

## THE MANNER OF REMOVAL OF PROTEINS FROM NORMAL JOINTS\*

By WALTER BAUER, M.D., CHARLES L. SHORT, M.D., AND GRANVILLE A.  
BENNETT, M.D.

*(From the Medical Clinic of the Massachusetts General Hospital and the Department  
of Pathology, Harvard Medical School, Boston)*

(Received for publication, October 8, 1932)

Our knowledge concerning the physiology of normal joints is very meager. Fisher (1) concluded from certain of his experiments that true solutions are absorbed from normal joints by the lymphatics and capillaries alike. Key (2) and Ryneerson (3) state that particulate matter (India ink) is removed from normal joints by the lymphatics with the aid of fixed and wandering phagocytic cells. Except for a few publications of this sort, review of the literature reveals little concerning the physiology of normal articular cavities. Yet it is obvious that if we had a clearer conception of the factors involved in the interchange of true and colloidal solutions in normal joints, our knowledge of the physiology of these structures would be greatly enhanced and we would better understand the production and maintenance of joint effusions.

The protein which is present in normal and pathological synovial fluids is obviously an important factor in the interchange of normal synovial fluid and the maintenance of articular effusions by reason of its osmotic pressure. We have been unable to find any data in the literature bearing directly upon the removal of colloidal matter like protein from the synovial cavity, although the experiments of others (Lewis (4), Bolton (5), Field and Drinker (6)) have established that proteins such as those contained in horse serum are absorbed from the tissue spaces and serous cavities under normal conditions by lymphatics alone, a belief expressed by Starling (7) in 1896. The purpose of this communication, then, is to set forth experiments under-

\* This is Publication No. 10 of the Robert W. Lovett Memorial for the study of crippling disease, Harvard Medical School, Boston.

taken to determine: first, whether proteins are absorbed through the synovial lymphatics or blood vessels; second, the approximate rate of their removal, and whether exercise of the joint hastens the rate of removal.

#### *Materials and Methods*

All the experiments were performed on large young adult dogs under nembatal "844"<sup>1</sup> anesthesia, thus permitting no motion of the joints in the unexercised dogs. In the exercised dogs, passive flexion and extension of the injected knee joints were accomplished by attaching the paw to a crank traveling in a circular path at a rate of 50 to 60 revolutions per minute. The protein-containing substance was injected directly into the knee joint by means of a hypodermic needle<sup>2</sup> and syringe. Such an injection can be made with ease after some practice, and there was little doubt that the injected material was within the joint, a fact corroborated by aspiration of the joint at the end of the experiment. Such intraarticular injections were made once the animals were anesthetized and samples of blood and lymph for control precipitin tests had been obtained. The appearance of the proteins in the blood and lymph was determined by precipitin tests done at frequent intervals, using the serum of rabbits which had been immunized against the particular protein employed. In certain experiments the lymphatics of both the right and left side of the neck were carefully isolated and all those entering the veins were tied off. By using this method of Field and Drinker (6) all communications between the vascular and lymphatic systems are severed. Samples of lymph could then be taken from the thoracic duct and blood samples from the jugular vein or a leg vein, knowing that the latter were free of all constituents absorbed by way of the lymphatics.

Preliminary experiments had shown that the precipitin reaction would give an approximately quantitative measure of the concentration of foreign protein in the circulating blood at any one time. It was thought that this method was more reliable and less open to error than the complement fixation reaction used by Lewis (4) in experiments devised to study the route of absorption of horse serum from tissue spaces. Immune sera of high titre (positive in dilutions of antigen of 1/100,000 or over) were prepared from rabbits, by the method described by Dienes and Schoenheit (8), with successive injections of small quantities of the antigen into subcutaneous, non-virulent tuberculous lesions. The precipitin reactions were done by the ring test method, with the serum or lymph dilutions overlying the undiluted antiserum. Readings were made after the tubes had stood for 1½ hours at room temperature. In each case successive dilutions of the antigen with normal saline were used, thus giving a dependable indication of the relative amount of foreign protein present in the sample tested. In each rack, the reac-

<sup>1</sup> Sodium-ethyl (1-methyl-butyl) barbiturate.

