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The State of Water in Muscle and Blood and the Osmotic Behaviour of Muscle.

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I. Introduction.

In the course of the investigation described in the preceding paper by Hill and Kupalov it became necessary to determine the amount of "free" water in muscle, *i.e.*, the weight of water per gramme of muscle which is capable of dissolving in a normal manner (with the normal depression of vapour pressure) substances added to it. For many years, chiefly on the evidence of the experiments of Overton (1902), it has been commonly supposed that a large proportion of the water of muscle exists in some "bound" form, incapable of taking part in the osmotic changes which occur when the tissue is immersed in hypo- or hyper-tonic solutions. From the fact that a muscle swells to much less than twice its initial weight when immersed in a solution of half the initial osmotic pressure Overton concluded "dass nicht das gesammte im Muskel befindliche Wasser in der Form eines Lösungsmittels enthalten sein kann." I have confirmed Overton's experiments (see below) but believe that a very different explanation of them is necessary.

Various other definitions of, and methods of determining, the "free" water of a solution (or a tissue) are possible, and these do not necessarily coincide with each other. Jensen and Fischer (1910) and Jensen (1912) determined the "bound" water by making cooling curves for muscles and calculating the

heat absorbed from the areas of these curves. A comparison of the results with those obtained with solutions of NaCl was believed to allow a calculation of the "bound" water. In fresh muscle they found 4 p.c. of the total water to be "bound," in muscle killed by freezing and thawing 14 to 17 p.c., in muscle heated to 100° C. 22 p.c.

A very obvious objection was raised by Rubner (1922), viz., that thermal conduction may be quite different in muscle and in solutions of NaCl. Rubner proposed an alternative method based on the same general idea, viz., that "bound" water may be defined as that which cannot be frozen out by cooling the tissue to such temperatures as − 20° C. The material to be investigated was cooled to a low temperature for two hours and then dropped into a water calorimeter, the heat required to melt it being measured. He found that 1 gramme of dry substance was associated with the following amounts of "bound" water :—

Egg-white	0·33 g.
Blood corpuscles	0·63 g.
Elastic tissue	0·44 g.
Blood vessels	0·45 g.
Beef muscle (dead)	0·76 g.
Beef heart-muscle (dead)	0·64 g.
Frog's muscle (alive)	0·90 g.

The frog's muscle was cooled to − 18° C. and 100 g. of muscle contained 61·7 g. of "free" water (mean of 11 observations) ; similar muscle contained 79·8 p.c. of total water.

Rubner's method also is not free from possible objection :—

(i) It assumes that the "bound" water is the same at − 20° C. as at the ordinary temperatures in which we are interested ; the "binding" of water by a hydrophilic colloid is likely to be an exothermic reaction, in which case it might proceed appreciably further at a low temperature than at a high.

(ii) It assumes that no reactions other than the melting of ice occur when the temperature rises.

(iii) The specific heats of the solid constituents of muscle may not be very accurately known, or the heats of their solution negligible. It is possible, moreover, that water prevented from freezing by association with hydrophilic colloids may nevertheless be capable of dissolving substances present in the tissue.

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Rubner's method was employed by Thoenes (1925). The latter found in a gelatin jelly about 2 g. of water " bound " by each 1 g. of dry material; in agar jelly about 4 g. In the muscles of young animals he found about 2 g. of " bound " water per 1 g. of dry substance, in those of old animals about 1 g. The muscles were frozen and thawed, and after passing into rigor were frozen again and the heat of thawing measured. " Auf diesem Wege," he claimed, " gelingt der Nachweis einer Änderung der Wasserbindung in Zustande der Starre mit grösserer Sicherheit."

Robinson (1928) applied the same method to investigating the hardiness of insects exposed to low temperatures during winter. In some insects as much as one-half of the water they contain may be " bound," in the sense that it is not frozen by cooling to — 20° C. In hardy insects (Promothea) exposed to low temperatures the proportion of water " bound " may increase from about 8 p.c. at the start to over 40 p.c. after two or three weeks' exposure. The method employed by Thoenes and by Robinson is discussed in detail by Gortner (1929).

Another definition of, and another method of determining, the " free " water were suggested by Newton and Gortner (1922), who added a known amount of cane sugar to expressed plant juice and measured the resulting depression of freezing point. By comparing this with the depression caused by adding the same amount of sugar to an amount of water equal to the *total* quantity contained in the juice, they were able to show that an appreciable fraction of the water was " bound," in the sense that it took no part in the solution of the cane sugar. In one case in which the solids made up 0·178 g. per 1 g. of juice, of the total water (0·822 g.) 0·130 g. was found to be " bound." Newton (referred to by Gortner (1929)) has employed the same method of studying the state of water in the sap of winter wheat, in drought-resistant crops, and in the press juice of grasses. The method is of general application, and similar in principle to the one employed in the present investigation. It requires, however, greater quantities of fluid, and for accuracy apparently greater concentrations of solute, and it is open to the possible objection that at ordinary temperatures the amount of water " bound " may be less than at the freezing point. A similar method was used by Straub (1927) in his investigations of milk.

Another method depends upon the fact that a soluble substance which is capable of penetrating the cells of a tissue will finally attain equality of concentration in the water of an external solution and in the " free " water of the tissue. A quantitative determination of the amount of the substance in

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question present in 1 g. of the tissue, when in diffusion equilibrium with a given external concentration, allows an estimate to be made of the " free " water. This method, suggested by P. Eggleton and H. V. Horton, was applied by them to the case of urea distributing itself between Ringer's solution and muscle ; their results they have kindly allowed me to report here. Not many substances, however, if any, are really suitable for the purpose : (*a*) They must be capable of penetrating the tissue rapidly and completely ; (*b*) they must not be dissolved in, or adsorbed by the solid material of the tissue—(*a*) and (*b*) are an unusual combination of properties ; (*c*) they must be in the same physical state in the solution as in the tissue—CO_2 is inadmissible because it produces HCO_3 ions in a buffered medium ; (*d*) they must be susceptible of accurate quantitative estimation ; (*e*) they must be non-electrolytes, otherwise the assumed equality of concentration will be prevented by the Donnan membrane effect.

An analogous method, however, can be employed if pieces of tissue are available which are small enough to allow *water* to redistribute itself in a reasonable time between them and a small quantity of solution in which they are immersed. Imagine, for example, that four small muscles are soaked for some time in normal oxygenated Ringer's solution (R). Let them be carefully blotted and weighed, and let their weight be (say) 1 g. Let them be mixed with a small weighed quantity of a solution, containing the constituents of Ringer's fluid in twice the normal concentration (2R) ; let the amount of water in this 2R solution be 0·8 g. Let the muscles and the solution be stirred together for (say) 16 hours in a stoppered tube containing oxygen ; in that time the osmotic pressures of muscles and solution will have been equalised by the diffusion of salt inwards and of water outwards. Let the depression of vapour pressure of the solution be equal finally (say) to that of a solution 1·5R. By water passing out and by salts passing in, therefore, the osmotic pressure of the muscle has risen 50 p.c., while that of the solution has fallen 50 p.c. This can only be the case if the " free " water of the original muscle was the same in amount as the water of the original solution ; hence the " free " water of the muscle in this case was 0·8 g.

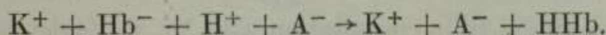
We have taken a simple case for purposes of illustration, but the method is general and requires no substance of peculiar properties as its basis. The only special requirement is an accurate means of measuring the depression of vapour pressure of a small amount of solution. This is available in the thermal method recently described (Hill, 1930) and referred to in the preceding paper

The practicability of the method as applied to muscles was not realised at

first; blood, therefore, and solutions of casein and egg-white, were first studied, and their "free" water determined, in the hope that the results might throw light upon the analogous problem of muscle. The method used was to add various soluble substances to the solution in question and to compare the depression of vapour pressure caused thereby with that due to adding the same amount of the substance to a known amount of salt solution. For example, let us add 1 g. of cane sugar to 10 g. of blood and measure the difference of vapour pressure between the resulting solution and the original blood; let this difference be 125 (arbitrary) units. Let us now add 1 g. of cane sugar to 10·1 g. of 1 p.c. NaCl solution (containing 10 g. of water) and measure the difference of vapour pressure between the resulting solution and the original 1 p.c. NaCl; let this difference be 100 units. It is clear then that the "free" water in the 10 g. of blood is 100/125 of that in the 10·1 g. of NaCl solution; it is 8 g. Various substances, and in various concentrations, have been added to the solutions in question, and the results are described in subsequent sections.

