55. A SIMPLIFIED ESTIMATION OF LACTATE IN MUSCLE

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EDWARDS [1938], modifying the method of Friedemann *et al.* [1927], improved the apparatus and simplified the method for estimating lactate in normal human blood. Treatment of blood extracts with copper sulphate and copper hydroxide, preliminary boiling or aeration of extracts and cleaning out the apparatus or adding fresh reagents between runs were all found to be unnecessary, since none of these procedures had any significant effect either on the estimation of pre-formed lactate or on the recovery of added lactate. We have applied these modifications to deproteinized extracts of muscles, and have also used a simple apparatus for preparing such extracts. The present method can be used with any apparatus suitable for the method of Friedemann *et al.* [1927].

Materials

These are prepared according to Friedemann *et al.* [1927] except as noted below. Instead of sodium bisulphite, a 5 % aqueous solution of sodium metabisulphite is used. This gives slightly better recoveries, especially in warm weather. Excess bisulphite in the titration is removed with $N/3$ iodine in KI. Technical grades of iodine and KI are satisfactory and cost about one-half as much as CP grades. Hydrated disodium phosphate (USP powdered) is used for the adjustment of alkalinity in estimating bound bisulphite. If the product as bought does not contain enough water of crystallization to lower the temperature of water as it dissolves, it is dissolved in an equal weight of hot water and allowed to crystallize with constant stirring. The crystals so obtained need not be pulverized.

A stock solution of trichloroacetic acid, made by dissolving one part of acid by weight with 1.5 parts of water, is stored in the ice box and diluted one to ten as needed. As Wendel [1931-32] pointed out, this reagent may have a high blank. Baker's CP product usually has a blank no higher than that of distilled water, although some samples have a small, but not troublesome, blank. Trichloroacetic acid can be purified by sublimation, but this is a wasteful procedure.

Method

The tissue grinder described by Potter & Elvehjem [1936] was found by Newman [1938] to be well suited for quantitative extraction of lactate from muscle. It consists of a pyrex test tube of 30 ml. capacity, graduated at 25 ml. and fitted with a revolving glass pestle that has a 15 cm. glass shaft on its upper end.¹ The shaft fits by means of a rubber stopper to the shaft of a 1/50 H.P. electric motor that has a built-in reducing gear of $8\frac{1}{2}$ ratio and a suitable rheostat.

A tube is set in a pan of solid CO_2 and 2 ml. of 4 % trichloroacetic acid are frozen in the bottom. The muscle is frozen in powdered CO_2 and a piece weighing

¹ These may be bought from Macalaster-Bicknell Co., Cambridge, Mass., U.S.A.

about 250 mg. is weighed, while frozen, with a rapid torsion balance. It is dropped into the tube, covered with 3 ml. of ice-cold 4 % trichloroacetic acid and the whole mass is frozen solid. The tube is dipped into cold water until melting just begins, the pestle is inserted, the motor started and the muscle is ground to a fine frozen pulp. This takes about 2 min. The pestle is withdrawn and washed off quantitatively into the tube with ice-cold 4 % trichloroacetic acid. The tube is filled to 25 ml. with the same reagent, a clean rubber stopper is inserted and the mixture is shaken. It is then centrifuged for 5 min. at about 2500 r.p.m.

A suitable aliquot of the supernatant fluid is taken for lactate estimation according to Edwards [1938], with certain precautions. The water leaving the condensers should range from 30° to 40°. Below 30° aldehyde is retained; above 40° aldehyde is lost. This has been pointed out by Friedemann & Kendall [1929] and by Friedemann & Graeser [1933]. They have also advised cooling the tube in which the titration takes place when the room temperature is above 26°. Otherwise blanks are high and recoveries of standard lactate low. It is for this reason that we use completely hydrated disodium phosphate which has a negative heat of solution [Lehnartz, 1928].