<sup>2</sup> No. 25 hypodermic needle was used in all experiments.

tions were controlled by one tube with normal saline overlying the immune serum. As noted above, in every experiment, control samples of blood and lymph were taken before the foreign protein was introduced into the joint. The importance of this procedure was borne out when we found in several experiments that these control samples gave slightly positive precipitin reactions, presumably of a non-specific nature.

The usual procedure in an experiment was as follows: After anesthesia was obtained, the lymphatics were isolated and tied off at their entrance into the vessels of the neck and control samples of blood and lymph were taken. The protein material (egg white or horse serum) was injected into the knee joints of each of two dogs. Not more than 3 cc. were injected, which probably caused little distention of the joint capsule or increase in the intraarticular pressure. The injected knee joint of one dog was kept motionless while the injected knee joint of the other was passively flexed and extended 50 to 60 times a minute by means of the apparatus described above. Following this, samples of blood and lymph were taken at regular intervals and the approximate amount of foreign protein present determined by means of the precipitin test.

#### *Experiments in Which Egg White Was the Protein Used*

Several preliminary experiments were performed in which the communications between the vascular and lymphatic systems were not tied off, in order to determine whether egg white injected into the knee joint can be detected in the blood stream. If so, how soon does it appear and does passive exercise of the joint hasten its appearance? 3 cc. of egg white with a protein content of 11.54 gm. per 100 cc. were injected into the knee joints of each of two dogs. It is very evident from Fig. 1 that egg white can be detected earlier in the blood stream of the exercised dog and that it is present in higher concentrations than in the case of the unexercised dog. It can be seen that a positive precipitin reaction was obtained with a serum dilution of 1/32 at the end of the 2nd hour in the exercised dog, whereas in the unexercised dog it was detectable only in a serum dilution of 1/4. Thus, one can conclude from this type of experiment that egg white can gain entrance into the blood stream from normal dog joints and can be recognized there by precipitin reactions, using immune rabbit serum. Such experiments further demonstrate that the absorption of protein is much more rapid from an exercised than from a non-exercised joint.

In order to determine whether or not results obtained in the preceding experiments were in part due to the accumulation of egg white in the blood as it escaped from the joint, small successive doses of egg

## 422 REMOVAL OF PROTEINS FROM NORMAL JOINTS

white were injected intravenously at hourly intervals in four dogs. Before each injection a sample of blood was obtained and a precipitin reaction carried out with immune rabbit serum. Fig. 2 illustrates the results obtained and shows that there is little tendency for the accumulation of egg white in the circulating blood. The serum dilutions positive, 1/16, obtained in Dogs 3 and 4, 5 minutes after the injection of

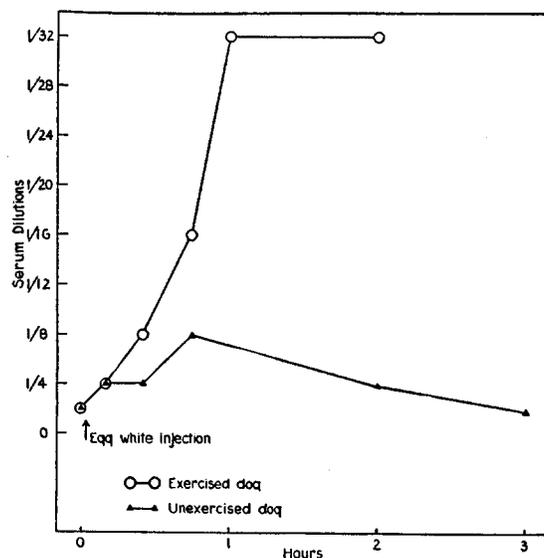


FIG. 1. Experiment I. The concentration of egg white in the blood stream following injection into the knee joint of 3 cc., showing the relative absorption from passively exercised and unexercised joints.

the egg white, agree quite closely with the theoretical concentration in the blood stream at that particular moment, as obtained from calculations. The rapid fall in the next 55 minutes is probably largely due to excretion through the kidney. This explanation is in accord with the finding of Opie (9) that egg white injected subcutaneously soon appears in the blood stream and shortly thereafter can be demonstrated in the urine. This rapid removal of foreign protein from the blood stream may well explain the inconsistent results obtained in later experiments, in some of which we observed no significant appearance of egg white in the blood stream of either exercised or non-

exercised dogs. Therefore, if one takes into account the rapidity with which a foreign protein can be removed from the blood stream, one realizes that even greater amounts have appeared in the blood stream than one detects with the precipitin tests. Such experiments further prove that our results as shown in the first experiments are not due to mere accumulation of the egg white in the blood stream.

