proteins from the circulating plasma was followed for 7 or more days. The specific activity of serum protein in the recipient dogs decreased rapidly at first, but after 2 days it decreased at a much slower rate. This slow phase is considered to represent the metabolic degradation of the labeled proteins, whereas the most reasonable explanation for the initial rapid phase is that reversible exchange occurs between plasma protein molecules in the blood stream and similar molecules in other regions of the animal.

In order to justify the above interpretation, it was felt that the rapid phase should be further investigated. Thus, the present communication deals with the exchange of labeled serum protein between blood and lymph. It is shown here that intravenously injected, S^{35}-labeled serum proteins appear very rapidly in the thoracic duct lymph, and that a continuous circulation of protein exists between blood and lymph.

EXPERIMENTAL
Labeled Serum Proteins

Preparation of sterile solutions of S^{35}-labeled serum proteins, obtained biosynthetically from a donor dog, is described in the preceding paper (1). The amount of labeled protein injected was approximately 0.012 per cent of body weight, in the case of the rats, and 0.021 per cent of body weight, in the case of the dogs. This labeled protein was dissolved in isotonic saline at a concentration of 6 gm. of protein per 100 ml., as described previously (1).

Animal Procedures

Rats—Three male rats of the Long-Evans strain, weighing from 400 to 500 gm., were used. They received a diet consisting of 68.5 parts of

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ground whole wheat, 5 of raw casein, 10 of fish meal, 10 of alfalfa leaf meal, 5 of sardine oil (fortified with vitamins A and D), and 1.5 of NaCl. This diet contains 19.3 per cent protein and is considered to supply adequate amounts of the known dietary factors. The diet was continuously available to the rats before and after the operation.

The rat was kept in a restraining cage (2) after introduction of a cannula into the thoracic duct (3). Lymph samples were collected in graduated centrifuge tubes containing dry heparin.

**Dogs**—Although the entire flow of thoracic duct lymph was collected from the rats, a special cannula was used with the dogs, which returned the lymph back into the blood stream via the left external jugular vein. This cannula consisted of two parts which could be separated for the collection of a lymph sample. Whenever the two parts of the cannula were disconnected, a syringe containing isotonic saline was attached to the venous part by means of a needle of large diameter. This served to prevent the entry of air into the blood stream; the introduction of small amounts of saline also reduced the possibility of clot formation in the cannula. The parts of the cannula were disconnected for short periods to permit collection of individual lymph samples, but at all other times the lymph was recirculated.

Dogs were under barbital anesthesia for the duration of the operation and the period in which lymph samples were collected. Their body temperatures were maintained approximately constant by the application of heat as required. A glucose-saline solution was administered by continuous intravenous infusion at a rate of 2 ml. per hour per kilo of body weight. This rate was estimated to replace the fluid lost, and no attempt was made to force the rate of lymph flow to abnormally high values. In order to minimize stagnation of lymph, the limbs of the anesthetized animal were frequently massaged and their positions changed during the entire experiment.

Dogs received Purina fox chow as their regular food, but meat and milk were given 3 hours before each experiment.

**Preparation of Cannulae**

The cannulae were cut from flexible, plastic tubing of the desired diameter. These tubes were carefully cleaned and dried, and their inner surfaces were coated with a thin layer of a liquid Silicone. The Silicone was purified before use by heating for several days at about 150°. This preliminary step may be necessary if HCl (produced in manufacture) or other

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1 This was a sterile solution containing 5 gm. of glucose and 0.9 gm. of NaCl per 100 ml. of non-pyrogenic, distilled water. It was obtained from the Cutter Laboratories, Berkeley.
volatile impurities are present. The thin coating of Silicone was applied
by introducing one or more drops, with a micro pipette, into the upper
end of a tube that had been fixed in a vertical position. Under the force
of gravity, the liquid slowly flowed through the tube, and the excess
drained from the lower end. About 2 days were required for complete
drainage of the excess when a viscous Silicone was used. If the cannula is
cut after its preparation, it is important to coat the new ends with Silicone,
because these ends are the most likely places for clots to occur. At no
time was a clot observed, with blood or lymph, that could be attributed
to contact between the fluid and the surface of the cannula.

