A QUANTITATIVE TECHNIQUE FOR PERFORMING PLASMAPHERESIS

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Repeated bleedings of an animal, followed immediately by the reinjection of corpuscular elements of the blood suspended in Locke's solution, results in depletion of the plasma. This procedure, a modification of that used by Morawitz (1), was first employed by Abel, Rowntree and Turner (2), and was called plasmapheresis by these investigators.

This technique was successfully used by Whipple and his associates (3–5) in studying the effects of diet and fasting on the curve of serum protein regeneration. Recently the Whipple group (6–8) has attempted to evaluate dietary factors as specific agents for promoting the formation of new serum protein. However, in these studies (6–8) plasmapheresis does not appear to have been performed quantitatively; judging from the data as reported (7, 8), the blood protein level was not constant but varied from 3.18 to 4.69 per cent. Thus the intensity of the stimulus for serum protein regeneration during each of the dietary periods in all probability was not the same.

In any quantitative approach to the study of the influence of diet upon the regeneration of serum protein it is essential to maintain the basal level of serum protein constant. In our investigation of this problem, plasmapheresis was performed by what we believe to be a highly quantitative method. By daily preliminary determinations

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of the serum protein concentration and weekly preliminary determinations of the blood volume, the size of each bleeding was calculated to reduce the serum protein concentration of the dog to 3.5 per cent. To avoid the withdrawal of insignificant quantities of blood, plasmapheresis was conducted only on those days when the serum protein level reached or surpassed 4.2 per cent. During the initial week, when the "reserve serum protein store" of the animal was depleted and the concentration of the blood protein reduced to the basal level, bleedings of one-fourth of the blood volume were performed daily.

The Bleeding Phase

The method usually employed in the clinic for withdrawing blood proved to be unsatisfactory in the present study for several reasons. For our purpose it was desirable to withdraw large volumes of blood accurately to within ± 1 per cent of the calculated amounts. Furthermore, it was essential to bleed the experimental animals with the least possible injury to the femoral artery so that on the succeeding days there would be no difficulty in palpating the vessel due to any excessive production of scar tissue or hematomas as a result of leakage at the sites of previous punctures. Therefore, a method, whereby the blood could be collected rapidly through a needle of relatively small bore with the artery having been entered but once, was sought. Finally, the tendency exhibited by the dogs' blood to clot rapidly, before the desired amounts were obtained, prevented quantitative blood withdrawals in our preliminary trials. This occurred frequently in spite of coating the inner surface of the rubber tubing with paraffin and even after preliminary rinsing of all the tubing with the anticoagulant solution.

For the present study a special apparatus and technique were finally devised, allowing one to overcome the difficulties enumerated above. The method permits the rapid removal of large quantities of blood aseptically, accurately, with a minimum of hemolysis and without any danger of clot formation during the process. A photograph of the apparatus is shown in Fig. 1.

The 500 cc. graduate (F) is calibrated and correction made for the volume occupied by the stirrer (G). 1 per cent of the volume read on the graduate is due
Fig. 1. The bleeding apparatus.
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to the presence of the stirrer. The motor driven stirrer (A) rotates at about 150
to 200 revolutions per minute. This feature not only permits the system to be
relatively compact, giving less play of rubber tubing, but also enables two individ-
uals to conduct a bleeding in a highly efficient manner. The minimal agitation
produced by the rotating stirrer, the spiral form of the active stirrer (G) and the
construction of the inlet tube (E), causing the blood to flow gently down the side
of the graduate, reduce hemolysis to a minimum. Determinations of the extent
of hemolysis produced during this stage of plasmapheresis were carried out as a
preliminary step in a later study. The value of 2.5 mg. of hemoglobin per 100
cc. of citrated plasma appears to be a valid expression of the hemolytic effects of
this initial stage of plasmapheresis. The mercury seal (D) allows efficient stirring
with the system under a negative pressure of 230 to 240 mm. of mercury. The
negative pressure is maintained by suction at the mouthpiece (C). The burette
(B) contains the anticoagulant, a 3 per cent solution of anhydrous sodium citrate
being used; the volume of the citrate solution used is regulated so that the final
concentration will be 0.3 per cent, or a 1 to 10 dilution. At the three-way tube
(H) the anticoagulant comes in contact with the blood. The arm of the tube lead-
ing to the burette has a capillary bore so that the greatest part of the negative
pressure in the system is exerted on the incoming blood. To the three-way syringe
(I), which is of the B-D Yale Kaufman, Luer-lok type, is attached a No. 19 plati-
num needle. This needle has proven to be very satisfactory inasmuch as its bore
is equivalent to that of a No. 18 steel needle.1 Due to the weight of the motor the
apparatus is unstable, but accidents due to this instability are easily prevented by
clamping (J) the supporting stand to a firm surface, the table, for example.