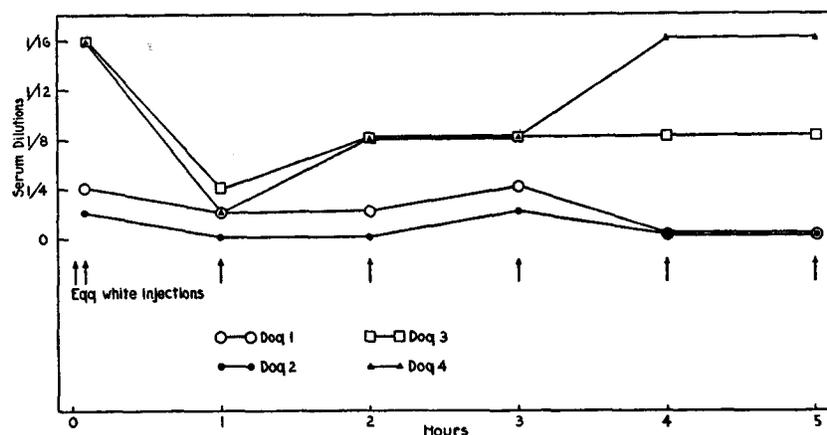


FIG. 2. Experiment II. The effect on the concentration in the blood stream of repeated small intravenous injections of egg white. Dogs 1 and 2 received 0.0027 cc. of egg white per kilo body weight, whereas Dogs 3 and 4 received 0.011 cc.

In the next series of experiments an attempt was made to determine if possible the route of removal of the injected protein.

Two dogs were used for each experiment; all the lymphatics emptying into the great vessels of the neck were tied off, thus eliminating all communications between the lymphatic and vascular systems. Cannulae in the thoracic ducts enabled us to collect frequent samples of lymph. Blood samples were taken from each dog at the same time intervals, thus allowing us to determine whether any protein can gain entrance into the blood stream if the lymphatics entering the neck veins are completely tied off. The injected knee joint of one dog was exercised as before. The abdomens of both were massaged in order to hasten the flow of lymph. The injected joint of the other dog was not touched.

From Fig. 3, one notes that the control blood samples did not give a positive precipitin reaction, but what is of more interest is that no

## 424 REMOVAL OF PROTEINS FROM NORMAL JOINTS

one of the blood samples from either dog gave a positive precipitin reaction after the intraarticular injection. It is further seen that the egg white appears in the thoracic duct lymph of the exercised dog in a significant dilution (1/16) in 30 minutes and thereafter rises rapidly, being detected in a dilution of 1/512 at the end of 2 hours. No trace of egg white was detected in the lymph collected from the unexercised

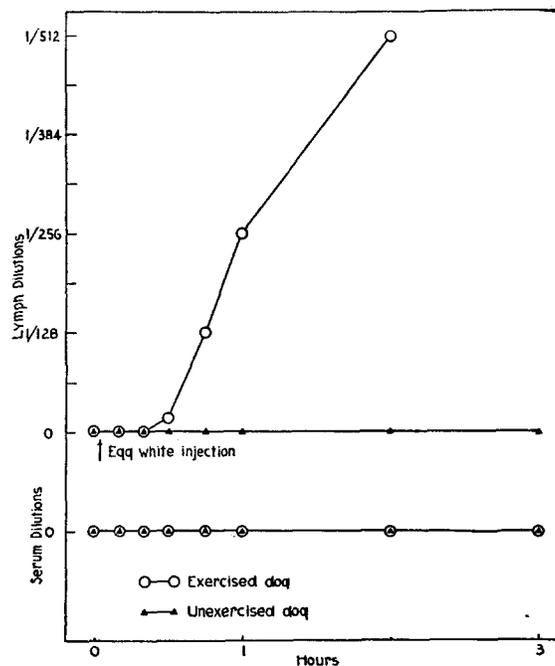


FIG. 3. Experiment IV. The concentration of egg white in the blood stream and thoracic duct lymph following injection into the knee joint of 3 cc. In this case, the leg of the unexercised dog was not massaged.

dog. From these experiments we conclude that proteins such as are contained in egg white are removed from the joint only by way of the lymphatics and if all connections between the lymphatic and vascular systems are eliminated, none of the injected protein will appear in the blood stream. We also believe that this type of experiment demonstrates very clearly the importance of exercise in the removal of proteins from a normal joint.