For the experiments with dogs, the recycling cannula made from Trans-
flex tubing was used and found to be very satisfactory. The two parts of
the cannula may be connected (a) by widening the end of the part of
the cannula that leads into the jugular vein, or (b) by using a larger size of
tubing for the venous part. The second procedure was most satisfactory
and was used with Dog 2.

In dogs, with properly prepared cannulae, no interruptions of lymph
flow, from thoracic duct to jugular vein, were observed. With the rats,
coagulation of protein tends to occur at the lymph-air interface, and clots
occasionally formed there.

Treatment of Blood and Lymph Samples

Blood samples were taken from the left external jugular vein, with
heparin as an anticoagulant. For each determination, a 1 ml. aliquot of
plasma was diluted with 1 ml. of water, and 2 ml. of 10 per cent trichloro-
acetic acid (TCA) were added, with vigorous stirring, to precipitate pro-
etins. Dilution of plasma, prior to TCA addition, discourages the forma-
tion of large clumps in the protein precipitate.

Lymph samples from the thoracic duct cannula were collected in cen-
trifuge tubes containing heparin. After centrifugation, aliquots of the
cell-free supernatants were mixed with equal volumes of 10 per cent TCA
to precipitate proteins. Unlike the plasma samples, lymph samples were
not diluted before the addition of TCA, since the protein concentration in
lymph is less than that in plasma.

All protein precipitates (plasma and lymph) were washed twice with 5
per cent TCA. The remainder of the procedure for the determination of
total protein sulfur and protein S86 has been described (4). No carrier
sulfur was added, since aliquots containing approximately the standard
amount of protein sulfur (25 μM) were taken.

Separation of Methionine and Cystine—Plasma and lymph proteins were

*Transflex tubing is a product of the Irvington Varnish and Insulator Company,
Irvington, New Jersey.
precipitated and washed with TCA as described above. The precipitates were hydrolyzed with constant boiling HCl, and the methionine and cystine were separated according to the procedure of Simpson and Tarver (5).

Results

Observations with Rats

Cannulae were introduced into the thoracic ducts of three rats anesthetized with ether. As soon as a steady flow of lymph was established (about 10 minutes after operation), each rat received an intravenous injection of 1 ml. of a sterile solution containing 6 per cent of the reconstituted, S\textsuperscript{35}-labeled dog serum protein. Lymph collection was begun immediately, and all lymph flowing through the thoracic duct was collected for the duration of the experiment. Lymph receivers were changed repeatedly so that a series of samples was obtained; the collection period for each lymph sample was 2 hours or longer.

Data from a male rat (Rat 18) weighing 490 gm. are considered typical and will be reported here. This rat was chosen because lymph flowed continuously through its thoracic duct cannula for 11 days. Results on other rats confirm the initial data, but lymph production did not continue for that length of time. The animals were not fasted; food and water were always available.

Time \((t)\), in the following experiments, is measured from the time of injection of the labeled protein. The cumulative volume output of the collected lymph samples as a function of time is shown in Fig. 1. This relation is well represented by three straight line sections with successively greater slopes. The slope of this cumulative function is the rate of flow of thoracic duct lymph in ml. per hour. This rate of flow was determined graphically for each section, and the following average values were obtained: first section \((t = 0\) to 52 hours), 0.74 ml. per hour; second section \((t = 60\) to 127 hours), 2.5 ml. per hour; and third section \((t = 131\) to 250 hours), 3.4 ml. per hour. The rate of flow of lymph increased suddenly between 52 and 60 hours. The rat was observed to increase its intake of food and water during this period.

The cumulative total protein output of the collected lymph as a function of time is also shown in Fig. 1. This curve is quite smooth, and its slope increases without abrupt changes during the 11 days. The slope of this curve is the rate of flow of protein through the thoracic duct. Initial and final values for this rate were determined graphically, and are 0.34 and 0.65 mg. of protein sulfur per hour, respectively.

