Acid-Labile Carbon Dioxide in Muscle: Its Nature and Relationship to Intracellular pH.* (32252)

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Conway and Fearon(1) reported that less than half of the acid-labile CO_2 in alkaline extracts of mammalian muscle could be removed by precipitation with $BaCl_2$. The fact that carbonate from an external source added to the alkaline extract was quantitatively precipitated was adduced as evidence that $BaCO_3$ was not held in fine suspension or protected from precipitation. The interpretation was that the "barium-soluble" fraction of CO_2 was not present in the alkaline extract as carbonate or originally present in muscle as bicarbonate or free CO_2. By subtraction of the barium-soluble fraction, Conway and Fearon calculated values for intracellular bicarbonate concentrations, which they used to support the contention that the muscle membrane is permeable to potassium, hydrogen, chloride, and bicarbonate ions and that the ratios of these ions conform to a Donnan relationship. The intracellular pH of muscle so calculated is 6.0, about one full pH unit lower than values calculated from the distribution of CO_2 without the subtraction of the barium-soluble fraction or than most values arrived at by other methods of measurement. For a review of values reported prior to 1956, see(2). Because of the importance of this question in relation to intracellular pH and because no satisfactory suggestion as to the chemical identity of the barium-soluble CO_2 fraction has been offered, the present investigation was undertaken.

Methods and results. Extracts of rat muscle were prepared essentially in the manner described by Conway and Fearon. The extracts were carried through all procedures in such a way as to minimize exposure to air. The method of Van Slyke and Neill(3) was used for determination of CO_2.

Rats were decapitated, and muscles of the thighs were removed as quickly as possible. The muscles were chopped with scissors into small pieces, which were dropped into CO_2-free 0.2 N KOH. For each gram of muscle, 2.5 ml of KOH solution was used. The preparation was kept at 5°C for 1 hour with occasional shaking, after which it was centrifuged at 2500 g for 20 minutes. The supernatant, which will be referred to as the "extract", was withdrawn for further experiments. All CO_2 concentrations to be reported below are corrected for the dilutions introduced in the successive experimental steps so as to refer to the concentrations in the muscle water (assumed to be 75% of the muscle weight). Values for CO_2 after barium precipitation are corrected for the solubility of $BaCO_3$ as determined after precipitation of carbonate from a 0.2 N KOH solution by a half volume of saturated $BaCl_2$.

The total CO_2 content of several extracts corresponded to 12 to 15 mM in muscle water, values in the range previously reported by other workers. When 0.5 ml of saturated $BaCl_2$ was added to each ml of extract and the mixture centrifuged for 90 minutes at 2500 g, the supernatant remained turbid. The concentration of CO_2 referred to muscle water was from half to two-thirds of that as determined from the original extract. Shaking for 24 hours at room temperature or letting stand at −20°C for 24 hours before centrifugation at 2500 *g* did not prevent turbidity in the supernatant or have much effect in reducing its CO_2 content. Centrifugation at 2500 g after heating for 1 hour at 100°C yielded a clear supernatant with a CO_2 content 70% that of a parallel preparation that had not been heated. In an extract originally containing 12.7 mM CO_2 referred to muscle water, barium precipitation followed by centrifugation at 2500 g for 30 minutes yielded a turbid supernatant in which the CO_2 content referred to muscle water was 8.3 mM.

* This investigation was supported by USPHS Research Grant GM 13606, from Nat. Inst. of General Med. Sciences, and by Research Career Program Award 5-K6-GM-19,429 to T. C. Butler from Nat. Inst. of General Med. Sciences.

Subsequent centrifugation at 100,000 g for 30 minutes yielded a clear supernatant having a CO_2 content 70% of that after the first centrifugation.

Several experiments were performed in which the muscle extract was acidified, freed of CO_2 by evacuation, and realkalinized. Bicarbonate was then added and precipitation with $BaCl_2$ carried out as before. In a typical experiment the original extract had a CO_2 content corresponding to 14.7 mM in muscle water. To 20 ml of the extract was added 0.4 ml of concentrated HCl. The acidified extract was evacuated in portions in the Van Slyke apparatus. To 19 ml of the evacuated extract was added 1.22 ml of 5 N KOH to restore the alkalinity to the original level. The small amount of carbonate introduced in the 5 N KOH gave the realkalinized extract a CO_2 content of 1.3 mM referred to muscle water. To one portion of the degassed and reconstituted extract was added 0.25 mg/ml and to another 0.50 mg/ml of $NaHCO_3$. The CO_2 contents referred to muscle water were measured as 15.5 and 27.3 mM, respectively. Saturated $BaCl_2$ was added in the same proportions as before. After centrifugation at 100,000 *g* for 30 minutes, the CO_2 contents of the clear supernatants after correction for $BaCO_3$ solubility were 3.5 and 5.5 mM respectively, referred to muscle water.