The results of a companion experiment are shown in Fig. 4. This experiment was carried out exactly as the preceding one, the abdomens massaged, etc., except that the leg of the unexercised dog was massaged without moving the joint. Again no egg white could be detected in the blood stream of either dog. It will be seen that the curve representing the appearance of egg white and its concentration in the tho-

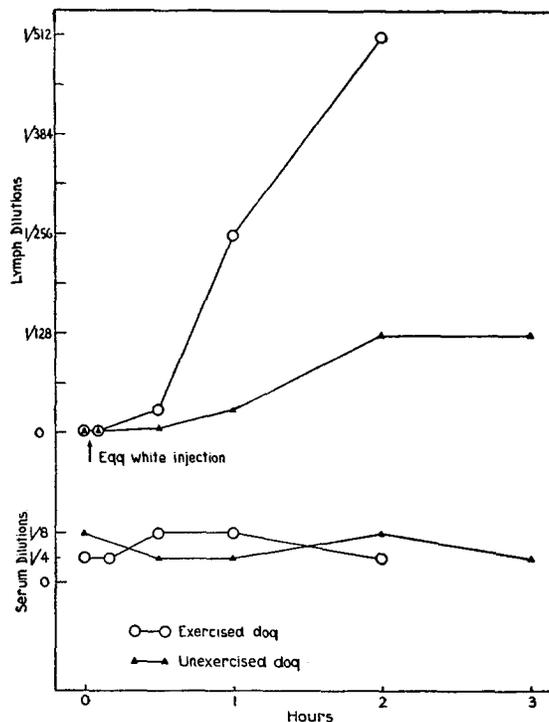


FIG. 4. Experiment V. The concentration of egg white in the blood stream and thoracic duct lymph following injection into the knee joint of 3 cc. In this case, the leg of the unexercised dog was massaged.

racic duct lymph in the exercised dog is almost an exact duplicate of that obtained in the exercised dog of the preceding experiment. However, in the case of the unexercised dog in which the leg was constantly massaged, the egg white appeared in the lymph later and did not reach as high a concentration (1/128 at the end of the 2nd hour). It must be remembered that these figures represent the appearance

time in the thoracic duct lymph and that the actual removal from the joint must take place much sooner. Thus, in the preceding experiments, where the lymphatics were not tied off, any appearance of egg white in the blood stream must represent a prior passage through the lymphatics to their point of entrance into the great vessels of the neck. Furthermore, the apparent stagnation of the egg white in the lymphatics of the non-massaged, unexercised leg affords further explanation of the inconsistencies in the first group of experiments dealing with the appearance of egg white in the blood stream alone. In such experiments, no attempt was made to massage either the abdomen or leg. Therefore, we can further conclude that the amount of protein in the thoracic duct lymph in a dog with an unexercised injected knee joint is greatly increased if the leg muscles are massaged.

*Experiments in Which Horse Serum Was the Protein Used*

*Preparation of the Immune Sera.*—In the preceding experiments, the egg white used represented of course a mixture of several proteins, with no clue presented as to any possible qualitative or quantitative difference in ease of removal among them. It was determined, therefore, in the case of horse serum, to study comparatively the egress of certain of the protein fractions, by preparing rabbit sera immune not only against horse serum as a whole, but also against its constituents.

Three protein fractions were prepared according to the method of Doerr and Berger (10), with saturated ammonium sulfate from 500 cc. of horse serum.