II. The State of Water in Blood.

Mammalian blood contains about the same relative amount of water as amphibian muscle, viz., 80 g. of H_2O per 100 g. of blood. Its chief protein, moreover, hæmoglobin, is an efficient buffer, being the ionised alkali salt of a weak acid, the undissociated protein being formed when a stronger acid is added :—



$$K^+ + Hb^- + H^+ + A^- \rightarrow K^+ + A^- + HHb.$$

In both respects blood, regarded as a physico-chemical system, is similar to muscle. Its hydrogen ion concentration is about the same. Very precise data are available as to the chemical constitution of normal mammalian blood; its freezing point (— 0·56 to — 0·57° C.) is accurately known, and experience has demonstrated that "mammalian Ringer-Locke's solution" of the same freezing point is isotonic, at any rate approximately, with the tissues which were previously in equilibrium with the blood. It is instructive, therefore, to calculate the sum of the molal concentrations (gramme-molecules per 1000 g. of *water*) of the constituents of blood, and to compare this sum with the molal concentration of a NaCl solution which is known to be isotonic with the blood.

Abderhalden's (1898) analyses of the blood of two cattle, two sheep, two horses, two dogs, one goat, one pig, one rabbit and one cat, allow the following mean values to be calculated. It is realised that by taking the mean of his 12 values for each constituent we obtain a result which is true in detail for no

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particular animal; since, however, our only object is to find the *sum* of the molal concentrations for mammalian blood, no error is introduced by taking the mean, and the result is more accurate.

Table I.—Mean Values calculated from Abderhalden's Data for Mammalian Blood.

Substance.	g. to 100 g. blood.	g. to 100 g. H_2O.	Molal concentration.	Remarks.
H_2O	80·2	—	—	—
Hb	12·5	15·6	0·0023	Molecular weight assumed 67000 (Adair (1925), (1928)).
Sugar	0·0790	0·0980	0·0054	—
Na	0·2460	0·3060	0·1331	—
K	0·0790	0·0980	0·0252	—
Ca	0·0044	0·0055	0·0010	Assumed 70 p.c. as free Ca ions.
Mg	0·0031	0·0039	0·0016	—
Cl	0·2890	0·3604	0·1015	—
Total P	0·0314	0·0392	0·0088	See note.
		Sum	0·2789	

Note.—The molal concentration for total P assumes that the number of dissolved molecules containing phosphorus is 70 p.c. of the number of phosphorus atoms. According to private information from Mr. P. Eggleton the phosphorus distribution in blood is approximately as follows, expressed as mg. P. per 100 g. blood. Inorganic phosphate, 4½; pyrophosphate, 2½; adenylic acid, 12; hexosemonophosphate, 1½; hexose diphosphate, 4; the remaining phosphorus being present in lipins, probably not dissolved.

Abderhalden's list, however, although it makes up nearly 87 p.c. of the sum of the molal concentrations, must be supplemented by the following, data for which have been obtained, in consultation with Mr. P. Eggleton, from various sources.

Table II.—Constituents of Mammalian Blood.

Substance.	Concentration assumed.	Molal concentration.	Remarks.
Protein other than Hb	One-third of the value for Hb	0·0008	A rough estimate, but the value is practically negligible in any case.
Bicarbonate	50 c.c. combined CO_2 per 100 g. blood	0·0280	—
Lactate	16 mg. per 100 g. blood	0·0022	—
SO_4	....	0·0003	—
Urea	0·030 p.c. in blood	0·0062	—
Amino acids	0·006 p.c. N in blood	0·0053	One N atom assumed to each molecule.
Creatine	0·007 p.c. in blood	0·0006	—
Creatinine	0·001 p.c. in blood	0·0001	—
Uric acid	0·002 p.c. in blood	0·0002	—
	Sum	0·0437	

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The sum, therefore, of the molal concentrations of all constituents, obtained by adding the results of Tables I and II, is 0·3226. This is equal to the sum of the molal concentrations of the ions of a 0·1613 molal NaCl solution, which is 0·943 g.* in 100 g. H_2O. Mammalian Ringer-Locke's solution, as given by Bayliss (1924, p. 211), is isotonic with 0·971 g. NaCl in 100 g. H_2O, while the freezing point of human blood corresponds to 0·96 g. NaCl in 100 g. H_2O. If we assume that about 2 p.c. of the water of blood is bound by the protein, the remaining 98 p.c. being free to dissolve the constituents of blood in a normal manner, then the sum of the molal concentrations of the constituents of blood agrees exactly with the observed freezing point, and very closely with the usual constitution of mammalian Ringer-Locke's solution. It seems probable (i) that the water of blood is almost entirely "free," in the sense of being able to dissolve chemical substances in a normal manner, and (ii) that the constituents of blood are to be regarded as normally dissolved, and as exerting their normal osmotic pressures, in the water of the blood.

This conclusion has been tested experimentally by finding the depression of vapour pressure caused by adding various substances to blood, and comparing this with the depression of vapour pressure caused by adding the same substances in similar amounts to a 1 p.c. NaCl solution. The depression of vapour pressure has been reckoned in both cases *per gramme of substance added to* 100 *grammes of water*, the water content of the blood being measured by drying to a constant weight on a water bath at 100° C. Results have been expressed in terms of the ratio :—

$$\frac{\text{(Vapour pressure depression caused by adding 1 g. of solute to 100 g. of water in 1 p.c. NaCl solution)}}{\text{(Vapour pressure depression caused by adding 1 g. of solute to 100 g. of water in blood).}}$$

If the ratio be unity we conclude that the whole of the water of blood is free to dissolve in a normal manner chemical substances added to it. If the ratio be less than unity we must conclude that some of the water of blood is bound by the colloidal or other bodies there present, and so is unable to assist in the solution. If it be greater than unity we must assume that the substance added is somehow removed from free solution by the presence of other bodies, *e.g.*, by surface adsorption, or by "solution" in or combination with the protein or lipins.

* [*Added in proof.*—This is almost precisely the mean value for the NaCl solution which has the same vapour pressure as human blood exposed to 5 per cent. CO_2, as found by Margaria in recent experiments here.]

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A further test can be applied by adding to blood, not chemical bodies as described above but water. If we regard as fixed the total number of ions or molecules present in a given amount of blood, and add water to it, the depression of vapour pressure observed at any stage should be inversely proportional to the total volume of " free " water present at that stage. It is found by experiment that the reciprocal of the difference of vapour pressure between water and a mixture of blood and water is a linear function of the amount of water added to a given amount of blood. This is an expression of the relation, true for dilute solutions, $PV = a$ constant, P being the total osmotic pressure of all the dissolved constituents and V the volume of water in the solution. By plotting the relation between $1/P$ and V and extrapolating it backwards to the axis of V it is possible to determine the amount of " free " water in the original blood. The results so obtained agree with those found by the previous method.

The depression of the vapour pressure of a solution below that of pure water, or the difference of vapour pressure between two solutions, was measured by the thermal method described recently (Hill, 1930). Two similar pieces of filter paper dipped in the two fluids and allowed for a few moments to drain are placed on the two opposite faces of a symmetrical thermopile. The instrument is mounted in a moist chamber and placed in a thermostat. The temperature of the present observations was 20·25° C. The difference between the rates of evaporation of the two fluids is proportional to the difference between their vapour pressures ; the former causes a difference of temperature between the two faces, which becomes steady in 30 to 45 minutes ; this may be used as a measure of the latter. Thus the difference of vapour pressure required is directly proportional to the electromotive force developed in the thermopile, as read by a potentiometer and a sensitive galvanometer, and can be expressed in absolute units by calibrating the apparatus with solutions of known vapour pressure, *e.g.*, of cane sugar, NaCl or KCl. With the instrument hitherto employed,* if the difference to be observed was not too small, the average error of a single reading was about $1\frac{1}{2}$ p.c. The sensitivity was such that the difference of vapour pressure between water and 1 p.c. NaCl solution gave a deflection of about 1200 mm. on the galvanometer scale.

