

CALCULATION OF INTRACELLULAR pH FROM THE DISTRIBUTION
OF 5,5-DIMETHYL-2,4-OXAZOLIDINEDIONE (DMO). APPLICA-
TION TO SKELETAL MUSCLE OF THE DOG *

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Estimations of hydrogen ion activities in the interior of cells have been attempted through a number of different experimental approaches. The methods that have been employed are of several general types: measurements on preparations of broken cells, measurements on fluid withdrawn from cells, observations with visible indicators, measurements with micro-electrodes introduced into cells, measurements of the intracellular and extracellular concentrations of weak organic acids and bases, and calculation of the balance of ionic charges. The last named method has been proposed by Conway (1). The other methods are discussed in the review of Caldwell (2), which contains a tabulation of the results of measurements on various types of cells with different experimental methods. Some of the methods that have been used are of quite limited applicability, and most of the values arrived at by any of the methods are subject to question.

Potentially, the method utilizing the distribution of weak organic acids and bases has a wider range of applicability than the other methods, and it is not subject to some of the theoretical objections that can be raised to other methods. There is a great deal of evidence that membranes of animal and plant cells in general are characterized by free permeability to the undissociated forms of weak organic acids and bases and by impermeability or apparent impermeability to the ionic forms. If the pH of the interior of a cell differs from that of the external medium in contact with it, there will be a corresponding difference in the concentration of an organic ion. The assumption

that the undissociated form of an acid or base is in equal concentration on the two sides of the cellular membrane permits the calculation of intracellular pH from measurements of extracellular pH and the extracellular and intracellular concentrations of the acid or base. In most of the work that has hitherto been done based on this principle, carbon dioxide has been the compound used as the indicator. It is assumed that the cellular membrane is permeable to carbon dioxide and that the tension of carbon dioxide inside the cell is equal to that measured in an external phase in equilibrium with the cell. The total carbon dioxide released from the cells after addition of acid is determined and is assumed to come from dissolved carbon dioxide, carbonic acid and bicarbonate ion. These data are sufficient for calculation of the intracellular concentration of bicarbonate ion and of the intracellular pH. The pK_1' of carbonic acid is such that its ionization should furnish a sensitive measure of intracellular pH. However, the validity of pH measurements made with the carbon dioxide method has been questioned. Conway and Fearon (3) concluded from their experiments that a large part of the carbon dioxide released from muscle cells by acid is derived from acid-labile material other than bicarbonate. If this should be so, the pH values calculated from the assumption that all acid-labile carbon dioxide comes from bicarbonate would be seriously in error.

In the course of a study of the tissue distribution of 5,5-dimethyl-2,4-oxazolidinedione ("DMO"), the product of metabolic N-demethylation of the antiepileptic agent, trimethadione (Tridione®) (4), we have found that the attributes desirable in a compound to be used for the measurement of intracellular pH are combined to an almost ideal degree in DMO. These properties will be dis-

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cussed in detail below. The advantages of DMO over carbon dioxide are in the facility of analytical determination and in the absence of any question as to the chemical identity of the material determined by the analytical procedure. The present report comprises a theoretical consideration of the estimation of intracellular pH from the distribution of acids and bases and an application of the DMO method to the estimation of intracellular pH of skeletal muscle of the dog under various conditions. We have been particularly interested in muscle because other methods of estimating intracellular pH have been applied more extensively to muscle than to other mammalian tissues, and because changes of pH in muscle are of great importance in buffering on account of the large bulk of the tissue. With muscle, unlike some other tissues, it is possible to take several successive samples from a single animal during the course of an experiment without disturbing the conditions. Furthermore, there is evidence that the chloride of muscle is largely extracellular (5), and determination of the chloride space affords a method of measuring extracellular water that is convenient and probably sufficiently accurate for this purpose. Extracellular water of some other tissues cannot be estimated with as great ease or certainty.

THEORETICAL CONSIDERATIONS

Mathematical formulations. We shall consider a tissue containing intracellular water of volume V_i and extracellular water of volume V_e , the intracellular phase being contained within membranes freely permeable to the undissociated form of a weak organic acid or base (to be called the "indicator compound"). V_e can be derived from the distribution of a substance confined to extracellular water, and V_i from the difference between V_e and total tissue water. Neither the intracellular nor the extracellular tissue phase is individually accessible for analysis, but the concentration, C_t , of the indicator compound in the water of the entire tissue can be determined. By determinations in plasma with corrections for the Donnan equilibria, the pH and the concentration of the indicator compound in the extracellular tissue water (pH_e and C_e , respectively) can be derived. It is assumed that the aqueous phases are homogeneous and that the indicator compound is fully equilibrated, the concentration of the undissociated form being equal in intracellular water, extracellular tissue water and plasma water. If this equality of concentration of the undissociated form is maintained regardless of pH differences that may exist between the two sides of the cellular membrane, it is im-