The apparatus is rendered aseptic by consecutive washings with a mercuric
cyanide solution, sterile water, alcohol and ether. To dry the system completely,
air is sucked through a concentrated sulfuric acid bath, in place of the syringe,
and through a sterile cotton plug inserted in the mouth of the burette, the stop-
cock being open. The suction from a house line is applied at the mouthpiece (C).
Bacteriological analyses indicated that this technique does indeed give satisfactory
asepsis.

From 3 to 5 cc. of the calculated amount of the sterile 3 per cent sodium citrate
solution is first sucked through the needle into the graduate; the remaining citrate
is poured into the burette. The needle is inserted into the femoral artery, and
the plunger of the syringe withdrawn beyond the side arm. The system is main-
tained under negative pressure. Simultaneously the citrate is added slowly from
the burette at a rate which roughly approximates 1/10 of that of the blood flow.
The stirring completely mixes the blood with the anticoagulant. About 5 cc. of
the citrate is left in the burette. When the citrated blood has reached a volume
10 cc. below the desired amount, the needle is withdrawn. The amount of blood
in the syringe and rubber tubing together with the remaining citrate used to flush

1 We are indebted to Dr. Daniel C. Darrow of the Department of Pediatrics
for this suggestion.
out the tubing brings the volume of citrated blood to that desired. 400 cc. of blood may be collected easily within a 3 to 5 minute interval, the needle having been placed in the artery only once. The operator handles the syringe and nothing else. His assistant can easily operate the apparatus, pet the dog and signal when to withdraw the needle from the artery. It is very convenient to have the graduate marked with crayon at the reading corresponding to the desired blood volume and the mark 10 cc. below this. With a little experience, the volumes of blood obtained are accurate and consistent.

The method for calculating the volume of blood to be removed is exceedingly simple. The only assumption involved is that the plasma volume and the serum volume are equal. Any error arising from the volume occupied by the fibrinogen must be exceedingly small, being undoubtedly less than 1 per cent. By elementary algebra it is easily shown that the hematocrit, or the volume per cent of blood representing the plasma or serum, does not enter into the calculation.

If we let

- \( x \) = the blood volume in cubic centimeters,
- \( y \) = the fraction of the blood represented by the plasma,
- \( xy \) = the plasma volume in cubic centimeters,
- \( z \) = the serum protein concentration in grams per cent as determined daily,
- \( 3.5 \) = the serum protein level in grams per cent to which the concentration is to be lowered each day that bleeding occurs,

then

\[
\frac{z - 3.5}{y} \cdot xy = \text{plasma volume in cubic centimeters to be removed},
\]

and

\[
\frac{z - 3.5}{y} \cdot xy = \text{blood volume in cubic centimeters to be removed},
\]

or

\[
\frac{z - 3.5}{y} \cdot x = \text{blood volume in cubic centimeters to be removed}.
\]

The formula last given is the one to be used. A representative experiment will serve to illustrate all the details in the calculations.


\[
5.04 - 3.50 = 1.54 \text{ per cent serum protein increment to be removed}.
\]
Then
\[
\frac{1.54}{5.04} \cdot 1425 = 437 \text{ cc. of blood to be removed},
\]
and
\[
437 \cdot \frac{1}{9} = 49 \text{ cc. citrate to be added},
\]
since
\[
486 \text{ cc. total of citrated blood},
\]
and therefore
\[
5 \text{ cc. volume occupied by stirrer, or about 1 per cent},
\]
therefore
\[
491 \text{ cc. reading on the graduate.}
\]

The graduates were always marked with a crayon to the nearest 5 cc. mark; in the above case, 490 cc. In Table I are listed representative bleedings, as obtained experimentally and as calculated, to show the extent of agreement. These were obtained with dog 4. Using our technique, the average deviation is easily kept within ± 1 per cent; in the series listed in Table I it is ± 0.6 per cent. The omissions on June 2, 9, 16, and 23 were due to the fact that no bleedings were conducted on Sundays.

For the most part the volumes of blood withdrawn were not so large as to cause physiological disturbances (6, 9, 10), because subsequent to the hemorrhage a red cell suspension was injected immediately.