Defibrinated slaughter-house blood was used. In some cases this was centrifuged and the corpuscles alone employed. It appeared to make no

* [*Added in proof.*—In Dr. Margaria's hands the same instrument, and other similar ones, employed with human blood are considerably more accurate than originally claimed.]

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difference whether the blood was laked or not. If cane sugar, for example, be added to intact blood, water is immediately withdrawn from the corpuscles in amount sufficient to make the osmotic pressure of the external fluid equal to that of the internal; the rise of osmotic pressure is the same as if the corpuscular envelope were absent. This would not be the case were the osmotic pressure normally existing inside the red cells different from that of the serum. The absence of such a difference was demonstrated as follows.

A mass of centrifuged corpuscles was carefully oxygenated and separated into two parts, each being placed in a stoppered bottle. One part was laked, without exposure, by freezing to about − 15° C. and thawing. The difference of vapour pressure between the two fluids, laked and unlaked, was then measured and found to be negligible. In this respect, at any rate, a true equilibrium appears to exist across the corpuscular membrane.

Various substances have been added to blood, or to blood corpuscles:— NaCl, KCl, $CaCl_2$, cane sugar, urea, creatine, succinic acid, lactic acid, and water. In the case of the acids, in order to avoid if possible any error due to driving out carbon dioxide, the blood was shaken previously at 38° C. for half an hour, with continual changes of air. The lactic acid was prepared for me free from lactide by Mr. P. Eggleton.

In addition to the experiments given in Table III below, three series of experiments were performed in which water was added to blood corpuscles in varying amounts, and the free water content computed from the relation between observed vapour pressure depression and amount of water added. In these three series the ratio (free water)/(total water) had the following values:—0·93, 0·92, 1·00; mean 0·95.

We see that for NaCl, KCl and cane sugar, substances which are incapable of penetrating living tissues in their normal state, the ratio is about unity, having a mean value of 0·97. The addition of water led to almost exactly the same number, viz., 0·95. For $CaCl_2$ in a single experiment the value was rather greater, 1·06, but we should expect a certain amount of the calcium to be precipitated. Urea and creatine gave a greater number, the mean value being 1·17. It will be seen later that in casein solutions and in egg white the ratio for urea is not far from unity. In blood, therefore, the lower value is probably due to the removal of part of the urea from solution, perhaps by adsorption to, or combination with, the colloidal material or the lipins present; urea is singularly capable of penetrating living cells, which is probably a sign of its readiness to be combined or adsorbed. Succinic acid and lactic acid

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Table III.

The ratio given is B/A where A = the depression of vapour pressure caused by adding 1 g. of the substance in question to 100 g. of total water in blood, and B = the depression of vapour pressure caused by adding the same quantity of the substance to 100 g. of water in a NaCl solution containing 1 g. of NaCl in 100 g. of water. For a substance which is not adsorbed B/A is a measure of the ratio, "free" water/total water. The total water in the blood was determined by drying at 100° C. on a water bath.

Fluid.	p.c. H_2O.	Substance added.	g. to 100 g.	g. to 100 g. H_2O.	Ratio B/A.
Blood	78·5	NaCl	1·03	1·313	0·99
Blood	82·5	NaCl	0·25	0·303	0·95
Corpuscles	70·2	NaCl	0·283	0·404	0·955
Blood	82·5	KCl	0·40	0·485	0·93
Corpuscles	71·1	KCl	4·50	6·33	0·97
Blood	78·5	Cane sugar	10·70	13·63	1·02
Blood	82·5	Cane sugar	2·50	3·03	1·02
Corpuscles	70·2	Cane sugar	2·50	3·57	0·94
Blood	82·5	$CaCl_2$	0·40	0·485	1·06
Blood	78·5	Urea	2·00	2·55	1·13
Blood	78·5	Urea	0·50	0·637	1·24
Corpuscles	71·0	Urea	0·513	0·723	1·11
Corpuscles	71·0	Urea	0·355	0·500	1·18
Corpuscles	71·1	Urea	7·138	10·035	1·09
Corpuscles	70·1	Creatine	0·80	1·140	1·25
Blood	78·5	Succinic acid	0·98	1·248	1·70
Blood	78·5	Succinic acid	0·49	0·624	2·08
Blood	78·5	Lactic acid	0·455	0·580	1·34
Blood	78·5	Lactic acid	0·321	0·410	1·24
Blood	78·5	Lactic acid	0·317	0·403	1·24
Blood	78·5	Lactic acid	0·228	0·289	1·39
Corpuscles	71·8	Lactic acid	0·201	0·280	1·32
Corpuscles	71·8	Lactic acid	0·248	0·345	1·35

also gave a greater number; for the latter the mean value was 1·31. This low value is partly due to two factors:—

(*a*) In the solution of the acid in unbuffered NaCl solution a certain small degree of ionisation occurs; the effect of the H-ions is added to that of the lactate (or succinate) ions, consequently the numerator of the ratio is rather too large. In the solution of the acid in blood, however, the powerful buffering prevents any appreciable quantity of hydrogen ions from appearing. The total effect, however, is not great. To allow for it might decrease the ratio to 1·25.

(*b*) A small amount of bicarbonate remaining in the blood may have been broken up by the acid, and CO_2 driven off. For such lactate ions as simply replaced bicarbonate ions no vapour pressure change at all would be registered; consequently the ratio would be lowered. No

reasonable estimate, however, of the CO_2 driven off will account for all the effect observed ; it is more likely that a certain amount of the lactic or succinic acid is adsorbed, or removed from solution, in the same way as urea and creatine appear to be.

On the evidence, therefore, of the results obtained by adding NaCl, KCl, cane sugar and water, we may conclude that nearly the whole of the water of blood, say, 97 p.c. of it, is free to exert its normal behaviour as a solvent. This agrees with the deduction made above from the analyses and the observed freezing point. Some substances, however, such as urea and creatine, and probably lactic and succinic acids, fail to exert their full effect on the vapour pressure owing to a slight degree of adsorption on, or combination with, the colloidal constituents or the lipins of the blood. It would, of course, be possible to argue that some of the NaCl, KCl, or cane sugar also is adsorbed, the effect being exactly compensated by the demobilisation (*qua* solvent) of part of the water. It is not easy, however, in that case to explain the fact that the addition of water leads to the same value of the ratio as the addition of any of these three bodies. It is much simpler to suppose that nearly all the water is free and that these substances are normally dissolved in it. This view is confirmed by the experiments on casein solutions, on egg yolk and on egg white to be described next.

III. The State of Water in Protein Solutions.

The method described above for the case of blood was applied with little change to (*a*) a strong solution of casein in N/10 NaOH, (*b*) egg-white concentrated by evaporation, and (*c*) egg-yolk. The total water content was determined by drying at 100° C. on a water bath and finally at 110° C. in an oven. The stronger casein solutions were very viscous. The strips of filter paper, therefore, were left in them for some time to become properly moistened. They were then removed and laid on a glass plate, and the excess of solution was rapidly cleaned off with a pair of forceps and a wire. In spite of the difficulty in manipulation, apparently very reliable results were obtained. The egg-white was left in a vacuum desiccator for three days over calcium chloride. In that time half the water evaporated and the depression of vapour pressure was doubled.

From Table IV we see that for casein solutions and egg-white the mean value of the ratio "free" water/total water for *all* substances added is 0·98. Apparently, therefore, at room temperature only about 2 p.c. of the water is

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Table IV.

Change of vapour pressure due to adding various substances to casein solution, egg white and egg yolk. The ratio given is B/A, where A = the depression of vapour pressure caused by adding 1 g. of the substance in question to 100 g. of water in the solution investigated, and B = the depression of vapour pressure caused by adding the same quantity of the substance to 100 g. of water in a 1 p.c. NaCl solution. For a substance which is not adsorbed B/A is a measure of the ratio, "free" water/total water.