material to the theory through what mechanism the pH gradient is brought about or whether the membrane is considered to be truly impermeable to the ionic form of the indicator compound. The apparent ionization constant and the activity coefficient of the ion are assumed to be equal in all the aqueous phases. Then, if the indicator compound is a weak organic acid with apparent ionization exponent, pK' , the pH of the intracellular water, pH_i , can be expressed in terms of measurable quantities or values mathematically derived from measurable quantities:

$$pH_i = pK' + \log \left\{ \left[\frac{C_t}{C_e} \left(1 + \frac{V_e}{V_i} \right) - \frac{V_e}{V_i} \right] \times [10^{(pH_e - pK')} + 1] - 1 \right\}. \quad 1)$$

If the indicator compound is a weak organic base with apparent acid ionization exponent, pK'_a ,

$$pH_i = pK'_a - \log \left\{ \left[\frac{C_t}{C_e} \left(1 + \frac{V_e}{V_i} \right) - \frac{V_e}{V_i} \right] \times [10^{(pK'_a - pH_e)} + 1] - 1 \right\}. \quad 2)$$

The partial derivative of the ratio (C_t/C_e) with respect to pH_i is an index of the precision with which pH_i can be calculated from measured concentrations of the indicator compound. For an acid,

$$\frac{\partial(C_t/C_e)}{\partial(pH_i)} = \ln 10 \left(\frac{V_i}{V_i + V_e} \right) \left(\frac{10^{pH_i}}{10^{pH_e} + 10^{pK'}} \right). \quad 3)$$

For a base,

$$\frac{\partial(C_t/C_e)}{\partial(pH_i)} = - \ln 10 \left(\frac{V_i}{V_i + V_e} \right) \left(\frac{10^{-pH_i}}{10^{-pH_e} + 10^{-pK'_a}} \right). \quad 4)$$

These partial derivatives as functions of the ionization exponents of acids and bases are shown in Figures 1 and 2, respectively. In these figures, pH_e is assumed to be 7.4 and pH_i 7.0 (approximately the pH_i of muscle as found in this study). The functions are without maxima, increasing to approach asymptotic limits with decreasing values of pK' for acids and increasing values of pK'_a for bases. If pH_i is less than pH_e , as is assumed in these figures, the limiting value is greater for a base than for an acid. The stronger an acid or a base, the more sensitive an indicator it is of intracellular pH.¹ If intracellular pH is less than plasma pH, a base of sufficient strength can be a more sensitive indicator than any acid.

For an acid of the strength of DMO and for the values of pH_i , pH_e , V_i , and V_e assumed in Figures 1 and 2, an error of 0.01 unit in the calculated value of pH_i would be introduced by an error of 1.4 per cent in the ratio (C_t/C_e) or by an error of 7.7 per cent in V_e . If the ionic strength of intracellular water should differ from that of extracellular water by as much as 0.05, the pK'

¹ This theory is not applicable to compounds that are completely dissociated such as sulfonic acids and quaternary nitrogen compounds.

values in the two phases as predicted by the Debye-Hückel theory would differ by as much as 0.03 unit, and an error of that magnitude would be introduced in the calculated value of pH_i .

The assumption made above that all aqueous phases are homogeneous is undoubtedly not altogether valid, particularly with respect to the intracellular water. If the cell interior is nonuniform with respect to pH , or if the cells in the mass of tissue analyzed differ among themselves, the value calculated for intracellular pH is not a mean value in the mathematical sense. It has been termed an "aggregate" pH by Irving, Foster and Ferguson (6) and an "overall" pH by Conway and Downey (7). Despite the fact that the calculated intracellular pH may not have a simple mathematical significance, it is a value intermediate between the values for the regions of highest and lowest pH in the intracellular water of the tissue and thus furnishes some information, however limited, concerning the conditions in the cells. Of even greater interest than the calculated pH for the normal cell are the changes that this value undergoes under physiological and pathological conditions, for these changes can be interpreted in terms of the buffering capacity of cells and the gain or loss of hydrogen ions by the cells.