Soon after intravenous injection of the S\textsuperscript{35}-labeled serum protein, the thoracic duct lymph was found to contain labeled protein. The cumulative
output of protein $S^{35}$ in the thoracic duct lymph as a function of time is shown in Fig. 2. Each point on this curve includes the sum of all protein $S^{35}$ collected from the thoracic duct after time zero. It can be seen that the early rapid appearance of $S^{35}$ in lymph becomes more gradual with time, and after 6 days the curve approaches a plateau corresponding to
the output of a total of about 32 per cent of the injected $^{38}S$. Since this curve was drawn from cumulative data, local irregularities in the actual rate of output of labeled protein are minimized. For this reason another curve is presented in Fig. 2 which elucidates the more intimate aspects of the process under study. This curve shows the rate of output of $S^{38}$-protein in the thoracic duct lymph as a function of time. Each collection period is represented by a point, and two approximations were used: first, the ordinate of each point is the average of all infinitesimal rates occurring during the collection period represented by that point; second, this average rate is plotted at the mid-time of the particular collection period. Neither approximation influences the shape of the curve appreciably except during the first few hours, when the rate is changing rapidly.

If time zero is defined as the instant immediately before injection of the labeled protein, the rate of appearance of $S^{38}$-protein in the thoracic duct is zero at that time. The rate increases very rapidly after injection, and the highest rate (0.83 per cent per hour) is plotted in Fig. 2 at $t = 3.4$ hours, the mid-time of the second collection period. The downward trend of the curve is quasiexponential; however, superimposed upon this trend are periodic increases that recur at intervals of 1 day. These increases are clearly shown in Fig. 2, and, since they occur at night, they are probably related to the nocturnal habits of the rat.

In the studies with rats, all of the lymph from the thoracic duct was collected for the duration of the experiment. Those with cannulae continually lose water, protein, salts, etc., and this fact must be considered in interpreting an experiment of this kind. Loss of protein is particularly important in the present study. It was calculated that Rat 18 lost each day a quantity of plasma protein equal to two-thirds the total amount present in its blood stream. Thus, it is unlikely that the animal was in a steady state with respect to its plasma proteins for more than 1 hour of lymph collection.

The loss of protein observed above must lead to a fall in the plasma protein concentration, and continued survival of the animal, despite this serious loss, could have been accomplished only by very rapid synthesis of new protein. By this device the animal probably attains a new steady state at a more rapid rate of turnover of plasma proteins.

**Observations with Dogs**

In the above experiments with rats, all of the thoracic duct lymph was collected, and the steady state of the animal with regard to serum proteins was soon disrupted. With dogs, however, the lymph was returned to the general circulation in an essentially normal manner by means of the specially prepared cannula, except during the short periods required to
obtain lymph samples. The volume of lymph taken in these samples was small compared with the total flow of thoracic duct lymph.

Cannulae were introduced into the thoracic ducts of two healthy, normal dogs. Barbitral anesthesia was used for the operation and for the experimental period thereafter. At time zero, each dog received an intravenous injection of the reconstituted, sterile serum containing $^{35}S$-labeled protein. Blood and lymph samples were taken repeatedly, and the protein sulfur and protein $^{35}S$ contents of each sample were determined.

Similar results were obtained with both dogs, and Dog 2 will be con-

**FIG. 3.** Dog 2. Specific activities of plasma and lymph protein $^{35}S$ following intravenous injection of labeled serum protein. Determinations were made in duplicate on all plasma samples and on the last three lymph samples.

considered as representative. This dog was a female and weighed 11.5 kilos. Data obtained from it are shown in Figs. 3 and 4, and the following may be observed. (a) The specific activity$^3$ of plasma protein $^{35}S$ decreases rapidly at first and then more slowly. This is consistent with the observation of an initial rapid phase reported in the preceding paper (1). In that study, the decline in specific activity of plasma protein was followed for 7 days after intravenous injection of the same labeled protein used here. The rapid component of this decline, the subject of the present study, was observed to disappear, for practical purposes, after 2 days. The most obvious explanation for these findings is that the rapid phase results from the reversible exchange (mixing) of the injected labeled protein with sim-

$^3$ Specific activity is expressed as counts per minute per mg. of sulfur of protein origin.
ilar unlabeled protein outside the blood stream. (b) The intravenously injected protein appears rapidly in the thoracic duct lymph, and the specific activity of the protein of the first lymph sample (35 minutes after injection) was close to the highest observed value. (c) The specific activity of protein in the thoracic duct lymph remained significantly less than that in plasma throughout the experiment (19 hours). This difference is probably the result of unlabeled protein coming from all parts of the animal and diluting the labeled protein.

The use of "specific activity" in the experiments with dogs is not subject to the limitations of its use in the experiments with rats. These limitations resulted from (a) continual loss of S38-protein, and (b) synthesis of new unlabeled protein to replace that lost. With the dogs, by recirculating the lymph and by carefully limiting the volumes of the blood and lymph samples removed, the steady state of the plasma proteins was preserved.