Fluid.	p.c. H_2O.	Substance added.	g. to 100 g.	g. to 100 g. H_2O.	Ratio B/A.
Casein solution	85·2	NaCl	0·803	0·942	0·94
Casein solution	85·2	Urea	1·946	2·284	1·00
Casein solution	85·2	KCl	1·034	1·213	0·97
Casein solution	85·2	Cane sugar	8·22	9·65	0·97
Casein solution	83·1	NaCl	0·826	0·994	1·00
Casein solution	83·1	Urea	1·616	1·945	1·06
Casein solution	83·1	KCl	0·889	1·070	0·92
Casein solution	81·3	NaCl	2·84	3·49	0·99
Casein solution	80·56	NaCl	1·95	2·424	0·98
Egg white	78·7	KCl	2·12	2·70	0·98
Egg white	78·7	Cane sugar	1·412	17·95	0·94
Egg white	78·7	Urea	2·83	3·60	1·01
Egg yolk	47·0	KCl	1·189	2·53	0·85

"bound." In the single experiment on egg yolk there was about 15 p.c. of water "bound" by 53 p.c. of solid. The two protein solutions, therefore, give practically the same result as blood, in which we concluded that only about 3 p.c. of the water is "bound" by the solid constituents.

IV. The State of Water in Muscle.

(*a*) *The Solution of Urea in Muscle.*—The following experiments were suggested and carried out by Mr. P. Eggleton and Mr. H. V. Horton, who have kindly allowed me to report the results. One, two, or three sartorius muscles, from large Hungarian frogs, were soaked for about 4 hours in a small quantity of Ringer's solution containing initially 0·14 p.c. urea. Each sartorius weighed about 250 mg. and the weight of solution employed was about twice that of the muscles. The opposite muscles from the same animals were similarly soaked in Ringer's solution without urea. Urea was chosen (*a*) owing to the extreme readiness with which it penetrates the tissues; a comparison of the results obtained after 3 and 5 hours' soaking showed that diffusion was complete in the shorter time, so 4 hours' soaking was taken as standard; (*b*) because it is not known to react in any way with the constituents of muscle; and (*c*) because the amount of it in the solution can be measured with considerable

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accuracy as total nitrogen. The muscles were weighed before and after soaking; no important change of weight occurred. A measurement of the total nitrogen was made four times in every experiment, once each on the two solutions before soaking, once each after soaking. From the results the quantity of urea which had disappeared from the solution was calculated; assuming that it was dissolved in the "free" water of the muscle, attaining there a concentration equal to that in the fluid outside, the quantity of "free" water in the muscle was computed and expressed as a fraction of the weight of the muscle. The results are given in Table V.

Table V.

"Free" water of frog's muscle, calculated from the distribution of urea between muscle and external solution, and expressed as a percentage of the weight of the muscle. The number in brackets after each result is a weighting factor, depending partly upon the quantity of material used, partly upon an estimate by the observers of the general reliability of the experiment.

74 (1); 75½ (1); 67 (1); 91 (1); 90 (1); 83½ (2); 79 (3).

Weighted mean = 80.

Accepting the assumptions made in the calculation the mean value corresponds to a quantity of "free" water equal to 80 p.c. of the weight of the muscle, equal, that is to say, to the total water; apparently none of the water of muscle is "bound" by the solids. It is true that the same result might have been found if part of the water were "bound," and a corresponding part of the urea adsorbed. If urea be added to blood a small fraction of it—as shown, above—does not go into solution in the water of blood; in egg-white and casein solutions, however, the whole of the added urea is apparently simply dissolved. The urea experiments, therefore, cannot be regarded as a final proof that all, or nearly all, the water of muscle is "free"; they have been confirmed, however, by those to be described in the next section, which involved a different procedure and different substances.

(*b*) *The "Free" Water of Muscle determined by Vapour Pressure Measurements.*—Two methods have been employed to determine the "free" water of muscle, the "single" and the "differential." Both are applicable to the case of a resting muscle; for muscles fatigued or in rigor, however, the latter only can be used.

In the "single" method several small muscles (preferably the gastrocnemii and the vasti interni (Marshall) of one or two small frogs, which can be prepared practically without injury) were first soaked for several hours in normal

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oxygenated Ringer's solution (R), isotonic—see the preceding paper by Hill and Kupalov—with 0·703 p.c. NaCl. Since, apart from CO_2, the muscles were already practically isotonic with this solution, 5 or 6 hours of soaking should bring them fairly accurately into equilibrium with it. They were then withdrawn one by one from the solution, freed from all adherent fluid by rapidly blotting with filter paper, and dropped into a weighed glass tube of capacity about 10 c.c. When the muscles were all in the tube the whole was weighed, and so the weight of the muscles obtained to the nearest 1 mg.

To the muscles in the tube was now added an accurately weighed amount of Ringer's solution of twice the normal strength (2R), which is isotonic with 1·406 p.c. NaCl. It can be shown mathematically that the method is most sensitive and accurate when the quantity of 2R-solution added contains an amount of water equal to the amount of "free" water in the muscles. Since the latter is about 80 p.c. of their weight it has proved convenient in practice to add a quantity of 2R-solution containing an amount of water precisely equal to 80 p.c. of the weight of the muscles. The tube was then filled with oxygen and stoppered, and the muscles and the 2R-solution were left 15 to 20 hours, so as to come into diffusion equilibrium with one another; during that time the tube was kept slowly revolving so as to ensure adequate mixing. The final result was that muscles and solution attained an osmotic pressure about equal to that of 1·5R. The muscles weighed finally 10 to 20 p.c. less than they did at the start, but were usually in excellent condition and very excitable.

It is essential that a sufficient period should be allowed for equilibrium to be attained; see the last section of this paper; with muscles of 0·2 to 0·3 g. it is best to leave the tube rotating during the night and to complete the observations next day. If sufficient time be not allowed the osmotic withdrawal of water from the muscles will not be complete, the osmotic pressure of the solution will be too high, and the "free" water of the muscle calculated from it too low. It is *not* advisable to use muscles weighing more than 0·3 g., otherwise the time required for osmotic equilibrium to be attained (which for muscles of similar shape varies as the 2/3 power of the weight) will be so great that the muscles may have depreciated; moreover there is the difficulty of an adequate supply of oxygen to the interior (Hill, 1928) if the muscles be too large. The process of equilibration consists partly of a diffusion of water from the muscle into the solution outside, and partly (so far as semi-permeable membranes allow) of a diffusion of salt from the solution into the muscle. It is impossible to imagine a difference of osmotic pressure to exist between two different parts

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of the system once equilibrium has been attained, for the membranes involved are far too thin to be able to stand any considerable mechanical stress.

After 15 to 20 hours mixing the tube was opened, the muscles removed, and the difference of vapour pressure measured between the fluid left in the tube and the original Ringer's solution (R). Two or three readings of this vapour pressure difference were generally made, and on the same day the apparatus was calibrated with the solutions R and 2R on its two faces. To take an example, 2 g. of muscle after equilibration with R was mixed with an amount of 2R containing 1·6 g. of water. After 18 hours mixing the final vapour pressure difference between the fluid in the tube and the solution R was 127 (arbitrary units). On the same day the vapour pressure difference between R and 2R gave 249 units. Thus the final osmotic pressure of the mixture in the tube was the same as that of a solution $(1 + \frac{127}{249})\ R = 1{\cdot}51\ R$.

We may argue as follows, if x be the amount of "free" water per 1 g. of muscle :—

$$(x \times \text{weight of muscles})\ R + (\text{weight of water in 2R added})\ 2R$$
$$= (x \times \text{weight of muscles} + \text{weight of water in 2R added})\ 1{\cdot}51R.$$

This merely expresses the fact that when equilibrium has been attained the dissolved substances originally present (*a*) in the muscle at osmotic pressure R, and (*b*) in the fluid added at osmotic pressure 2R, have been redistributed to give a uniform osmotic pressure (observed) 1·51R. Hence

$$x = \frac{1 - 0{\cdot}51}{0{\cdot}51} \times \frac{\text{weight of water in 2R added}}{\text{weight of muscles}}$$
$$= 0{\cdot}49 \times 0{\cdot}8/0{\cdot}51 = 0{\cdot}77.$$

Therefore 1 g. of muscle contained 0·77 g. of "free" water.

The first six experiments of Table VI were made by this method. It is valid so long as the condition of the muscle may be assumed to remain constant during equilibration with 2R. It cannot be applied when the initial osmotic pressure is not accurately known (as in fatigue) or when the osmotic pressure alters of itself during equilibration (as when anaerobic conditions are necessary, in order to prevent recovery). In such cases the "differential" method must be employed, in which an unknown initial osmotic pressure or progressive osmotic changes during equilibration are automatically allowed for.