Properties to be desired in a compound to be used for measurement of intracellular pH in animal tissues. To be an adequately sensitive indicator of intracellular pH , the compound must be a sufficiently strong acid or base. An acid with pK' less than about 7.0 or a base with pK'_a greater than about 7.0 will be satisfactory for the physiological values assumed in Figures 1 and 2. In concentrations that are analytically determinable the compound should produce no pronounced pharmacological effects,

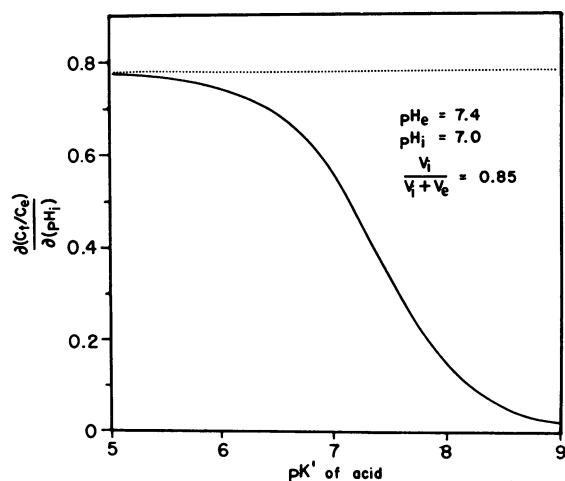


FIG. 1. THE PARTIAL DERIVATIVE OF THE RATIO OF CONCENTRATION IN TOTAL TISSUE WATER (C_T) TO CONCENTRATION IN EXTRACELLULAR TISSUE WATER (C_E) WITH RESPECT TO INTRACELLULAR pH (pH_i) AS A FUNCTION OF THE APPARENT IONIZATION EXPONENT FOR WEAK ORGANIC ACIDS

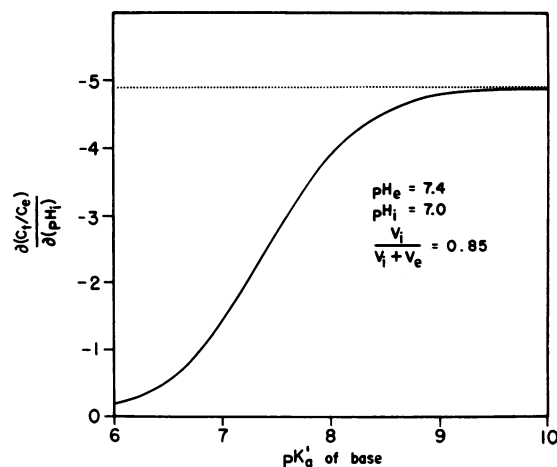


FIG. 2. THE PARTIAL DERIVATIVE OF THE RATIO OF CONCENTRATION IN TOTAL TISSUE WATER (C_T) TO CONCENTRATION IN EXTRACELLULAR TISSUE WATER (C_E) WITH RESPECT TO INTRACELLULAR pH (pH_i) AS A FUNCTION OF THE APPARENT ACID IONIZATION EXPONENT FOR WEAK ORGANIC BASES

toxic or otherwise, and should not of itself affect acid-base equilibria inside or outside of cells. The compound should not interact with plasma proteins or with any constituents of the cell to a degree that would significantly affect its distribution. It should not be localized to a significant extent in nonaqueous phases of cells or plasma. Although not essential, it is desirable that the compound be slowly eliminated so that after a single dose the plasma concentration will change very little during the period required for equilibration of the compound between plasma and tissues. Finally, an analytical method must be available that is specific and sufficiently sensitive for concentrations that have no disturbing effect.

Properties of DMO. The compound is an acid with pK' of 6.13 at 37° C. and ionic strength of 0.16.² As shown in Figure 1, this value of pK' gives almost as sensitive an indication of intracellular pH as is theoretically possible for an acid. We have used plasma concentrations of DMO no higher than 250 $\mu g.$ per ml. in dogs for measurement of muscle pH . There are no

² The pK' of DMO has not been measured in protein solutions. The pK' of carbonic acid, which is nearly equal to that of DMO, is not affected to a great extent by the presence of protein in solutions of egg albumin, in muscle homogenates, or in erythrocyte hemolysates (8, 9). The assumption that the pK' of DMO is equal in intracellular water, interstitial fluid, and plasma probably does not involve a serious error in the estimation of intracellular pH . The error in the calculated value of pH_i contributed by an error in the value of pK' for DMO in intracellular water is equal to the latter error and is constant. The magnitude of a change in pH_i occurring under experimental conditions would be measured without error.

grossly observable pharmacological effects at this level, while the concentrations in plasma and muscle are high enough to permit analytical measurements of sufficient accuracy. Patients receiving trimethadione may have plasma concentrations of DMO as high as 1 mg. per ml. without any evidence of toxic effect. DMO is not bound to plasma albumin (10). As reported in this paper, DMO is not bound to an homogenate of dog muscle to a measurable degree. The concentration of DMO in fat is very low relative to that in plasma, as evidenced by the finding that the apparent volume of distribution of DMO in omental fat of the dog is nearly equal to the chloride space and to the total volume of water of the tissue. It is unlikely that DMO is contained in lipid phases in the cell in proportions of any importance. There is no reason, then, to believe that the distribution of DMO is significantly affected by any interactions or by any localizations in nonaqueous phases that would invalidate the calculation of intracellular pH. DMO is among the most persistent drugs that have been studied. It is not metabolized, and excretion is very slow in acid urine (10). In the normal dog after equilibration, the hourly decline in plasma concentration is only about 1 to 2 per cent. For measurements of tissue pH, a single dose of DMO can be administered to an animal, and the equilibration of tissues with plasma is not complicated by a rapidly falling plasma concentration. DMO is determinable in plasma and tissues by ultraviolet spectrophotometric methods of adequate sensitivity and of demonstrated specificity.