**Preparation of Cells for Reinjection**

Plasmapheresis necessarily involves the reinjection of the corpuscular elements of the blood. The details of the preparation of the cells in accordance with our technique are given below.

The citrated blood is transferred to calibrated 100 cc. centrifuge bottles which had previously been graduated accurately to the nearest cubic centimeter. These containers are sterilized either by the same procedure as that described above or by having cleaning solution (potassium dichromate in concentrated sulfuric acid) replace the mercuric cyanide solution. The rubber stoppers are so cut as to retain a flange extending about 6 mm. beyond the mouth of the bottle. This feature prevents the stoppers from being driven into the tubes during centrifugation and also aids in the asepsis. A small glass plunger, running through the center of the stopper, also with a flange, permits the complete filling and stoppering of the bottles with a minimal production of pressure. The stoppers are sterilized in boiling water. These and the tubes are then kept for 10 minutes in an oven at 105°C. to drive off the last traces of alcohol and ether. The mouths of these bottles are always swabbed with alcohol whenever they are opened or stoppered.

Centrifugation for 1 hour at 2100 r.p.m. is satisfactory for the determination of the cell volume. After correcting for the citrate present, the hematocrit is calculated. Following the removal of the citrated plasma by suction, physiological saline is added in approximately equal volume. The saline suspension of cells is then stored in the refrigerator at 5–8°C. until the next plasmapheresis. The
longest time interval, during which we have stored these cell suspensions, has been 4 days; on injection these proved to be utilized satisfactorily. After a serum protein determination, which indicates the necessity for a plasmapheresis in order to maintain the basal level constant, the saline suspension of cells is centrifuged (1500 R.P.M.) for 1 hour, the saline removed by suction to be replaced by an approximately equivalent volume of modified Locke's solution (5). The Locke's solution is made daily from water freshly distilled (11) from pyrex glassware (12) and contains 0.900 per cent sodium chloride, 0.042 per cent potassium chloride and 0.020 per cent sodium bicarbonate.2

### TABLE I

<table>
<thead>
<tr>
<th>Date</th>
<th>Bleeding Calculated</th>
<th>Bleeding Experimental</th>
<th>Deviation per cent</th>
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<td>1935</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>May 31</td>
<td>346</td>
<td>347</td>
<td>+0.3</td>
</tr>
<tr>
<td>June 1</td>
<td>335</td>
<td>334</td>
<td>-0.3</td>
</tr>
<tr>
<td>3</td>
<td>437</td>
<td>436</td>
<td>-0.2</td>
</tr>
<tr>
<td>4</td>
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<td>392</td>
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</tr>
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<td>5</td>
<td>286</td>
<td>295</td>
<td>+3.2</td>
</tr>
<tr>
<td>6</td>
<td>339</td>
<td>340</td>
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</tr>
<tr>
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<td>+0.3</td>
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<td>334</td>
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</tr>
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<tr>
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<td>383</td>
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</table>

Average deviation.................. ±0.6%

### Reinjection of the Cells

At the initial plasmapheresis, subsequent to the hemorrhage, a suspension of cells obtained from one of the donor animals is used.

2 This solution is easily made by dissolving 9.62 gm. of modified Locke's mixture, composed of 93.55 per cent NaCl, 4.37 per cent KCl and 2.08 per cent NaHCO₃, in 1 liter of freshly distilled water.
Thereafter the cells removed in the preceding plasmapheresis are reinjected immediately after the bleedings. Smith, Belt and Whipple (5) had conducted plasmapheresis in which the injection was simultaneous with the hemorrhage. By such a procedure a considerable portion of the bleeding is due to the passive withdrawal of the injected cell suspension. Later (6-8) this was modified so that the injection phase followed immediately after the bleeding. In the present study such a procedure has been followed.

The apparatus employed for the reinjection of the cells is shown in Fig. 2.