In the "differential" method the muscles were divided into two lots, those from the right leg of an animal being allotted to one, those from the left leg to the other. The procedure was much as before, except that twice as many

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frogs and two tubes—instead of one—were used. In one tube (A) was placed one lot of muscles together with a weighed quantity of Ringer's solution (R), containing an amount of water equal to 0·8 of the weight of the muscles. In the other tube (B) was placed the other lot of muscles, together with a weighed quantity of 2R-solution containing the same relative quantity of water. After prolonged equilibration the vapour pressure difference was measured between the fluids in tubes A and B, and expressed as a fraction f of that between R and 2R. As before, if x be the quantity of "free" water in 1 g. of the original muscles, it can be shown that

$$x = \frac{1-f}{f} \times \frac{\text{weight of water in 2R added}}{\text{weight of muscles}}.$$

There are several advantages in the "differential" method :—

(*a*) A preliminary soaking in R-solution is unnecessary ; the initial osmotic pressure of the muscles need not be known, all that is necessary is that it should be the same in both lots.

(*b*) Changes occurring in the muscles during the prolonged second equilibration, which would be fatal in the first method, balance out exactly in the second, since they affect the contents of tubes (A) and (B) alike.

(*c*) If the muscles be first equilibrated with R-solution, and if a further observation be made of the difference of vapour pressure between R and the contents of tube (A), we can calculate not only the "free" water of the muscles but the increase of their osmotic pressure, resulting (say) from fatigue or heat rigor. If, for example, the final difference of vapour pressure between R and the contents of tube (A), expressed as a fraction of that between R and 2R, be f', the rise of osmotic pressure in the contents of tube (A) must be equal to that of a solution f'R. Let us suppose the osmotic change in question to have occurred in the muscle alone, and not to have been shared with the fluid around it ; then the rise of osmotic pressure would have been greater in the ratio

$$\frac{\text{total "free" water in tube}}{\text{"free" water in muscle}},$$

which can be shown to lead to the simple expression,

Rise of osmotic pressure in muscle = that of a solution $f'\mathrm{R}/(1-f)$.

In a few experiments, instead of using 2R as the test solution, a solution of urea in Ringer's fluid was employed. This gave very consistent and accurate results, the muscles being in excellent condition after prolonged soaking even

in fairly strong solutions. The value, however, of the "free" water found by the use of urea tended to be rather higher than by that of the solution 2R, and it was recalled that in blood—see Table III above—values for the "free" water obtained with urea were slightly, but definitely, too high. Urea is a peculiar substance, its extreme ability to penetrate living cells may well imply a high solubility in the lipins of the tissue, or a great liability to adsorption; and if some of the urea in the test fluid were removed from free solution by adsorption or otherwise it would appear as if the "free" water of the tissue were greater than it really is. Hence, although the results were in good agreement with those of Eggleton and Horton described above, and although the method employing urea seemed to work so well, it was thought wiser to avoid its use for fear of introducing a small constant error. There is little danger of this with the 2R-solution, the chief constituent of which is NaCl, a substance very unlikely not to remain in free solution.

In Table VI are given the results for resting muscles, every experiment made

Table VI.—Experiments to determine the "free" water of resting muscles, made with Ringer's solution of twice normal strength (2R).

D = "differential" method; S = "single" method; v = very; g = good; p = poor.

The weighting factor is adjusted to take account (*a*) of the final state of the muscles as determined by stimulation, and (*b*) of the general reliability and type of the experiment. The equilibration was at room temperature (about 16° C.).

Three experiments with urea instead of 2R are included at the end of the table, but no account is taken of them in the mean value.

Date, 1930.	Average weight of muscles, grammes.	Period of equilibration, hours.		Type of Experiment.	Final condition.	Weighting factor.	Free water fraction.
		Preliminary in R.	In 2R.				
19–20 Feb.	0·27	6·9	15½	S	v.g.	3	0·73
19–20 ,,	0·23	6·5	16	S	v.g.	3	0·73
21–22 ,,	0·22	5	16½	S	v.g.	3	0·77
21–22 ,,	0·27	5	17	S	g.	2	0·79
21–22 ,,	0·19	5	17½	S	g.	2	0·82
21–22 ,,	0·19	5	18	S	v.g.	4	0·80
5–6 Mar.	0·29	2	17	D	v.p.	1	0·93
5–6 ,, ...	0·26	1	19½	D	p.	2	0·82
21–22 Mar.	0·29	6¾	18	D	v.g.	6	0·76
21–22 ,,	0·30	6¾	19½	D	v.g.	6	0·735
6–7 Mar.	0·34	1	(urea) 20½	D	v.g.	—	0·83
17–18 Mar.	0·25	3	(urea) 17	D	v.g.	—	0·83
17–18 ,,	0·45	2½	(urea) 19½	D	v.g.	—	0·80

The mean value of the "free" water fraction, taking due account of the weighting factors, is 0·77.

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being recorded. Some of the observations were, on technical grounds, *e.g.*, state of muscles at end, consistency of vapour pressure readings, type of experiment (" differential " being preferable to " single "), etc.—more reliable than others, and the relative reliability of the result was assessed by the observer and expressed by a weighting factor given in the penultimate column. The mean value of the " free " water fraction, taking due account of these weighting factors, is 0·77. It is possible that the true value is slightly greater than this: any error due to incomplete equilibration would make the result too low, and it is not certain that equilibration is quite complete even in 18 hours. The true value cannot, however, be higher than 0·80 or 0·81, which is that of the total water fraction. There is obviously very little water " bound " in the resting muscle. So far as they go the urea experiments gave a slightly higher value than those made with 2R; it may be that they give the truer value, owing to the rapid penetration of urea; for reasons, however, which we have discussed above no account was taken of them in calculating the mean.

In Table VII are given results for fatigued muscles, and for muscles in heat rigor. The " differential " method alone was used, and a weighting factor was allotted, as described above.

The experiments on heat rigor were the simpler. The procedure was identical with that for resting muscles, except that :—

(*a*) The solutions R and 2R contained M/50 or M/100 NaCN to prevent oxidation and partial recovery.

(*b*) The tubes were filled with nitrogen instead of oxygen for the same reason.

(*c*) When all was complete, and the tubes filled and stoppered, they were immersed for half-an-hour in water at about 45° C., so that the muscles inside then went into heat rigor.

After prolonged equilibration the " free " water was determined as before, and in addition, in some experiments, the osmotic change due to rigor was measured by comparing the vapour pressure of the final fluid in the R-tube with that of the original R-solution (for details see above). The result was expressed in the following way :—The osmotic change in the fluid in the R-tube was measured; this is entirely due to the muscles; assume the whole change to be concentrated in the muscles, and express it in terms of p.c. NaCl added to R-solution. To say that the muscles, originally isotonic with 0·703 p.c. NaCl, have undergone an osmotic change equivalent to 0·455 p.c. NaCl, implies that if all the products of rigor had been kept inside the tissue and not

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Table VII.—Experiments to determine the "free" water of fatigued muscles, or of muscles in heat rigor, made with Ringer's solution of twice normal strength (2R).

All experiments by "differential" method. The weighting factor is adjusted to express the general reliability of the experiment. The osmotic change is expressed in terms of NaCl as explained in the text. The lactic acid produced is calculated as a fraction of the original free water of the muscles (assumed to be 77 p.c. of their weight). Two experiments with urea instead of 2R are included at the end of the table, but no account is taken of them in the mean value.

Ri = heat rigor ; Ex = exhaustion.