METHODS AND MATERIALS

Synthesis of DMO. Ethyl α -hydroxyisobutyrate was condensed with urea in the presence of sodium ethylate by the method of Stoughton (11). DMO melts at 78 to 79° C.

Experiments on dogs. In some early studies of the pH of tissues under normal conditions, dogs were anesthetized with ether or were killed by intravenous injection of air without anesthesia. In all of the experiments to be described in this report in which the effects of acidosis and alkalosis were studied, the dogs were anesthetized with 125 mg. per Kg. of phenobarbital administered intravenously as a solution of the sodium salt. DMO was given intravenously in saline solution in a dose of 100 mg. per Kg. at least an hour before the first muscle sample was taken. This time has been found to be more than sufficient for equilibration of DMO between plasma and muscle. Both renal pedicles were ligated tightly so as to occlude the renal vessels and the ureters. CO₂-O₂ mixtures were administered with an anesthetic gas machine. Overventilation was produced with a respiration pump. HCl was administered by slow intravenous infusion of a 0.30 N solution. NaHCO₃ was given intravenously in 0.30 or 0.95 M solution. Samples were taken from the muscles of the thighs. The muscle samples were blotted with cotton gauze, and all visible fat and connective tissue were trimmed away. Blood samples were taken from the femoral veins, about 3 mg. of potassium oxalate per ml. of blood being used as anticoagulant. Blood used

for determination of pH and CO₂ was handled with strict anaerobic precautions.

Total water of muscle and plasma was determined by drying weighed samples in an oven at 120° C. for at least 12 hours. There was found to be so little variation in the water content of plasma that this determination was omitted for some samples, and the value of 0.93 Gm. per Gm. was used in the calculations.

Determination of DMO. For plasma from unanesthetized dogs or dogs anesthetized with ether, the method of Butler (12) was used. For plasma from dogs anesthetized with phenobarbital the method was modified in that the extract in the buffer of pH 9.0 was divided into two parts and the subsequent procedures were carried out as described below for the method for muscle. For muscle, a sample of 5 to 10 Gm. was homogenized with 10 ml. of distilled water with the homogenizer described in a previous study (13). A weighed sample of 1 to 3 Gm. of the homogenate was transferred to a glass stoppered tube, in which it was shaken with 4 ml. of 5 M NaH₂PO₄ and 25 ml. of redistilled reagent grade absolute ethyl ether previously saturated with water. After centrifugation, 20 ml. of the ether phase was removed and extracted with 5 ml. of the borate buffer of pH 9.0 as in the original plasma method. Of the buffer extract, 2 ml. portions were transferred to each of two tubes. To one was added 2 ml. of the buffer of pH 9.0 and to the other 2 ml. of 0.1 N HCl. Absorbancies of both preparations were measured at 215 and 220 m μ , the blank for the first being the buffer, and for the second, equal volumes of buffer and 0.1 N HCl. The absorbancies at pH 9.0 were corrected by subtracting the corresponding absorbancies in acid solution. The concentration of DMO was calculated from the difference between the corrected absorbancy at 215 m μ and that at 220 m μ . When this method was applied to muscle from dogs that had had no drug, the calculated concentrations of DMO were between 1 and 2 μ g. per Gm. When DMO was added to normal muscle in concentrations of 50 to 100 μ g. per Gm., the recoveries were 96 to 104 per cent. Since the blank values are low relative to the concentrations to be determined, and since DMO is not metabolically altered in the body, the material determined as DMO in muscle can be considered to consist almost entirely of that compound. Ether and phenobarbital in anesthetic concentrations do not interfere with this method for muscle.

Determination of chloride. For plasma the method of Van Slyke and Hiller (14) was used. For muscle a sample of about 15 Gm. of the same homogenate prepared for the DMO determination was weighed into a glass stoppered graduated centrifuge tube. To the tube were added 5 ml. of a solution containing 150 Gm. of Na₂WO₄·2H₂O and 250 ml. of concentrated H₃PO₄ per L., about 0.5 Gm. of AgIO₃, and sufficient distilled water to make the total volume to the nearest multiple of 5 ml. The tube was shaken for 1 hr. and centrifuged. The supernatant liquid was filtered through filter paper, and a measured volume of the filtrate was titrated with thiosulfate as in the method for plasma.

