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A METHOD FOR ESTIMATING THE FRACTION OF THE VOLUME OF A MUSCLE CONTAINED IN THE VASCULAR SYSTEM

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When a muscle is perfused with a solution containing a substance X which can penetrate into the muscle cells, the substance first enters the vascular system, then penetrates into the intercellular spaces and finally penetrates the cell membrane of the muscle fibres. Thus, at least three distinct diffusion spaces are to be recognized: (1) the vascular system, (2) the space between the muscle fibres not included in (1), (3) the intercellular space. The first step in calculating the permeability of the muscle fibres must therefore be to determine what fraction of the amount of X present in the muscle at a given time is present in the vascular system. The *concentration* of X in the vascular system will, for most substances, be substantially the same as in the perfusion fluid. Hence the amount of X in the vascular system can be calculated if the volume of the vascular system is known.

A convenient procedure for finding the volume of the vascular system is to add a second substance Y (in this paper, haemoglobin) which penetrates the muscle fibres very slowly, if at all. The substance Y will first flood the vascular system and then penetrate into the intercellular spaces. From the time relationships of the increase in the amount of Y, the volume of the vascular system can then be obtained. It is necessary that Y should not cause either dilation or contraction of the vessels, and desirable that it should be easy to estimate in small amounts.

Since the results given here are designed to estimate the accuracy of the method for finding the volume of the vascular system, substance X is omitted and only the non-penetrating substance Y (haemoglobin) studied.

Experimental methods

Haemoglobin solutions were prepared by osmotic haemolysis of ox red cells (thrice washed in 1 % NaCl), followed by addition of salts to bring the salt concentration to that of frog's Ringer (6·75 g. NaCl, 0·15 g. KCl, 0·2 g. $CaCl_2$, 0·02 g. Na_2HPO_4 per litre). This solution was filtered through a Whatman no. 1 filter paper.

Preparations were made of the hind legs of the frog and these perfused with the haemoglobin solution, through the aorta. Hungarian frogs were used. Frogs of similar size were chosen and where the gastrocnemius muscle weighed less than 1·95 or more than 2·05 g. the experiment was discarded. They were first perfused for 15 min. with 3½ % gum acacia solution, to wash out the erythrocytes already present. The gastrocnemius muscle from one leg was removed and used as a control, and the other leg then perfused with haemoglobin solution for a definite time, after which the gastrocnemius was removed. The muscles were dried by rolling on filter paper and weighed, then roughly minced and extracted twice by stirring for 30 min. with 10 c.c. of distilled water. The extract was centrifuged for 10 min. at 2000 rev./min. and the volume made up to 25 c.c. The haemoglobin was estimated with a photoelectric colorimeter. In the 1938 experiments a special green-sensitive cell was used, and in the 1939 experiments an ordinary cell with a green filter. Extracts of frog muscle, e.g. a gastrocnemius, made after perfusion with gum acacia, contain little haemoglobin, but have a good deal of suspended matter with marked light-scattering powers, which gives an 'apparent haemoglobin' value, as estimated by the photoelectric colorimeter, sometimes nearly as much as one-third of that of a second gastrocnemius muscle (from the same frog) perfused with haemoglobin. There is considerable variation from frog to frog in this respect, but if two gastrocnemius muscles of the same frog are compared, the 'apparent haemoglobin' values do not differ from each other by more than 10 %.[1] Thus, in a muscle perfused with haemoglobin the haemoglobin value given by the photoelectric method is made up of (*a*) true haemoglobin, and (*b*) 'apparent haemoglobin'. The amount of the latter is given by the control which was perfused with acacia only. The maximum error in the determination of true haemoglobin, i.e. (true haemoglobin + apparent haemoglobin) in the haemoglobin perfused limb, minus (apparent haemoglobin) obtained from the acacia perfused limb, is not greater than

[1] Seventeen frogs were studied. All except two had muscle pairs agreeing to 5 %; the remaining two lay between 5 and 10 %.