Date, 1930.	Average weight of muscles, grammes.	Type of experiment.	Period of equilibration, hours.		Weighting factor.	Free water fraction.	Osmotic change, per cent. NaCl.	Lactic acid, per cent. of "free" water.
			Preliminary in R.	In 2R.				
24 Feb. ……..	0·24	Ri	1	5½	1	0·82	—	—
24–25 Feb.	0·24	Ri	2½	14	2	0·83	0·37	—
26–27 ,,	0·27	Ex	0	19¼	4	0·79	—	—
27–28 ,,	0·26	Ex	0	17½	4	0·76	—	—
20–21 Mar.	—	Ri	2	17½	4	0·73	0·455	0·655
20–21 ,,	—	Ri	2	19	4	0·73	0·43	0·665
26–27 ,,	0·29	Ex	3½	15	5	0·79	0·44	—
27–28 ,,	0·22	Ex	1½	18	6	0·79	0·45	—
27–28 ,,	0·21	Ex	4	21	6	0·73	0·49	0·81
19–20 Mar.	—	Ri	4	(urea) 15	—	0·84	—	—
19–20 ,,	—	Ri	4	(urea) 16½	—	0·81	—	—

The mean value of the free water fraction, taking due account of the weighting factors, is 0·77 ; the mean value of the osmotic change is the equivalent of 0·45 p.c. NaCl.

allowed to diffuse out into the R-solution, the muscles would finally have been isotonic with 0·703 + 0·455 = 1·158 p.c. NaCl.

Two experiments were made with urea ; they were good experiments, but the results are not included in the mean, for the reasons given above.

The experiments on fatigue were really experiments on rigor. Muscles severely fatigued will not long survive if deprived of oxygen ; it was necessary, in order to make sure that equilibration was complete, to subject them to 15 hours or more of mixing ; this means—since recovery would obviously have spoilt the experiment—a long period of oxygen want, during which they invariably passed into rigor. To ensure that the fatigued muscles should be alive at the end of it, equilibration would need to last not longer than about 4 hours. Only by using exceedingly small muscles, weighing, say, 50 mg. apiece (see fig. 2, p. 503), would it be possible so to quicken diffusion that

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equilibration would be complete in that time. The difficulty of preparing a sufficient mass of such small muscles without injury was too great.

It is open, therefore, to any who will, to argue that the "free" water of *fatigued* muscles has not been measured at all. Strictly speaking this is so. The muscles could be properly described as fatigued during the earlier part of their equilibration, but during the latter part they were certainly in rigor. Since, however, the "free" water of muscles in rigor appears to be the same as that of muscles at rest, it is very unlikely that in fatigue, which in many respects may be regarded as an intermediate condition, the case is seriously different.

Fatigue was induced in two ways. In the first two experiments the intact legs were exhausted by induction shocks and then skinned; the muscles were dissected, placed immediately in the two tubes and weighed. In the last three experiments the muscles were dissected and soaked in Ringer's solution, blotted, placed in their tubes and weighed; they were then exhausted by induction shocks led through the contents of the two tubes in series. Finally in both cases the R and the 2R solutions were added. As before these contained NaCN, and the tubes were filled with nitrogen.

In three experiments the lactic acid production was measured. I am much obliged to Miss M. Kerly for making the determinations. The total lactic acid found in a tube is expressed in Table VII as a percentage of the initial "free" water of the muscle in the tube.

The mean value of the "free" water fraction for fatigued and rigor muscles, taking due account of the weighting factors, is 0·77. This is identical with the mean value for resting muscles. Again it may be slightly too low, owing to equilibration being not quite complete.

The osmotic change in fatigue and rigor has a mean value equivalent to 0·45 p.c. NaCl. The lactic acid actually produced, in the three experiments in which it was measured, averaged 0·71 p.c. calculated in the "free" water of the muscle. This is osmotically equivalent to 0·23 p.c. NaCl, which is 0·23 p.c. less than the mean osmotic change observed in the same three experiments. If we further suppose that all the phosphagen was broken down, liberating creatine equivalent to 60 mg. p.c. P, the extra osmotic effect would be equivalent to 0·07 p.c. NaCl. There is still an excess of osmotic pressure to account for, as found in the previous paper. This excess (equivalent to 0·16 p.c. NaCl) may seem rather small, when compared with that described there; actually its *absolute* value is almost exactly the same, but its *relative* size is diminished by the much greater amount of lactic

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acid produced in the present experiments. We may recall also that in Table III, above, the vapour pressure depression caused by adding lactic acid to blood was shown to be appreciably less than that due to adding it to water; perhaps in muscle also the lactic acid is partly adsorbed by, or dissolved in, the solid constituents of the tissue. If so, the large amount of lactic acid produced in the rigor experiments would be osmotically equivalent in muscle to less than the amount of NaCl computed, and the unexplained excess of osmotic pressure still greater than calculated above.

The fundamental point brought out by the experiments of Tables VI and VII is that nearly the whole of the water of muscle is "free," in the sense that it can dissolve in a normal manner substances added to it. This is true, whether the muscles be at rest or in rigor. The mean value of the "free" water is 77 p.c. for both. The true value may be even slightly greater. It is important to define precisely what is meant by "free" water; the difference between the present results and those of others (*e.g.*, of Rubner) may be, in part at least, simply a matter of definition.

In dealing with the equilibria occurring in blood, or between blood and tissues, it is common to express concentrations in grammes (or mols) per litre, or per 1000 g. of tissue. It is clearly better, and likely to lead to much simpler relations, to express all concentrations (where physico-chemical equilibria are involved) in grammes (or mols) per 1000 g. of "free" water. If the "free" water be not accurately known, the total water is a close approximation, at least in muscles and blood.

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V. The Swelling and Shrinking of Muscles in Hypo- and Hyper-Tonic Solutions.

The experiments of Overton (1902) have been referred to in the Introduction above. They have been repeated and extended as follows. The object was to measure the "osmotically active water fraction," *i.e.*, the weight of water per gramme of living muscle which may be regarded as surrounded by semi-permeable membranes and liable therefore to osmotic increase or decrease in hypo- or hyper-tonic solutions.

It is necessary to consider for how long a time a muscle must be immersed in an aqueous solution of substances to which it is normally impermeable, in order to attain a sufficiently constant weight. The problem is one of diffusion, chiefly of water but partly also of salts, and is analogous to that discussed by Hill (1928, pp. 68–73, and fig. 5) in relation to the diffusion of oxygen into

a cylinder. The first stages of diffusion are rapid; the creep, however, to the final equilibrium is slow.

Fig. 1 illustrates the process graphically. Vertically is shown the change in weight of a cylinder composed of semi-permeable fibres, immersed in a hypo- or hypertonic solution, expressed as a fraction of the total change finally

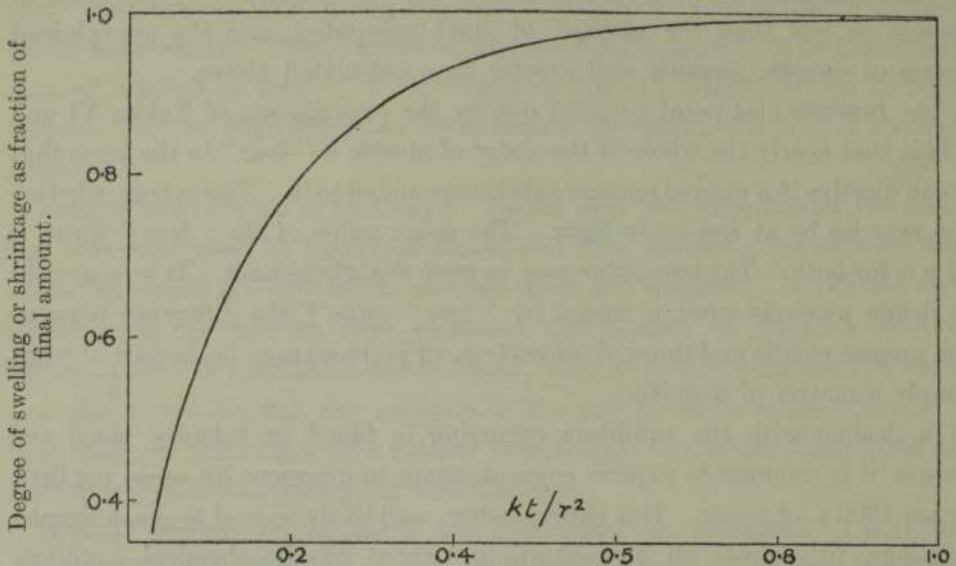

FIG. 1.—The swelling (or shrinkage) of a cylindrical tissue consisting of semi-permeable elements, when immersed in a hypotonic (or hypertonic) solution. Vertically, the degree of swelling (or shrinkage) as a fraction of that finally attained after long immersion; horizontally kt/r^2, where k is the diffusion constant of water in the tissue, t is time, and r is the radius of the cylinder. [Drawn from the data of Table IV, p. 71, of Hill (1928).]

attained after prolonged immersion. Horizontally is kt/r^2, where k is the diffusion constant of water into the tissue, t is the time, and r the radius of the cylinder. The curve was constructed from the data of Table IV in the paper just mentioned. The time needed for any given change is inversely proportional to the diffusion constant, and directly proportional to the square of the linear dimensions of the tissue; the factor kt/r^2 always occurs as a whole in dealing with a cylindrical tissue. Since the weight varies as the cube of the linear dimensions, for muscles of the same shape, we may conclude that the time required is proportional to the 2/3 power of the weight. As we shall see below, with muscles of the size used the time required to attain final equilibrium is so great that the values actually found are complicated by changes in the tissues. It is necessary to extrapolate from readings at an earlier stage.