Determination of plasma CO₂. Blood was centrifuged under mineral oil and the plasma CO₂ was determined by the method of Van Slyke and Neill (15).

Measurement of pH. The pH of blood was measured at 37° C. by the procedure described in another report (13).

Calculations. It was assumed that Donnan equilibria prevail between plasma and extracellular tissue water for the hydrogen ion, the chloride ion, and the DMO ion. The pH of the extracellular tissue water, pH_e, was assumed to be 0.02 unit higher than that of plasma and the concentrations of chloride and DMO ions in extracellular tissue water to be 1.05 times the corresponding concentrations in plasma water.³ On the assumption that all chloride in muscle is extracellular, the volume of extracellular water, V_e, as a proportion of total tissue weight was calculated by dividing the chloride concentration in tissue by the concentration in extracellular tissue water. The volume of intracellular water, V_i, as a proportion of total tissue weight was derived by subtracting V_e from the total proportion of water in the tissue. The concentration of DMO in extracellular tissue water, C_e, was calculated from the assumption that the concentration of the ion differs from that in plasma water by the Donnan ratio and that the concentration of undissociated form is the same in the water of the two phases. The CO₂ tension of plasma was calculated from the total CO₂ content of plasma and the pH of blood by use of the Henderson-Hasselbalch equation, the pK' of carbonic acid being taken as 6.10 and the solubility coefficient, α , of CO₂ as 0.53.

Binding of DMO to muscle homogenate. A sample of 4 Gm. of dog muscle was homogenized with 15 ml. of 0.2 M phosphate buffer of pH 7.0. With the procedure described in an earlier report (13), this homogenate was dialyzed in a cellophane bag against a large volume of the buffer containing 100 μ g. per ml. of DMO. The contents of the bag and of the outer chamber were analyzed for DMO by the method described above for muscle.

RESULTS

Binding of DMO to muscle homogenate

When the homogenate of dog muscle described under Methods was dialyzed at 37° C. against a buffer of pH 7.0 containing 100 μ g. per ml. of DMO, there was no measurable binding of DMO to the homogenate.

³ The value of the Donnan ratio varies with the pH of plasma as well as with the composition of plasma with respect to protein and inorganic ions. The value used here is a theoretical value calculated for protein-free extracellular tissue water equilibrated with plasma containing 160 mM per L. of anions other than protein and 17 mM per L. of base bound to protein. Experimentally determined ratios for different ions in different plasma

Intracellular pH of dog muscle

In 11 dogs that had received no treatment designed to disturb acid-base equilibria, of which seven had been anesthetized with 125 mg. per Kg. of phenobarbital, two anesthetized with ether, and two killed by air embolism, the calculated intracellular pH values for muscle were all in the range of 6.96 to 7.10 with an average of 7.04. This total range is only of the order of magnitude of perhaps twice the expected experimental error, and no effect of anesthesia on pH was apparent.

The effects of various treatments altering the pH or the pCO₂ of plasma were studied. In the experiments described below, two different treatments were applied successively to each dog. The combinations of treatments were chosen with the purpose of furnishing controlled comparisons of changes in pH and pCO₂ under various conditions. Table I shows the analytical data and derived values from which the intracellular pH is calculated. In addition to the experiments described in Table I, there were eight other satisfactory experiments in which at least one of these same treatments was studied in each dog. In all of these experiments, the effects of the treatments were essentially the same as in the corresponding experiments of Table I.

In Experiment 1 of Table I a dog inhaled 30 per cent carbon dioxide, and sodium bicarbonate was later administered intravenously while the carbon dioxide inhalation was continued. As the pCO₂ of venous plasma rose, the pH of venous blood fell by 0.43 unit and the calculated intracellular pH of muscle fell by 0.28 unit. Administration of 20 mM per Kg. of sodium bicarbonate restored the blood pH to its original value, but the plasma pCO₂ was increased somewhat and the muscle pH was further lowered to 6.61.

In Experiment 2 the same two treatments were given in reverse order. An intravenous dose of 10 mM per Kg. of sodium bicarbonate raised the pH of blood by 0.20 unit and the intracellular pH of muscle by 0.11 unit without significant effect

ultrafiltrates and transudates sometimes deviate significantly from theoretical values (5). There is no direct knowledge of the actual values of the ratios for the hydrogen, chloride, and DMO ions in interstitial fluid of muscle. The error in the estimation of the Donnan ratio probably does not introduce any large error in the calculation of intracellular pH.