$\frac{30}{70} \times 10$ %, i.e. about 5 %. To this must be added a maximum of 1 % error due to manipulation and the photocell, giving a 6 % maximum error.

Finally, there is an error due to incomplete recovery of haemoglobin, as part remains adsorbed on the minced particles. To correct for this, frog muscle was minced, portions of 2 g. each were weighed out, mixed with known volumes of haemoglobin solution, allowed to stand for 3 hr. and then extracted with water, as in the case of the perfused muscles.

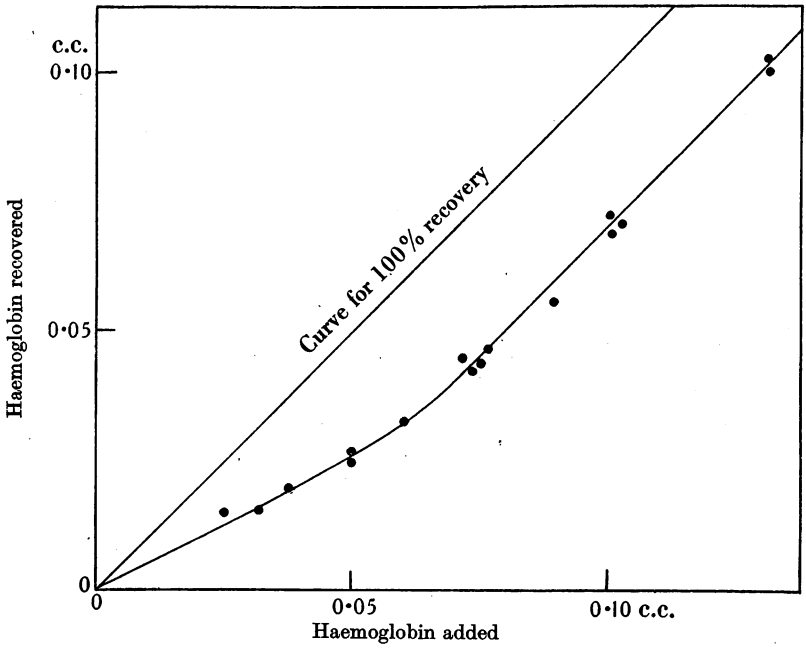

Fig. 1. Results of extraction of mixtures of minced muscle and haemoglobin solution. The figures are in units of c.c. of 7 % solution added to 2 g. of muscle.

The amount of haemoglobin recovered was plotted against the amount added (Fig. 1); this curve was then used to correct the values for perfused muscles. The possibility that this correction is inadequate will be discussed later. The uncertainty in the value of this correction involves a further error of $2\frac{1}{2}$ % in a determination of 0·1 c.c. of haemoglobin solution, giving a maximum possible error for a single determination of $8\frac{1}{2}$ %.

Chloride determinations were also made on the muscle extract, using the method of Sendroy [1937].

From the relationships between the volumes of undissolved material and extractant (2–2·5 c.c. of solid, twice extracted with 10 c.c.) it was

concluded that between 4 and 6 % of the chloride was not extracted. This was checked in some cases by grinding with sand the residue left after extraction, and extracting again with three 10 c.c. portions of distilled water (see Table 1). The amount of chloride obtained by the

TABLE 1

Exp. no.	% of chloride removed by first two extractions	% of chloride removed by three further extractions
7	95·4	4·6
8	96·1	3·9
15	94·9	5·1

first two extractions was therefore taken as 95 % of the chloride actually in the muscle. The chloride determinations were made in duplicate and never differed by more than 2 %; the corrected values are therefore not subject to an error of more than ±3 % for a given muscle.