1. 0-33 per cent saturation  $(\text{NH}_4)_2\text{SO}_4$  = euglobulin
2. 33-50 “ “ “ “ = pseudoglobulin
3. 50-56 “ “ “ “ = discarded
4. 56-66 “ “ “ “ = albumin

The euglobulin was precipitated in  $\frac{1}{3}$  saturation of the whole serum with ammonium sulfate. The filtrate from that was  $\frac{1}{2}$  saturated with ammonium sulfate to precipitate pseudoglobulin and so on. Each precipitate was washed with the corresponding concentration of ammonium sulfate, until the filtrate gave no test for protein. The wash water was thrown away each time. The fraction between 50 to 56 per cent was also thrown away, in order to separate globulin and albumin more carefully. Each precipitate was dissolved in 250 cc. distilled water and reprecipitated by the addition of the proper amount of saturated ammonium sulfate. They were again washed and the washings discarded. Then they were dissolved and dialyzed—euglobulin against 4 per cent sodium chloride changed twice a day and the others against tap water. When

the water outside the membranes gave no test for ammonia with Nessler's reagent, the solutions were placed in sterile containers with a little chloroform and kept in the ice box. Several of the fractions showed bacterial contamination which was removed by means of a Seitz filter.

Rabbits were immunized against the three fractions, as well as against whole horse serum according to the method previously used. As expected, precipitin tests after immunization failed to show entire specificity. The two globulin fractions demonstrated complete and equal cross-immunization. In the actual experiment, therefore, immune sera only against pseudoglobulin, albumin and horse serum were used. Titration of these against the antigens used in immunization gave the results shown in Table I.

From that table, it can be seen that the separation between pseudoglobulin and albumin was incomplete, with the former containing enough albumin finally to

TABLE I

Immune sera	Dilution of antigens positive (with protein content in basic solutions in gm. per 100 cc.)		
	Horse serum 7.13 gm.	Pseudoglobulin 1.81 gm.	Albumin 0.31 gm.
1. Against horse serum . . . . .	1/20,000	1/1,000	1/100
2. " pseudoglobulin . . . . .	1/20,000	1/1,000	1/100
3. " albumin . . . . .	1/20,000	1/10	1/1,000

immunize the rabbit slightly against it. We may assume the albumin fraction nearly free from pseudoglobulin, however, with the slightly positive reaction (1/10) gained with the albumin immunized rabbit serum explained on the albumin contamination of the pseudoglobulin fraction used as antigen.

As shown in the protocol (Experiment VI), this experiment was carried out like the two preceding ones, with horse serum containing 7.13 gm. of protein per 100 cc. injected into the knee joint of dogs prepared by tying off the lymphatics as they enter the veins of the neck; then control blood and lymph samples were taken. Again one knee joint was exercised and one kept quiet, although the leg was massaged. Precipitin tests were done on samples of blood and lymph removed at intervals, using the three immune sera described above.

Fig. 5 shows the relative appearance in the thoracic duct lymph of the two fractions. For the sake of clarity, the reactions with the rabbit serum immune to horse-serum are omitted, but are contained in the complete table in the protocol and serve to confirm the data on the chart. The first point which the chart clearly demonstrates is the rapidity and ease of the removal of the albumin fraction from the

## 428 REMOVAL OF PROTEINS FROM NORMAL JOINTS

joint, as shown by its high concentration in lymph (positive in a dilution of  $1/2,048$ ),  $\frac{1}{2}$  hour after the intraarticular injection in the exercised dog and after 2 hours in the unexercised dog. In contrast, the pseudoglobulin fraction makes only a very slight appearance in the