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It was thought desirable to avoid the use of solutions differing considerably in osmotic pressure from the muscles. The solutions employed were 0·8R and 1·2R, or 0·75R and 1·25R.* The muscles were always alive and normally excitable at the end of immersion. Since, however, progressive changes may occur in them, the simple procedure employed by Overton was not used, but a "differential" method was adopted. This automatically eliminates the effects of such unknown disturbances, each experiment being, so to speak, its own control.

The two gastrocnemii of a small frog were prepared and kept in oxygenated Ringer's solution (R) till required. Each was carefully blotted and weighed to the nearest 0·1 mg. on a Bunge air-damped balance. One was then hung in 0·8R solution, stirred by oxygen bubbling slowly through it, the other in 1·2R solution similarly stirred. After a sufficient period of immersion at room temperature the muscles were removed, blotted, and weighed to 0·1 mg. as before.

Consider a pair of similar muscles, *e.g.*, the two gastrocnemii of one frog. Let their initial weights be taken as unity, and assume that they have been brought, by soaking, into equilibrium with Ringer's solution R. Let (i) a be the solid plus "bound" water fraction; (ii) x the "osmotically active water fraction"; (iii) $(1 - a - x)$ the "osmotically inactive water fraction." Let us immerse one of the muscles in a hypertonic solution $(1 + r)$ R, and the other in a hypotonic solution $(1 - r)$ R, and leave them till a constant difference of weights sets in. Imagine that x changes in any way, owing to the processes of survival in salt solution, becoming $x(1 + \delta)$, where δ is positive or negative; causes of such change might be (1) a loss of semi-permeability in some of the membranes or fibres, in which case δ would be negative, or (2) a production of metabolites during survival, causing the tissue to swell, in which case δ would be positive, etc. Imagine further that $(1 - a - x)$ also changes as the result of immersion and survival, becoming $(1 - a - x)(1 + \delta)'$, where δ' is positive or negative; a cause of such change might be (1) an imbibition of water by the colloids of the tissue, or (2) a loss of semi-permeability in some of the fibres coming originally in the other fraction; in either case δ' would be positive. Let A be the final weight of the muscle in the hypotonic solution, B that of the muscle in the hypertonic solution. Since in the former the osmotic

* By a yR solution is meant, as before, an aqueous solution of the usual constituents of Ringer's fluid (except bicarbonate), in the usual relative proportions, but with $1/y$ of the usual amount of water.

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pressure of the environment was changed from R to R $(1-r)$, the "osmotically active water" must have increased in the ratio $1/(1-r)$; hence

$$A = a + (1 - a - x)(1 + \delta') + x(1 + \delta)/(1 - r).$$

Similarly

$$B = a + (1 - a - x)(1 + \delta') + x(1 + \delta)/(1 + r).$$

We see at once from these formulæ the danger of making "single" uncontrolled experiments; δ' is unknown, it may be relatively large and positive, so that the apparent osmotic increase of volume in hypotonic solutions may be partly due to swelling in no wise caused by osmotic forces. That this danger is not imaginary is shown by the fact that in all the experiments performed on pairs of muscles, as described above, the increase of weight in the hypotonic solution was considerably greater than the decrease of weight in the hypertonic solution.

It is simple, however, to eliminate δ' completely by subtraction together with a; we find

$$A - B = x(1 + \delta)\, 2r/(1 - r^2)$$

so that the *final* "osmotically active water fraction" is given by

$$x(1 + \delta) = (1 - r^2)(A - B)/2r.$$

There is no direct way of finding the *initial* "osmotically active water fraction"; the best that can be done is to employ muscles so small that the time of equilibration is reduced as much as possible. For the two sets of observations to be reported first the gastrocnemii of the smallest frogs available were used. In the longer series of observations (by Kupalov) then described various muscles of widely different sizes were used. By extrapolation to "zero weight" the true value can be approximately estimated.

The following experiment is instructive. The 10 gastrocnemii of five frogs were dissected from 9.45 to 10.15 a.m., and left in oxygenated R-solution. From 11.15 to 11.42 a.m. they were blotted, weighed, and transferred, one set to oxygenated 0·8R-solution, the opposite set to oxygenated 1·2 R-solution. From 6.29 to 6.49 p.m. they were blotted and weighed again. They were soaked, therefore, in R-solution for $1\frac{1}{2}$ hours, and in 0·8R and 1·2R for 7·17 hours.

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Frog.	In 0·8R-solution.		In 1·2R-solution.		A–B calculated.
	Initial weight.	Final weight.	Initial weight.	Final weight.	
	mg.	mg.	mg.	mg.	
1	143·7	163·0	140·4	132·9	0·1876
2	246·4	268·0	253·0	239·2	0·1424
3	187·8	206·4	193·2	180·3	0·1659
4	213·5	229·8	202·3	188·5	0·1448
5	214·5	235·0	211·5	199·2	0·1539

It should be noted that A and B are fractions (final weight) $\div$ (initial weight) in each case. The average value of (A — B) is 0·1589. The average initial weight of the muscles was 201 mg. Since in this case $r = 0 \cdot 2$, the "osmotically active water fraction" $(A - B)(1 - r^2)/2r$, is $2 \cdot 4\,(B - A) = 0 \cdot 382$.

In this experiment, however, there is evidence that a steady difference (A — B) had not been reached. On the same day a similar group of 10 gastrocnemii, soaked in oxygenated R-solution for 1·6 hours, was immersed, one set in 0·8R, the opposite set in 1·2R, for 6·16 hours. The average initial weight was 202 mg. and the average value of (A — B) was 0·1514. This is appreciably less (at 6·16 hours) than the value obtained for 7·17 hours. A third group of muscles, soaked in oxygenated R-solution for 1·7 hours, was immersed in 0·8R and 1·2R for 4·5 hours; the average initial weight was 194 mg. and the average value of (A — B), 0·1360. This is considerably less (at 4·5 hours) than the value at 7·17 hours. It is clear that a constant difference was not reached. We may, however, gain a rough idea of the true final value by extrapolation.

Our data are as follows :—

Time, hours	4·6*	6·16	7·17
A — B	0·1360	0·1514	0·1589

We have to choose three points on the curve of fig. 1 such that the abscissæ are in the ratio 4·6 : 6·16 : 7·17, and the ordinates in the ratio of the corresponding values of (A — B). If we take 4·6 hours as corresponding to $kt/r^2 = 0 \cdot 12$, then 6·16 and 7·17 hours correspond respectively to 0·161 and 0·187. The values of the percentage change read off from fig. 1 for these values of kt/r^2 are, 65·4, 72·6, and 76·5, from which the calculated final value of

* 4·6 hours is a corrected time, to allow for the fact that the muscles in this group weighed slightly less than in the others.

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(A — B) should be 0·136/0·654 = 0·208, 0·1514/0·726 = 0·209, and 1·1589/0·765 = 0·208, a satisfactory agreement. Thus the final value of (A — B) to which the muscles were tending is 0·208, which corresponds to an "osmotically active water fraction" of 0·50. To attain it, however, would have required about 24 hours, during which time the fraction in question would have diminished, owing to irreversible changes taking place in the muscles.