Edwards [1938] found that as many as eleven runs could be made without cleaning out the apparatus or adding fresh manganous sulphate and H_2SO_4 between runs. We have extended his findings on this point to muscle. Friedemann *et al.* [1927] stated that for relatively small amounts of lactate, such as are found in 200 mg. of muscle, satisfactory results can be obtained without the use of manganous sulphate, provided that the rate of oxidation is carefully adjusted.

In our battery of 10 units, each run contains a reagent blank, a standard of lithium lactate and 4 muscle extracts in duplicate. The recovery of the standard is always in the range 97–102 % of the theoretical amount. The blank is usually less than 0.04 ml. of 0.005 *N* iodine, and rarely as much as 0.06 ml. Duplicates of the extracts always agree to within 0.02 ml. of 0.005 *N* iodine. These blanks and recoveries compare favourably with Wendel's [1931–32], although our blanks are slightly higher than his. He got recoveries of only 95 % of the theoretical values when adding zinc lactate, which was thought to be impure. The sensitivity of the method in our hands is therefore about the same as in his, namely, down to about 0.01 mg. of lactic acid; corresponding to not more than 5 mg. of lactic acid per 100 g. of muscle, with duplicate estimations agreeing to within 2 mg. of lactic acid per 100 g.

By the present method, duplicate estimations on each of the 4 gastrocnemii of two frogs, along with a reagent blank and a standard solution, can be finished in from 70 to 90 min. after the animals are killed. With an accumulation of samples, one run is made while another is being titrated, so that 10 titrations can be finished every 30–40 min., and since cleaning out the apparatus, or adding fresh reagents, is unnecessary, there is no delay in beginning new runs.

Evidence justifying the various steps of the method

In methodological studies, it is necessary to have a supply of material so homogeneous that the concentration of the substance in question is constant from one sample to another. In the case of muscle, this is best achieved by powdering the muscle. We have used pills made from frozen frog muscle. In the course of preparation, all the equipment is cooled with solid CO_2 . The muscles are frozen and powdered in a mortar and the powder is sifted first through a coarse and then a fine sifter. The pills are made in a pill press so constructed that they are hammered solid by a steel plug fitting into a hole bored through a block

of steel. They weigh between 200 and 300 mg. Duplicate estimations are made on each pill. The pills can be stored for days in airtight containers surrounded by solid CO_2 without change in content of water, glycogen or lactate. The lactate concentration is satisfactorily constant from pill to pill, the maximum difference in any given batch being 5 mg./100 g.

In taking an aliquot from the supernatant fluid, the assumption is made that all of the lactate originally present in the muscle is distributed in equal concentration by volume throughout both the supernatant fluid and the mass of denatured protein. Experiment 1 (Table 1) shows that in the present method this assumption is valid. The experiments were carried out on pills of the same batch, divided into two groups. The first group of analyses was made on aliquots taken from the supernatant fluid. The pills of the second group were ground up and the supernatant fluid was saved. Then each pill residue was extracted with three 5 ml. portions of the trichloroacetic acid. The original supernatant fluid and the washings were pooled, made up to known volume, and aliquots taken for analysis. Data of Newman [1938] are also included. The same result was obtained by these two methods of lactate extraction.

The three reagents most commonly used for deproteinizing muscle are trichloroacetic acid, mercury salts and sodium tungstate. Trichloroacetic acid in concentrations varying from 4 to 10 % has been used by Cori & Cori [1932-33], Davenport & Davenport [1928], Flock *et al.* [1939], Gordon & Quastel [1939],

Table 1. *Various factors influencing the estimation of muscle lactate*

Results for solutions of lithium lactate are expressed as mg. lactic acid per 100 ml., and are the average of duplicate or triplicate estimations.

Results for muscle and muscle pills are expressed as mg. lactic acid per 100 g. tissue, and are the average of duplicate or triplicate estimations on each of two or three samples.