TABLE I

Effects of various procedures altering acid-base equilibria in dogs. Analytical data and derived values used in the calculation of intracellular pH of muscle

Time	Measured Values								Calculated Values						
	Muscle				Plasma				C _t	C _e	V _e	V _i	pH _e	pCO ₂	pH _i
	DMO	Cl ⁻	H ₂ O	DMO	Cl ⁻	CO ₂	pH	μg/Gm.							
Min.	μg/Gm.	mEq/Kg.	Gm./Gm.	μg/Gm.	mEq/Kg.	mM/L.		μg/Gm.	μg/Gm.				mm.Hg		
Experiment 1. Female dog, 4 Kg.															
0	99	24.7	0.75	192	114	25.8	7.35	132	217	0.19	0.56	7.37	44	7.02	
50-210	Inhalation of 30 % CO ₂ .														
120	109	24.6	0.76	168	122	30.0	6.92	143	190	0.18	0.58	6.94	126	6.74	
135-185	Intravenous infusion of NaHCO ₃ , 20 mM/Kg. (88 ml. of 0.95 M)														
205	82	19.8	0.76	226	87	92.5	7.35	108	255	0.20	0.56	7.37	157	6.61	
Experiment 2. Male dog, 15 Kg.															
0	94	13.1	0.76	190	111	23.6	7.34	124	215	0.10	0.66	7.36	41	7.04	
30-60	Intravenous infusion of NaHCO ₃ , 10 mM/Kg. (145 ml. of 0.95 M)														
200	83	11.6	0.75	202	104	38.5	7.54	111	228	0.10	0.65	7.56	43	7.15	
255-375	Inhalation of 34 % CO ₂ .														
370	75	11.4	0.76	192	105	36.2	7.32	99	217	0.10	0.66	7.34	66	6.89	
375-425	Inhalation of 50 % CO ₂ .														
420	83	12.6	0.75	185	100	39.8	7.10	111	209	0.11	0.64	7.12	116	6.71	
Experiment 3. Female dog, 9 Kg.															
0	94	13.5	0.76	184	112	24.0	7.35	124	208	0.11	0.65	7.37	41	7.07	
30-290	Overventilation														
110	91	20.4	0.76	204	114	17.0	7.56	120	230	0.16	0.60	7.58	18	7.15	
140-225	Intravenous infusion of HCl, 6 mM/Kg. (182 ml. of 0.3 N)														
285	81	14.7	0.77	155	129	9.4	7.30	105	175	0.10	0.67	7.32	18	7.02	
Experiment 4. Male dog, 16 Kg.															
0	90	13.1	0.75	190	112	20.9	7.35	120	215	0.10	0.65	7.37	36	7.03	
30-240	Intravenous infusion of HCl, 7 mM/Kg. (372 ml. of 0.3 N)														
300	99	17.9	0.75	141	122	12.7	6.99	132	159	0.13	0.62	7.01	46	6.90	
315-355	Intravenous infusion of NaHCO ₃ , 3.75 mM/Kg. (200 ml. of 0.3 M)														
425	92	16.6	0.74	168	116	14.9	7.19	124	190	0.13	0.61	7.21	36	6.95	
455-475	Intravenous infusion of NaHCO ₃ , 3.25 mM/Kg. (172 ml. of 0.3 M)														
555	81	15.1	0.75	166	117	14.2	7.25	108	188	0.11	0.64	7.27	30	6.94	

on pCO₂ of plasma. Inhalation of 34 per cent carbon dioxide lowered the blood pH to normal while raising the plasma pCO₂ and lowering the pH of muscle. Further increase in the inhaled concentration of carbon dioxide to 50 per cent caused additional changes in the same direction, the muscle pH finally reaching a value of 6.71.

In Experiment 3 the pCO₂ of plasma was lowered by overventilation with a respiration pump. Intracellular pH of muscle rose by 0.08 unit as the pCO₂ of plasma fell and the pH of blood rose. Subsequently, while overventilation was continued, 6 mM per Kg. of hydrochloric acid was infused

intravenously. The muscle pH as well as the blood pH fell somewhat below their initial levels without significant change in the plasma pCO₂.

In Experiment 4 the initial treatment was an intravenous infusion of 7 mM per Kg. of hydrochloric acid. There was only a slight rise of plasma pCO₂, which may have been due to depression of respiration consequent to deepening of phenobarbital anesthesia in acidosis (13). While the blood pH fell by 0.36 unit, the intracellular muscle pH fell by only 0.13 unit. After the injection of an equivalent amount of sodium bicarbonate, the blood pH rose by 0.26 unit while

the muscle pH rose by only 0.04 unit, an amount that probably does not exceed the experimental error. In two other dogs in which acidosis was produced by intravenous infusion of 10 mM per Kg. of hydrochloric acid, the intracellular pH of muscle did not fall below 6.90 even though the blood pH in one dog fell to 6.84.