The logarithm of the specific activity of plasma protein S38 was plotted as a function of time (Fig. 4). The first five points lie close to a straight line, and a line was fitted to these points by the method of least squares. The transformation constant obtained was 0.103 per hour. Thus, the rate of disappearance of labeled protein from the blood stream was 10.3 per cent per hour. The curve soon deviates from the straight line in a manner suggesting that a reversible process is at work. At any time, the extent of the reverse process, by which labeled molecules diffuse back into the blood stream, depends upon the number of such molecules outside of the blood stream at that time. During the early interval used for the above calculation, it is justifiable to ignore the reverse process. The
transformation constant, 0.103 per hour, compares well with similar values obtained with unanesthetized dogs (1). The values for the latter were 0.098 and 0.099 per hour for the rapid phase.

Identity of \( \text{S}^{35} \)-Protein Found in Dog Lymph

In the present investigation, as in those of other laboratories (6–8), it is natural to question the identity of the labeled protein found in the thoracic duct lymph after the intravenous injection of labeled serum protein. Alteration of the labeled protein molecules, after their intravenous injection, is a possibility that could seriously change interpretations based upon these measurements.

Table I contains evidence that the \( \text{S}^{35} \)-protein observed in lymph is the same as that in the blood stream. The last column shows the ratio of the specific activity of cystine sulfur to that of methionine sulfur for the total protein of plasma and of thoracic duct lymph. The values of this ratio are nearly equal: 0.824 for plasma and 0.811 for lymph. Since the rate of the rapid phase was almost identical for albumin and globulin (1), unfractionated protein appeared adequate and was used for the present purpose. If any fundamental change had occurred in the molecules of the injected protein, it is improbable that these ratios would be so nearly alike.

**DISCUSSION**

The labeled serum proteins used in this study were synthesized in a dog that had been injected with \( \text{S}^{35} \)-methionine. Dialyzable \( \text{S}^{35} \) had been removed. Thus, the \( \text{S}^{35} \) present in the injected serum may be considered as part of the structure of nearly normal molecules of dog serum protein. Proteins prepared in this manner contain \( \text{S}^{35} \) in both methionine and cystine; this fact was utilized in an attempt to identify the labeled protein in lymph with that in the blood plasma.
As previously reported, the rates of turnover are approximately the same for the labeled albumin and globulin fractions of dog serum (1). Fibrinogen, however, appears to be more labile than the other plasma proteins (9). If fibrinogen turns over relatively rapidly, it might easily attain a higher specific activity than the other proteins in donor plasma after injection of the labeled amino acid. Although the concentration of fibrinogen in plasma is small, a relatively high specific activity in this protein could complicate the interpretation of data obtained during the first few days following injection of labeled plasma proteins. For this reason, serum rather than plasma from the donor dog was used in the present study.

In the case of the rats, the behavior of the labeled dog serum protein may differ from that of rat serum protein, but in the experiments with dogs, it is reasonable to believe that the results obtained represent normally occurring physiological processes. In the experiments with rats, all of the thoracic duct lymph was collected; intravenously injected, labeled serum protein appeared rapidly in the lymph. As shown in Fig. 2, the rapid increase in rate of appearance of labeled protein in lymph was followed by an almost equally rapid decrease in this rate. With the dogs, however, the experiments were designed so that most of the lymph was returned to the blood stream. Intravenously injected protein also appeared rapidly in the thoracic duct lymph, but, unlike the results obtained with rats, this was not followed by a rapid disappearance. The latter experiments undoubtedly give a truer picture of the blood-lymph relationship.

The initial rapid rise in the specific activity of lymph protein, shown in Fig. 3 for the dog, stopped rather abruptly, with little continued rise. In spite of the constancy in the specific activity of lymph protein, between 0.5 and 6 hours, the specific activity of plasma protein continued to fall during this period. The rapid appearance of labeled protein in lymph suggests a relatively direct route for the passage of protein molecules between the blood stream and the thoracic duct. But the labeled proteins probably diffuse out of the blood stream into tissues in general, and it is the return of varying mixtures of endogenous protein with the injected labeled protein that must account for the constancy of specific activity observed in thoracic duct lymph.