To 1, 2, 3, and 4% solutions of bovine serum albumin in 0.2 N KOH was added $NaHCO_3$ in a concentration of 15 mM. To a portion of each solution was added a half volume of saturated $BaCl_2$. After centrifugation for 1 hour at 100,000 *g*, the CO_2 contents of the supernatants (with corrections for dilution) were, respectively, 0.2, 0.5, 0.7, and 1.0 mM. When account is taken of the factor for dilution in the experiments with muscle extracts, the CO_2 retained in solution by 4% albumin is comparable to that retained by a muscle extract.

The amino acids glycine, arginine, aspartic acid, cysteine, histidine, lysine, and tryptophan in concentrations of 18 mM all failed to show any inhibitory effect on the precipitation of $BaCO_3$ from 16 mM solutions of carbonate in 0.2 N KOH.

Discussion. While it is clear that there is something in an alkaline extract of muscle that protects a limited amount of acid-labile CO_2 from precipitation in the presence of an excess of the barium ion, the chemical nature of the protective process remains unknown. That serum albumin will likewise inhibit the precipitation of a limited amount of carbonate suggests protein or a polypeptide in the muscle extract as the inhibitory agent. However, it is conceivable that some constituent of a different nature may be of more importance as inhibitor. Various mechanisms of inhibition of crystallization of $BaCO_3$ might be conceived. Combination of CO_2 with the inhibitory substance in some acid-labile chemical form other than the free carbonate ion could remove this fraction from reaction with the barium ion. It is well known that foreign substances in minute amounts can exert large effects on the processes of nucleation and crystal growth(4). It is conceivable that the inhibitory substance in the muscle extract could interact with the nuclei of $BaCO_3$ or be adsorbed on the surface or enter into the lattice of crystals of submicroscopic size to prevent further growth. Mechanisms of this sort have been postulated to account for the inhibitory action of sodium hexametaphosphate on the precipitation of $CaCO_3$(5) and the inhibitory action of a substance in urine on the precipitation of calcium phosphate(6). Whatever the chemical form of the barium-soluble CO_2 may be, the particle size is too small to be sedimented at 100,000 *g*.

Regardless of the chemical mechanism of protection from precipitation, the present experiments demonstrate that bicarbonate from an external source is in part protected from precipitation as $BaCO_3$ in an alkaline muscle extract. Our conclusions as to the nature of acid-labile CO_2 of muscle are accordingly quite contrary to those of Conway and Fearon. Our experiments leave no basis for belief that any significant part of the acid-labile CO_2 in the extract is derived from any compound existing in the living muscle other than bicarbonate, dissolved CO_2, and carbonic acid.

In fact there are good reasons to believe that the subtraction of the barium-soluble fraction in calculation of intracellular pH

of muscle leads to erroneously low values. One reason for this belief is the close agreement of the distribution between extracellular water and intracellular water of total acid-labile CO_2 and that of another weak acid of almost identical pK', 5,5-dimethyl-2,4-oxazolidinedione (DMO), a substance proposed for the measurement of intracellular pH(7). The fact that the extracellular/intracellular concentration ratio of DMO is independent of the total amount of DMO in the system(8,9,10) furnishes convincing evidence that this ratio is determined by the pH gradient and not to any significant extent by interactions with intracellular or extracellular components or by "active transport," as has been suggested(11).

In a number of studies, intracellular pH values of muscle as calculated from the distribution of CO_2 on the assumption that total acid-labile CO_2 consists of bicarbonate and dissolved CO_2 (in equilibrium with carbonic acid) have been in general agreement with the results of other experiments in which pH values were calculated from the distribution of DMO. In one study(8) in which there was direct comparison in the same muscle preparation of the intracellular pH values calculated from the distribution of CO_2 and that of DMO, there was quite close agreement at a pCO_2 of 110 mm Hg. At a pCO_2 of 40 mm, the pH values calculated from CO_2 averaged 0.14 unit higher than those calculated from DMO. At a very low value of pCO_2 (10 mm) at which the assumption of equality of intracellular and extracellular pCO_2 might be expected to lead to an erroneously high value of intracellular pH as calculated from the CO_2 distribution, the discrepancy was 0.45 unit.

The most reasonable interpretation of our experiments and those of others would seem to be that the barium-soluble fraction of an alkaline extract does not represent a non-bicarbonate, non-ionized substance of mysterious chemical identity in muscle but that essentially all of the acid-labile CO_2 in the living muscle is bicarbonate, CO_2, and carbonic acid. A limited part of this CO_2 as it exists in an alkaline extract is protected from precipitation as $BaCO_3$. The intracellular pH of muscle calculated from the total acid-labile CO_2 and the pCO_2 and on the assumption of impermeability of the membrane to the bicarbonate ion is probably valid.

Summary. A portion of the acid-labile CO_2 in alkaline extracts of rat muscle is not precipitable by $BaCl_2$. Carbonate added to an extract that has been acidified, freed of CO_2 by evacuation, and realkalinized is only partially precipitated. It is concluded that barium solubility is the effect of some substance in the muscle extract in inhibiting crystallization of a limited amount of $BaCO_3$. Calculation of intracellular pH from total acid-labile CO_2 appears valid.

The expert assistance of Dorothy Johnson is gratefully acknowledged.

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Received April 7, 1967. P.S.E.B.M., 1967, v125.