DISCUSSION

In these experiments the largest changes in the intracellular pH of muscle are those brought about by increases in the carbon dioxide tension of blood. In severe metabolic acidosis with normal carbon dioxide tension the intracellular pH of muscle is not lowered to the extent that it is in conditions of elevated carbon dioxide tension. If the carbon dioxide tension of plasma is abnormally high, the pH of muscle is abnormally low even though the pH of blood may be normal. Both metabolic and respiratory alkalosis may cause some increase in intracellular pH of muscle, but the increases that can be produced by any treatment are small by comparison with the decreases resulting from increased tension of carbon dioxide.

The effect of carbon dioxide tension on the intracellular pH of muscle is to be expected if the muscle fibers are freely permeable to carbon dioxide, as cellular membranes in general have been found to be. That greater effects on the pH of muscle can be produced by raising the carbon dioxide tension above normal than by lowering it below normal is in accordance with the form of the carbon dioxide dissociation curve of muscle as described by Irving and co-workers (6). The study of Swan and Pitts (16) indicates that hydrochloric acid administered intravenously to nephrectomized dogs is neutralized in part by loss of intracellular sodium and potassium ions from tissues in exchange for hydrogen ions from extracellular water. Experiments of the same type carried out by Schwartz, Ørning and Porter (17) in dogs with intact kidneys led to similar conclusions. Tobin (18) found that intravenous hydrochloric acid in nephrectomized cats caused a shift of sodium and potassium into the extracellular space and a loss of sodium and potassium from muscle. However, in Tobin's experiments the average values of intracellular muscle pH as measured by the carbon dioxide method did not change after cats

had received doses of 3.5 to 9.6 mM per Kg. of hydrochloric acid. Wallace and Hastings (19) did not find significant changes of muscle pH as measured by the carbon dioxide method after intravenous doses of 1 mM per Kg. of hydrochloric acid to cats. In our experiments in dogs the intracellular pH of muscle was significantly lowered by intravenous administration of hydrochloric acid, but the fall of muscle pH was no more than about 0.15 unit even in severe metabolic acidosis. The ionic exchanges that have been postulated to occur in metabolic acidosis apparently can operate in muscle only to the extent that intracellular buffering is sufficient to prevent any large change of intracellular pH. Studies of the intravenous administration of sodium bicarbonate to human subjects by Singer and associates (20) and to nephrectomized dogs by Swan, Axelrod, Seip and Pitts (21) have been interpreted as indicating the disappearance of about a quarter of the injected sodium from the extracellular space. One possible mechanism that would produce this effect would be the entry of sodium ions into cells either accompanied by bicarbonate ions or in exchange for hydrogen ions. Either process would tend to raise the intracellular pH. Wallace and Hastings (19) found no significant elevation of intracellular pH of muscle of cats in response to sodium bicarbonate injection. In our experiments the effects of sodium bicarbonate administration on muscle pH were quite small. The extent to which sodium ions may pass into muscle cells during the period of these acute experiments cannot be estimated from the present data. Intracellular buffering and possibly other mechanisms such as the intracellular production of organic acids may suffice to counteract the effect of entry of sodium to the extent that any change of pH may be so small as even to be difficult to detect. If the administration of sodium bicarbonate is accompanied by an increase in the carbon dioxide tension of blood, the result may actually be a decrease in the intracellular pH of muscle.

The values for intracellular pH of frog and mammalian muscle under normal conditions as measured by other workers with the carbon dioxide method have for the most part been in the range of 6.9 to 7.3. References to the earlier reports of the use of the carbon dioxide method are to be found in the review of Caldwell (2). Ad-

ditional recent experiments in which the carbon dioxide method was used for measurement of the pH of mammalian muscle are those of Tobin (18) with cats and Nichols (22) with rats. Reports of the relationship between carbon dioxide tension of blood and intracellular pH of mammalian muscle as measured by the carbon dioxide method (6, 19, 22) are in general accord with the results of our present experiments with the DMO method. While our experiments showed some small effect of blood pH on muscle pH aside from changes of $p\text{CO}_2$ and those of Wallace and Hastings (19) and of Tobin (18) with the carbon dioxide method failed to do so, the results from the use of both methods are all indicative that muscle pH is not influenced to any great extent by blood pH *per se*. Although there has been no direct, simultaneous comparison of the two methods, it may be concluded that there is no conspicuous discrepancy between the results of the carbon dioxide method and those of the DMO method either as to the value for intracellular pH of normal muscle or as to the manner in which this value is affected by changes of blood chemistry.