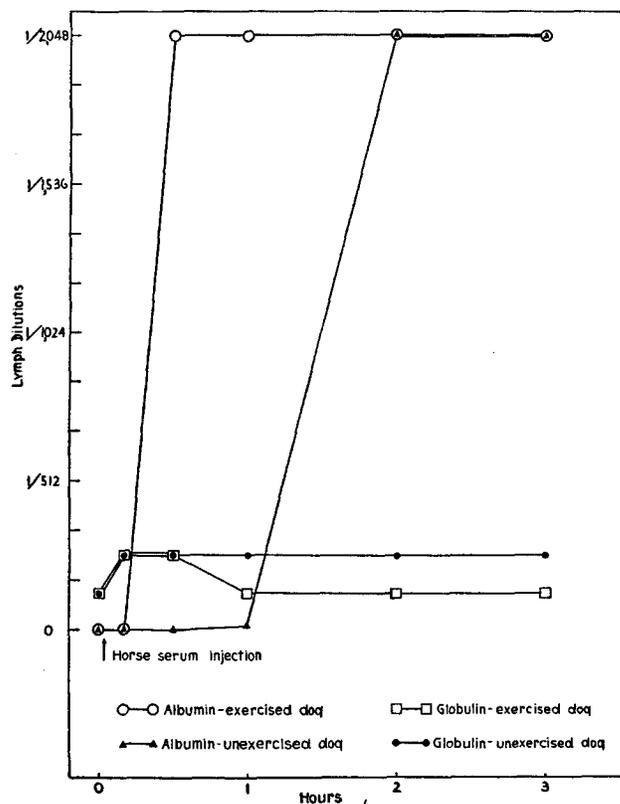


FIG. 5. Experiment VI. The concentrations of albumin and globulin fractions of horse serum in the thoracic duct lymph after injection into the knee joint of 3 cc. undiluted horse serum. Concentrations in the serum showed no significant rise and are omitted from the chart.

thoracic duct lymph of both dogs. This appearance, furthermore, may be only apparent, because the serum used, while acting preponderantly against pseudoglobulin, showed also a noticeable reaction with albumin, due to incomplete separation of the two fractions. Be-

cause of the equal cross-immunization of the two globulin fractions, this lack of egress from the joint applies also to the euglobulin fraction of the horse serum injected. We can say, then, at this point, without

*Results of Precipitin Reactions*

Immune serum used and time after injection	Dilutions positive			
	Serum		Lymph	
	Unexercised	Exercised	Unexercised	Exercised
<i>Anti-horse serum</i>				
Control	1/128	1/128	1/128	1/32
10 min.	1/256	1/128	1/128	1/32
30 "	1/128	1/128	1/128	1/64
1 hr.	1/128	1/128	1/128	1/128
2 hrs.	1/128	1/128	1/256	1/64
3 "	1/256	1/128	1/256	1/64
<i>Anti-globulin</i>				
Control	1/256	1/256	1/128	1/128
10 min.	1/256	1/256	1/256	1/256
30 "	1/256	1/256	1/256	1/256
1 hr.	1/256	1/256	1/256	1/128
2 hrs.	1/256	1/256	1/256	1/128
3 "	1/256	1/256	1/256	1/128
<i>Anti-albumin</i>				
Control	Negative	Negative	Negative	Negative
10 min.	"	"	"	"
30 "	"	"	"	1/2,048
1 hr.	"	"	1/8	1/2,048
2 hrs.	"	"	1/2,048	1/2,048
3 "	"	"	1/2,048	1/2,048

The results obtained with the serum immune against horse serum also demonstrate that these proteins are removed through the lymphatics and not the blood stream, and more rapidly from exercised joints. The fact that no higher concentrations were obtained is probably because the serum used proved to be weak against albumin, the protein readily removed, and strong against globulin, which apparently comes out with great difficulty, if at all.

reservation, that the albumin fraction of horse serum is removed readily from a normal dog's knee joint and is detectable in high dilutions of the thoracic duct lymph under the experimental conditions employed, while the globulin fractions are removed from a normal joint with

## 430 REMOVAL OF PROTEINS FROM NORMAL JOINTS

difficulty, if at all. The experiment also demonstrates the effect of exercise on the rapidity of removal of another protein, horse serum albumin. Finally, as shown by negative tests with the serum samples, horse serum albumin resembles the proteins in egg white in being removed from joints by way of the lymphatics alone. (See protocol, not on chart.)

*Experiment VI. (Fig. 5).—*

Dog 13, weight 21.8 kilos.

9:20 a.m. Anesthetized with 0.87 gm. nembutal intraperitoneally.

10:20 a.m. Control blood sample removed from right leg vein. Lymphatic trunks were then identified and tied off, and a cannula inserted into the thoracic duct.

1:20 p.m. Control lymph sample secured.

1:22 p.m. 3 cc. horse serum injected into left knee joint and leg exercised passively throughout experiment at rate of 40 to 60 revolutions per minute, and abdomen massaged.

Collections of blood and lymph were made as before at 1:32, 1:53, 2:22, 3:22 and 4:22 p.m.

Dog 14, weight 33 kilos (pregnant).

10:20 a.m. Anesthetized with 1.32 gm. nembutal intraperitoneally, lymphatics isolated and tied, and control samples obtained as with Dog 13.

2:13 p.m. 3 cc. horse serum injected into left knee joint. This dog was kept perfectly quiet throughout. The abdomen was massaged, as well as the left leg, with care taken not to move the joint.