In another experiment 14 pairs of gastrocnemii, again averaging about 0·2 g. in weight, were dissected and left in oyxgenated R-solution all night at about 18° C. On the following day they were blotted, weighed and immersed in 7R/6 and 5R/6 solutions for 7¼ hours. Finally they were blotted and weighed again. The average value of (A — B) was 0·115, which corresponds to an "osmotically active water fraction" of only 0·336. The low value is doubtless due partly to too short a time of immersion in the experimental fluids, partly to a genuine decrease—caused by long survival—of the fraction of the muscle contained within functioning semi-permeable membranes.

A series of similar experiments was performed by my colleague Dr. P. Kupalov, on various muscles of the frog dissected on the same day. These were the gastrocnemius, the sartorius, the semi-membranosus, and several other muscles of the upper leg. The period of immersion was about 5½ hours, after a preliminary soaking of about 2 hours in Ringer's fluid ; the solutions were either (*a*) 0·8R and 1·2R, or (*b*) 5R/6 and 7R/6. The muscles varied in weight from 30 mg. to 840 mg. Naturally with the smaller ones the experimental error was large, and moreover, in their dissection, there was risk of injury, which tended to increase the "scatter."

The results of these experiments are shown graphically in fig. 2, the value of the "osmotically active water function" being plotted against the initial weight of the muscle. The curve drawn through the points is a theoretical one, based on fig. 1, assuming the muscle to be cylindrical and r^2 (in kt/r^2) to be proportional to the 2/3 power of the weight. The extrapolated value for zero weight, viz., 0·47, is that to which, on the average, the muscles were tending at the time of observation (5½ hours). Apparently after 1 to 2 hours of preliminary soaking in Ringer's fluid and 5½ hours in the experimental solutions, the "osmotically active water fraction" is about 0·47 ; in other words, out of 0·77 g. of "free" water in 1 g. of muscle, 0·47 g. is confined within semi-permeable membranes and is subject to osmotic swelling or shrinkage in hypo- or hypertonic solutions, while 0·30 g. is unconfined.

Of the 0·77 g. of "free" water in 1 g. of muscle only a small part is present in the interspaces between the fibres ; certainly far more than 0·47 g. is con-

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tained within the fibres themselves. The reason why all the water is not "osmotically active" is not, as Overton supposed, that part of it is "bound";

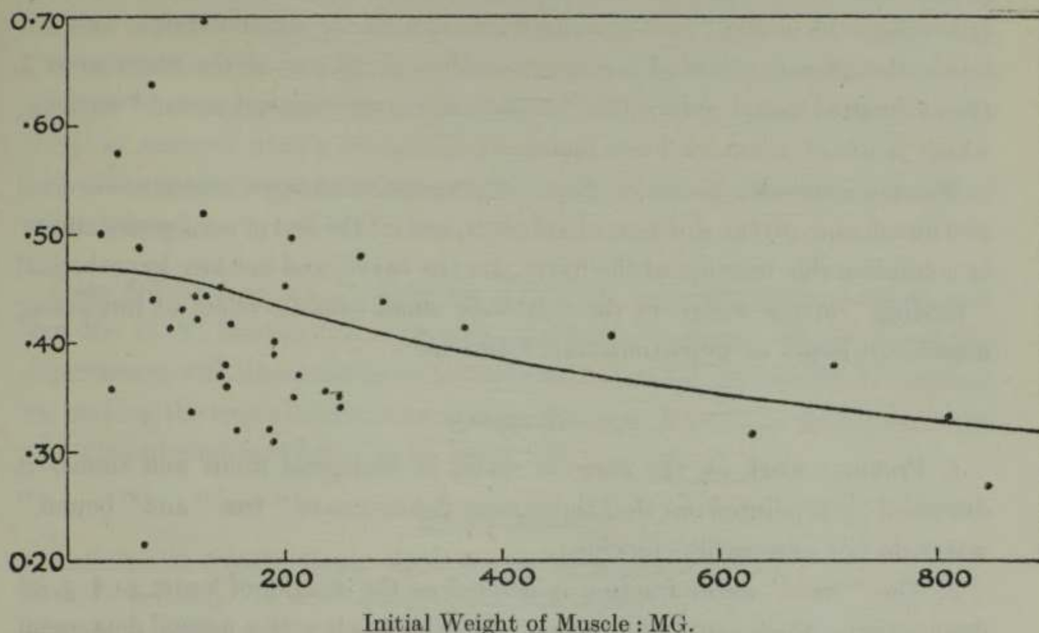

FIG. 2.—The "osmotically active water fraction" determined for a number of muscles of different sizes by the differential method described in the text, plotted against the weight. The curve drawn through the points was calculated from fig. 1, assuming (*a*) a constant time of immersion, (*b*) a constant coefficient k of diffusion, and (*c*) a cylindrical muscle, with r^2 varying as the 2/3 power of the weight. The true value, for the period of immersion considered, is that extrapolated to zero weight.

very little, if any, of it is "bound"; the simplest and most probable explanation is that, after prolonged survival without a normal blood supply, the membranes of some of the muscle cells have lost their semi-permeability. I am informed by Mr. H. V. Horton that a considerable fraction of its potassium may escape by diffusion from a muscle suspended in Ringer's solution, *e.g.*, 25 p.c. of it may be lost in 5 hours (see also Ernst and Scheffer (1928)). During life the membranes of a muscle are certainly impermeable to potassium. Apparently, however, the removal of their normal environment of blood plasma, or some other cause, somehow affects the membranes of a certain number of the fibres, rendering them permeable, allowing their K-ions to escape, and so making them presumably "osmotically inactive." The spontaneous onset of "reversible inexcitability" described by Dulière and Horton (1929) as occurring in an isolated surviving muscle is, so Mr. Horton informs me, almost

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certainly caused by the escape of potassium from the inside of a certain number of its fibres; its reversal by washing is due simply to the removal of this potassium. If the original "osmotically active water fraction" were 0·64 (allowing 0·13 of the "free" water fraction 0·77 for water between and not inside the fibres), a loss of semi-permeability in 25 p.c. of the fibres after 7 hours survival would reduce the "osmotically active water fraction" to 0·48, which is about what we have found.

We may conclude, therefore, that, (*a*) progressive changes, owing to survival and immersion, (*b*) the slowness of diffusion, and (*c*) the loss of semi-permeability in a considerable fraction of the fibres, are the cause, and not any hypothetical "binding" of the water, of the relatively small osmotic effect of immersing muscles in hypo- or hypertonic salt solutions.

Summary.

1. Previous work on the state of water in biological fluids and tissues is discussed; it is pointed out that the various definitions of "free" and "bound" water do not necessarily coincide.

2. The "free" water fraction is defined as the weight of water in 1 g. of fluid or tissue which can dissolve substances added to it with a normal depression of vapour pressure: this is analogous to Gortner's definition, substituting "vapour pressure" for "freezing point."

3. To measure the "free" water of a fluid, a weighed quantity of some suitable substance is dissolved in a weighed quantity of the fluid, and the depression of vapour pressure measured, and compared with that caused by adding the same substance to an approximately isotonic salt solution.

4. To measure the "free" water of a portion of tissue, a weighed quantity of the latter is stirred for a sufficient time with a weighed amount of a hypertonic salt solution, and the change of vapour pressure measured.

5. The "free" water of blood, or of centrifuged corpuscles, is practically equal to the total water, being perhaps 2 p.c. less. In dealing with the equilibria occurring in blood (or muscle), concentrations should be expressed, not in grammes (or mols) *per litre*, but in grammes (or mols) *per* 1000 *g. of "free" water.* The osmotic pressure of blood is exactly accounted for by supposing all the known soluble constituents of blood to be freely dissolved in the "free" water.

6. The "free" water of casein solutions, or of concentrated egg white, is almost exactly equal to the total water.

7. The "free" water fraction of frog's muscle, whether resting or in rigor,

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is about 0·77, or perhaps a little greater, the total water fraction being 0·80 or 0·81. Very little, if any, of the water of muscle is "bound."

8. The contrary conclusion, based upon Overton's experiments, is due to a variety of factors : (*a*) progressive changes caused by prolonged immersion in salt solutions ; (*b*) the slowness of reaching diffusion equilibrium ; and (*c*) the loss of semi-permeability in a considerable fraction of the fibres, as the result of removal from a normal environment.

9. The osmotic behaviour of muscles in hypo- or hypertonic salt solutions is considered.

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