The tube (H) is filled with the Locke’s solution, care being taken to eliminate all air bubbles. The suspension of cells is then filtered through 8 layers of sterile gauze into the gravity tube (F). The outside jacket (E) contains water at 40°C. to keep the cell suspension at approximately body temperature. The cells are injected under positive pressure maintained by an atomizer bulb (A). Due to the considerable pressure required for the injection of the cells, it is essential to have the stopper of the gravity tube held firmly in place by adhesive tape (C). The tube holds only 270 cc. so that the subsequent additions are made through the tube (B). The opening of this tube is so constructed that the blood flows gently down the side of the gravity tube with no foaming and the minimum of hemolysis. About 100 cc. of Locke’s solution is used to wash down the sides of the gravity tube subsequent to each injection. Thus, there are injected each day 100 cc. of fluid in excess of that withdrawn. On those days, when donors’ cells are injected in addition to the homologous cells in order to keep the hematocrit normal, the extra Locke’s solution is dispensed with. When these washings have passed the pilot tubes (I), the three-way syringe is closed, thus avoiding the possible injection of air bubbles. Most of the cell injections were made with a No. 18 needle inserted into the jugular vein; occasionally the radial and saphenous veins were resorted to. The injection of a cell suspension and extra Locke’s solution totaling 500 cc. required approximately 5 to 10 minutes. An animal can easily tolerate this rate of injection (13).

The injection of the suspension of cells is carried out aseptically. The gravity tube, stopper and tubing are sterilized in boiling water in a closed sterilizing dish. The entire system is rinsed with sterile physiological saline solution before the cells are added. In the early experiments a nonabsorbent cotton filter was inserted in the glass tube to which the pressure bulb was attached. This was omitted later because it interfered with the production of an adequate pressure. Undesirable symptoms as a result of this breach of asepsis were never observed in our dogs.

All rubber tubing and stoppers in both sets of apparatus are treated by boiling with N/2 sodium hydroxide, rinsing with water, boiling with N/2 hydrochloric...
Fig. 2. The cell infusion apparatus.
acid, and a final rinsing with water. Such a preliminary procedure eliminates any toxic reactions (14) which may follow blood transfusions involving the use of apparatus with rubber tubing.

For the determination of the serum protein concentration each day previous to a bleeding, in order to calculate the amount of blood to be withdrawn, a method was sought which would be rapid, reasonably accurate and would require a small amount of blood. The nephelometric method devised by Rona and Kleinmann (15) was found to be suitable. The technique involves the comparison of the turbidities produced by the standard and unknown when an aqueous solution of sodium sulfosalicylate is added to the dilute (1 to 200) serum protein solutions after they had been strongly acidified with hydrochloric acid.

The turbidities produced vary with the concentration of serum protein according to the nephelometric curve (16). The results of more than 400 of our own determinations in duplicate indicate that one may expect simultaneous estimations to agree to within ± 0.5 per cent of the mean.

Rona and Kleinmann (15) report that relatively large concentrations of ammonium sulfate, magnesium sulfate, Ringer's solution, thymol and decomposition products of proteins have only negligible effects as interfering factors in the method. In our own experience it has been found qualitatively that no turbidities are produced when the sulfosalicylic acid is added to solutions of proteose or peptone, obtained as products of peptic digestions. In fact, the reagent may be used as an indicator of the complete removal of acid meta-protein from a weak acid hydrolysate of protein. The precipitation of the protein presumably is not dependent on its large molecular size because solutions of dextrin or starch paste are not affected by the reagent. The only nonprotein substances which are precipitated by the reagent, as far as we know, are plant alkaloids.

The specificity of this method for the determination of the serum protein concentration in dogs subjected to plasmapheresis was confirmed by (a) determining the concentration of serum protein when present in a serum sample and subsequently when dissolved in physiological saline, and (b) observing that the addition of sulfosalicylic acid either to a suspension of the serum lipids in physiological saline or to a solution of the serum crystalloids fails to produce any turbidity.
As final proof of the validity of the technique, the results yielded by this nephelometric procedure were checked against those obtained by standard methods. The average percentage deviation of the nephelometric values from those obtained by Kjeldahl analyses (17, 18) on nine representative samples was ±4.0 per cent; the percentage deviations varied from +0.5 to −5.7 per cent. When compared with the results obtained gravimetrically (19, 20) on five other serum samples the average percentage deviation of the nephelometric values was found to be ±2.9 per cent, varying from −1.4 to +5.3 per cent.

For the estimation of blood volume, the dye method as improved by Hooper, Smith, Belt and Whipple (21) was employed. The exact validity of any method for the determination of the blood volume can, of course, be questioned (22). However, in our studies the same procedure was used throughout, so that any errors inherent in the method were reasonably constant during each of the experimental periods.

**SUMMARY**

1. A special apparatus and technique are described which permit one to conduct plasmapheresis quantitatively.

2. The validity of the methods employed, for determining serum protein concentration and blood volume as prerequisites for the calculation of the amount of blood to be withdrawn, are discussed.

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