Collections of blood and lymph were made similarly at 2:23, 2:43, 3:13, 4:13 and 5:13 p.m.

## DISCUSSION

Besides the rich subsynovial vascular supply, every joint has a similar system of lymph spaces. Magnus (11) has demonstrated that these spaces have no open connection with the joint cavity through "stomata," but are separated in every case by cells of mesenchymal origin, embedded in a matrix or ground-substance. Any passage into the lymphatic system from the interior of a joint, unless, as in the case of particles, mediated by phagocytosis, must then take place either through cells or intercellular substance. Since the lymphatic system is probably one of closed tubes (Drinker and Field, 1932), the lymphatic endothelium must also be passed in a similar manner. The last experiment described indicates that horse serum albumin readily

escapes from the interior of normal joints into the subsynovial tissues in the neighborhood of the lymphatic capillaries and is soon detectable in the lymph, whereas the globulin of horse serum does not escape readily from the interior of normal joints into the subsynovial tissues and therefore is not detected in the thoracic duct lymph. This type of experiment demonstrates that the size of a molecule which can be readily removed from a normal joint lies between that of horse serum albumin and horse serum globulin or between a molecular weight of 72,000 and 175,000 (Adair and Robinson (13)). Incidentally, the relative sizes of these two molecules are thus confirmed by *in vivo* experiments.

Lymphatic absorption from the peritoneal cavity is hastened by increased intraabdominal pressure (Bolton (5), Florey (14), Lemon (15)) and from the tissue spaces by massage (Lewis (4), Florey (14)). Similarly von Mosengeil (16) and Fisher (1) have demonstrated the value of joint exercise in absorption from the articular cavities. The experiments described above confirm the value, not only of exercise, but also of massage of the limb in furthering absorption of proteins. With the methods used, it is impossible to decide at present whether speeding up of lymphatic passage by massage and motion of the limb or actual changes in the intraarticular pressure, demonstrated by Smith and Campbell (17) during joint flexion, is the important factor.

The conclusion that the lymphatic system is the essential removal apparatus of the joint for protein seems justified by the experimental data presented. Any interference with this drainage, as by inflammatory changes of the synovial membrane and subsynovial tissues, should be an important factor in the production and maintenance of a joint effusion. Further work is in progress to study protein absorption when various types of inflammation have been produced in the synovial membrane. It is hoped, also, to find clinical application in the prognosis and treatment of joint effusions. Certainly it would seem that a simple test could be devised, enabling us to state whether or not the lymphatic drainage of a single diseased joint is interfered with. Such a test would allow for further study of the factors involved in the production and maintenance of joint effusions. We are at present attempting to work out a simple method of carrying out such a clinical test.

## SUMMARY AND CONCLUSIONS

1. Data are presented showing that precipitin tests can be used for the detection of the proteins contained in egg white and horse serum in the blood stream and thoracic duct lymph following injection into the knee joints of normal dogs. The sera of rabbits immunized against the particular protein employed were used in doing the precipitin tests.

2. Egg white is removed only by way of the lymphatics, appearing more rapidly if the leg muscles are massaged. The removal is even greater from passively exercised joints.

3. Horse serum albumin is readily removed from a normal dog's knee joint by way of the lymphatics alone and with greater rapidity from a passively exercised joint. Horse serum globulin escapes from normal joints with great difficulty, if at all, and therefore does not readily gain entrance into the underlying lymphatic capillaries.

4. The molecular size of a protein readily gaining egress from a joint through the lymphatics is thus defined.

5. The relative sizes of the albumin and globulin molecules contained in horse serum are confirmed by *in vivo* experiments.

6. The lymphatic system is the essential apparatus for the removal of protein from joints, and any interference with this drainage should promote the formation of intraarticular effusion.

The data presented suggest that a simple clinical test for determining the efficiency of the lymphatics draining a single joint can be devised.

Dr. C. K. Drinker and Dr. Madeleine E. Field were kind enough to prepare our dogs in the experiments in which all communications between the lymphatic and vascular systems were eliminated. We wish to take this means of thanking them. We are indebted to Drs. L. Dienes, H. Zinsser and Eliot Porter for their help and suggestions in preparing the immune sera used in these experiments.

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