RESULTS

Fig. 2 shows the results obtained with 'autumn' Hungarian frogs in 1938 and 1939, expressed as percentage of the total volume of the muscle occupied by haemoglobin solution. It was assumed that the haemoglobin solution in the vascular system is of the same concentration as the perfusion fluid. Each point on the curve represents results obtained by perfusion of a single frog. The haemoglobin volume rises to 5 % in 15–20 min., when perfused under a pressure of 15 cm. of water. Thereafter it remains almost constant, perhaps rising slightly, for up to 5 hr. The average volume of the vascular system of the gastrocnemius is obtained by exterpolating the linear portion of the curve to a time lying between 7½ and 10 min.—a volume in this case not significantly different from 5 %.

The chloride space is expressed as percentage of the total volume of the muscle which would be occupied by chloride solution if, in the muscle, it were present in all parts to which it has access in the same concentration as in frog's Ringer solution. This is the convention used by Fenn and Eggleton [see Fenn, 1936]. The average chloride space of the controls, i.e. of muscles perfused for 15 min. with 3½ % acacia solution, was 13·9 %. On changing to 7 % haemoglobin the volume of the chloride space fell sharply, settling to a value of about 10·5 %. This drop is due to the fact that 7 % haemoglobin solution has a much higher colloid osmotic pressure than has 3·5 % acacia solution, so that, on changing from acacia to haemoglobin solution, fluid is withdrawn from the intercellular spaces into the capillaries. This loss of fluid is also shown by the

drop in weight of the perfused preparation which is observed on changing from 3½ % gum acacia to 7 % haemoglobin. Roughly, half of the chloride space, under these conditions, is actually part of the vascular system.

For the purpose of attaining greater accuracy, it was decided to use as high a concentration of haemoglobin as possible. On the other hand, it was not desired to expose the preparations to these solutions for longer than necessary, although later experiments have shown that the haemoglobin solutions are not harmful.

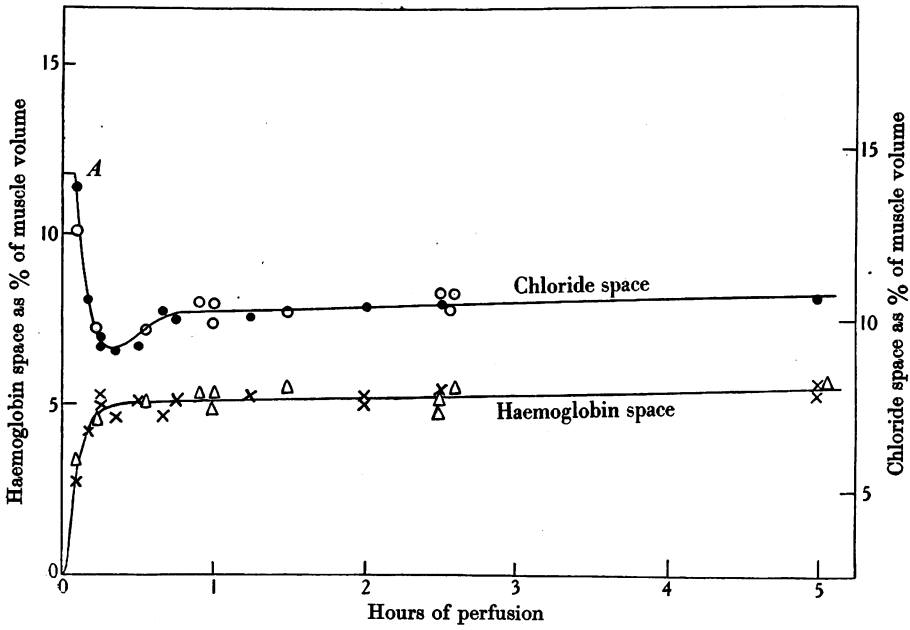

Fig. 2. The variation with time in haemoglobin and chloride space of perfused frog gastrocnemius muscle. Chloride space: ●, 1938; ○, 1939; haemoglobin space: ×, 1938; △, 1939. Point *A* is the average chloride space of the control muscles from the same frogs not perfused with haemoglobin.

A further series of experiments was made in which the initial perfusion with acacia solution was continued for 45 instead of 15 min. This made no significant difference to the volume of the vascular system.