It is clear from the above results that plasma protein molecules circulate within a much larger effective volume than that of the blood stream alone. In order to assess the significance of the thoracic duct as an agent for the directed transport of plasma proteins, an average rate of flow of protein in this duct was computed for the experiment with Dog 2. This experiment may be considered as a series of fifteen short lymph collection periods that alternate with a series of longer time intervals, between the periods, during which lymph was not collected. Let \( P_i \) represent the dura-
lations of the collection periods, in hours, where \( i = 1, 2, 3 \ldots 15 \). Let \( Q_i \) represent the durations of the intervals between collection periods, in hours, where \( i = 0, 1, 2 \ldots 14 \). Let \( R_i \) equal the rate of flow of protein in the thoracic duct during the \( i \)th period, and let \( m_i \) equal the mass of protein\(^4\) collected during that period. Then \( R_i = m_i / P_i \). Now, in order to find the total amount of protein, \( M \), passing through the thoracic duct during the entire experiment (19.43 hours), each interval \( Q_i \) (except \( Q_0 \)) was divided into two equal parts, and the calculation was performed as follows:

\[
M = Q_0 R_0 + P_1 R_1 + (\frac{Q_1}{2}) R_1 + (\frac{Q_1}{2}) R_2 + P_2 R_2 + \cdots + P_{15} R_{15} =
\]

\[
Q_0 R_0 + \sum_{i=1}^{14} P_i R_i + \frac{1}{2} \sum_{i=1}^{14} Q_i R_i + \frac{1}{2} \sum_{i=1}^{14} Q_i R_{i+1}
\]

Upon substitution of the various numerical values, \( M = 11.7 \) gm. of protein. Hence, the average rate of flow of protein in the thoracic duct was 14.4 gm. per day. The significance of this rate is emphasized when it is compared with an estimate of the total plasma protein in the blood stream of the animal. If the plasma volume of Dog 2 is assumed to be 575 ml. (5 per cent of 11.5 kilos), and the protein content of the plasma is 6 gm. per 100 ml., the blood stream of this dog contained about 34 gm. of plasma protein. Obviously, the 14.4 gm. of protein calculated above are highly significant. It means that about half of the total blood plasma protein of the dog passes through its thoracic duct each day under the experimental conditions. The anesthetized animal, however, forms lymph more slowly, and the rate in the normal dog may be greater than that observed here.

Rat 18 lost a total of 10.7 gm. of protein because the thoracic duct lymph produced by this animal was continuously collected during an 11 day period. This quantity of protein is about 7.3 times the normal total blood plasma protein of this rat. Furthermore, loss of protein occurred at an increasing rate: at the 11th day, the rate had increased 2-fold above the initial rate. Despite this great loss, the animal remained alive and active. Although there may exist collateral lymphatic circulation, it may be stated that newly formed plasma protein molecules can enter the blood stream without recourse to the thoracic duct.

**SUMMARY**

1. \( \text{S}^{35} \)-labeled serum proteins, prepared biosynthetically in a donor dog, were introduced intravenously into dogs and rats. At intervals, blood

\(^4\) The mass of protein was obtained by multiplying the protein sulfur by 76.92. This factor is an average value obtained experimentally.
and thoracic duct lymph samples were taken from the recipient animals for protein $^{35}$S determination.

2. In both the rat and the dog, labeled protein appeared rapidly in the thoracic duct lymph.

3. Thoracic duct lymph collected from the recipient rats was not returned to their circulation. These rats lost, via the thoracic duct, approximately two-thirds of the total blood plasma protein per day.

4. In recipient dogs, the thoracic duct lymph was returned to the general circulation by means of a special cannula. Thus, the steady state of plasma proteins was not disturbed. The importance of this recirculation of lymph is emphasized by calculation of the daily protein loss that would otherwise have occurred. Approximately half of the total blood plasma protein traverses the thoracic duct per day.

5. Rat 18 lived for 11 days, despite loss of 10.7 gm. of protein, which represents 7.3 times the estimated normal total blood plasma protein of this animal. It follows that the thoracic duct is not essential for the entry of newly formed plasma protein molecules into the blood stream.

6. The disappearance of labeled serum protein from the blood stream of the dog during the initial rapid phase appears to result from reversible exchange between plasma protein molecules in the blood stream and similar extravascular molecules. This interpretation is consistent with that made in our work previously reported.

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