The principal critic of the carbon dioxide method has been Conway (1, 3). Conway and Fearon (3) found that in alkaline extracts of muscle a large part of the acid-labile carbon dioxide is not precipitable by barium chloride. From their experiments they concluded that much of the acid-labile carbon dioxide of muscle is not bicarbonate. If the barium-soluble fraction is considered not to have come from bicarbonate in muscle, a much lower value for the calculated intracellular pH is obtained. The value so calculated is near 6.0, approximately that to be expected if the hydrogen ion ratio across the cellular membrane were governed by a Donnan equilibrium. This is also approximately the pH value estimated by Conway (1) from a balance of the ionic charges in muscle fibers. Conway concedes that the data from which this latter calculation must be made can at present only be considered approximate.

Hill (23) has argued that a Donnan equilibrium for hydrogen ions in muscle cannot be reconciled with his experiments with frog muscle. Muscles remained excitable in media of pH values such that the operation of a Donnan equilibrium would have resulted in intracellular pH values too low to be compatible with contractility. Conway (1) has

sought to refute Hill's argument in terms of the long time required for attainment of equilibrium.

In the determination by Danielson, Chu and Hastings (8) of the pK' of carbonic acid in muscle brei it was found that the calculated pK' values did not differ significantly among themselves in experiments in which the protein concentration and the pH varied over considerable ranges. These results could not have been obtained if any large part of the acid-labile carbon dioxide had been derived from a source other than bicarbonate. However, in these experiments the ground muscle had been diluted, sodium hydroxide and sodium bicarbonate had been added, and equilibration had been carried out with various tensions of carbon dioxide. Of the total carbon dioxide released by acid from the final preparations, only a small part had originally been in the muscle. An interfering substance that might constitute an important proportion of the carbon dioxide of the muscle itself might be in such small proportion in the final preparation that the effect of its presence might not exceed the experimental error. The experiments of Danielson and co-workers (8) are not, accordingly, conclusive evidence of the absence of a significant amount of acid-labile carbon dioxide other than bicarbonate in muscle.

There is no question of the chemical identity of the substance determined analytically in the DMO method. The fact that pH values calculated from the distribution of DMO and of carbon dioxide are in the same range supports confidence that these values are correct rather than the much lower values advocated by Conway. Whatever the chemical nature may be of the barium-soluble material in the alkaline extracts of Conway and Fearon, it appears likely that essentially all of the acid-labile carbon dioxide in the muscle cell itself is bicarbonate and that the pH values calculated on this assumption are correct.

The greater the number of indicator compounds found to give concordant results, the greater the confidence that can be placed in the values of intracellular pH calculated from the distribution of acids and bases. If unrecognized factors influence the distribution of one compound so as to invalidate the calculation of pH, it is unlikely that the same factors will be in operation with a compound of entirely different chemical structure. Studies of a number of indicator compounds of

diverse structures would therefore be of value. Bases would be of particular interest because they could furnish a more sensitive indication of pH than acids when used for tissues with intracellular pH values below the plasma pH. Comparison of bases with acids would also be of interest because the errors arising from differences in pK' values between intracellular and extracellular water due to differences in ionic strength would be in opposite direction for acids and bases. The only base the distribution of which has hitherto been used for calculation of intracellular pH is ammonia, which has the serious drawbacks of high toxicity and difficulty of analytical determination. The special requirements for a compound to be used as an indicator of intracellular pH exclude the great majority of known acids and bases, but a systematic search might disclose other appropriate compounds.

DMO has advantages over carbon dioxide for the measurement of intracellular pH, and at the present time it appears to have almost ideal properties for this purpose. Although this initial study has been limited to muscle, there is no reason why the method could not be applied to any tissue in which the determination of extracellular water is feasible. The analytical method described here for DMO in muscle has been shown to be suitable also for liver and brain. Since DMO is a foreign compound that is not attacked metabolically, an application is possible that would not be possible with carbon dioxide, *viz.*, the determination of an overall value for the intracellular pH of the tissues of the body as a whole. This could be carried out in human subjects since all that would be entailed would be the determination of DMO in plasma and urine after administration of a measured dose, together with simultaneous determinations of total body water and extracellular body water by means of other appropriate substances. The fact that DMO is not metabolized would also make possible the use of the isotopically labeled compound to study gradients of pH within small regions of a tissue or even within individual cells.

SUMMARY

The theory of estimation of intracellular pH by means of measurement of the distribution of weak organic acids and bases is discussed. 5,5-Di-

methyl-2,4-oxazolidinedione (DMO) is shown to have properties almost ideal for this purpose.

The distribution of DMO has been used to measure the intracellular pH of skeletal muscle of the dog under various conditions. The intracellular pH of normal, resting muscle averaged 7.04. The greatest changes of pH of muscle are those produced by raising the carbon dioxide tension of blood. The pH of muscle is lowered by a high tension of carbon dioxide whether or not the blood pH is lowered. Metabolic acidosis has less effect in lowering muscle pH than does high tension of carbon dioxide. Respiratory and metabolic alkalosis raise muscle pH only to a small extent.

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