Discussion

Krogh [1922] made an estimate of the fraction of muscle volume occupied by capillaries by counting the total number of capillaries in a muscle, and assuming their diameter equal to that of a frog erythrocyte —15μ: this gives 7·1 % of the muscle volume as capillary space. Later

he found that in resting frog sartorius muscle the average diameter was 4·3μ, and in stimulated muscle 7μ.[1] In the perfusion experiments described here the capillaries were probably mostly dilated (though not so much as in the presence of capillary poisons), so that 7μ may be taken as a rough value for the minimum capillary diameter. This gives 1·8 % as the minimum capillary volume in the sartorius muscle. If these figures are also applicable to the gastrocnemius (which may well be doubted) they suggest a rough value for the minimum capillary volume of about 2 %. This value does not contradict the value of 5 % obtained here, which includes the volume of arteries, arterioles, veins and venules. By another method, to be published later, a value of $5{\cdot}2 \pm 0{\cdot}5$ % has been obtained for the volume of the vascular system, in good agreement with that obtained here.

The results of Fig. 1 suggest that haemoglobin does not penetrate into the intercellular spaces. This, however, is not the case. On beginning a perfusion with haemoglobin the muscles flush a delicate pink in the first 20 min. and thereafter, during the next 5 hr., become deeply stained. The secondary slower deepening of colour must be due to haemoglobin penetrating the capillary walls. Microscopic observation of the surface of a stained muscle shows that the red colour (unlike carbon particles) is not confined to the capillaries, and the oedema fluid collecting between the muscles and the skin contains much haemoglobin. But it is impossible to remove this intercellular haemoglobin by two extractions of coarsely minced muscle with distilled water.

It has been mentioned above that part of the haemoglobin in the capillaries is adsorbed on the minced tissue, and a correction was made for this by extracting mixtures of minced muscle and haemoglobin solution. As extraction with water does not remove the intercellular haemoglobin, it may be doubted that the extraction of simple haemoglobin-minced muscle mixtures is a sufficient control on complete extraction from the interior of the vascular system. Two comments may be made on this: (1) by another method given in the second paper in this series, using galactose and maltose instead of haemoglobin, a value of $5{\cdot}2 \pm 0{\cdot}5$ % has been obtained, compared with 5 % for the haemoglobin method. This suggests that the method cannot be greatly in error; (2) coarse mincing does not break down the structure of a muscle, as is shown by microscopic observation of fragments of muscle perfused with a solution containing carbon particles; consequently the alternate compression and expansion due to stirring in extraction should secure

[1] The erythrocytes are greatly distorted during passage through these narrow capillaries.

efficient flushing of the vascular system but not necessarily of the intercellular spaces. Substances of high molecular weight removed from the intercellular spaces possibly must first diffuse into the capillaries—a very slow process in the case of large molecules such as haemoglobin. It is therefore reasonable to expect that the intercellular haemoglobin should be more difficult to extract than that present in the vascular system.

How far the chloride over and above that in the vascular system represents intercellular fluid is a matter of dispute [see e.g. Fenn, 1936; Eggleton, Eggleton & Hamilton, 1937; Conway & Kane, 1934]. But even if the whole of the chloride were intercellular, in the experiments reported here the volume of the intercellular space would be only 5 % of the muscle volume, in the case of a muscle after perfusion for an hour or more with haemoglobin solution, and about 9 % just prior to the entry of the haemoglobin solution. The volume of this intercellular, non-vascular, space is determined by the hydrostatic pressure in the vascular system, by the colloid osmotic pressures in the vascular system and in the intercellular space, by the rate of removal of fluid by the lymphatic system and by the volume and packing of the muscle fibres. It is therefore very susceptible to variation in experimental conditions.

Summary

1. A method is given for determining the volume of the vascular system of a perfused muscle, based on determination of a substance in the perfusion fluid.

2. Using haemoglobin as the determined substance, the volume of the vascular system of the frog gastrocnemius muscle is found to be 5 % of the muscle volume.

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