Type of sample and treatment		Lactate content	Lactate added	Lactate recovered	
		mg./ 100 ml. or g.	mg./ 100 ml. or g.	mg./ 100 ml. or g.	% of amount added
Exp. 1					
Batch 3 of muscle pills	{ quantitative extraction	55	—	—	—
	{ aliquots of supernatant	55	—	—	—
Batch 4 of muscle pills	{ quantitative extraction	48	—	—	—
	{ aliquots of supernatant	49	—	—	—
Rat muscle 1	{ quantitative extraction	42	—	—	—
	{ aliquots of supernatant	38	—	—	—
Rat muscle 2	{ quantitative extraction	47	—	—	—
	{ aliquots of supernatant	43	—	—	—
Rat muscle 3	{ quantitative extraction	39	—	—	—
	{ aliquots of supernatant	40	—	—	—
Exp. 2					
4 % trichloroacetic acid:					
No added lactate		49	—	—	—
Lactate added		76	28	27	96.5
Lactate added		102	52	53	102
Quantitative extraction with acid sodium tung-					
state, followed by treatment with copper-lime:					
No added lactate		48	—	—	—
Lactate added		96	48	48	100
Quantitative extraction with acid mercuric sul-					
phate followed by treatment with copper-lime:					
No added lactate		47	—	—	—
Lactate added		99	53	52	98

Table 1 (*cont.*)

Type of sample and treatment	Lactate content mg./ 100 ml. or g.	Lactate added mg./ 100 ml. or g.	Lactate recovered	
			mg./ 100 ml. or g.	% of amount added
Exp. 3				
Solutions of pure lithium lactate:				
In distilled water	—	1.91	1.91	100
In 4 % trichloroacetic acid	—	1.91	1.92	100.5
In 8 % trichloroacetic acid	—	1.91	1.89	99
In 16 % trichloroacetic acid	—	1.91	1.89	99
In distilled water	—	0.48	0.48	100
In 4 % trichloroacetic acid	—	0.48	0.49	102
In 8 % trichloroacetic acid	—	0.48	0.48	100
Pills of batch 4 ground in trichloroacetic acid:				
Ground in 10 times by wt. of 40 %, then diluted to 1 : 100 by wt.	54	—	—	—
Ground in 1 : 100 by wt. of 2 %	54	—	—	—
Ground in 1 : 200 by wt. of 2 %	58	—	—	—
Ground in 20 times its wt. of 4 %	54	—	—	—
Ground in 40 times its wt. of 4 %	53	—	—	—
Ground in 100 times its wt. of 4 %	57	—	—	—
Ground in 200 times its wt. of 4 %	55	—	—	—
Ground in 100 times its wt. of 8 %	57	—	—	—
Extracted quantitatively with 4 successive washings with 4 % trichloroacetic acid	55	—	—	—
Pills of batch 1 ground with 100 times their wt. of 4 % trichloroacetic acid:				
No added lactate	113	—	—	—
Added lactate	321	214	208	97
Pills of batch 2 ground with 100 times their wt. of 4 % trichloroacetic acid				
No added lactate	139	—	—	—
Added lactate	247	106	108	101.5
Pills of batch 5 ground in 100 times their wt. of 4 % trichloroacetic acid:				
No added lactate	49	—	—	—
Added lactate	111	61	62	101.5
Added lactate	73	24	24	100
Exp. 4				
4 % trichloroacetic acid extract of frog muscle, analysed with potassium permanganate by present method:				
No added lactate	383	—	—	—
Added lactate	591	211	208	98.5
Same extract, analysed with ceric sulphate according to Gordon & Quastel [1939]:				
No added lactate	383	—	—	—
Added lactate	698	320	315	98.5

Long & Grant [1930] and Long & Horsfall [1932] and others. Mercuric salts have been used by Lehnartz [1928], Kerly & Ronzoni [1937], Ronzoni & Wallen-Lawrence [1927], Ronzoni *et al.* [1928] and Lundsgaard [1933]. Sodium tungstate has been used much more with blood than with muscle.

Exp. 2 demonstrates that muscle extract is adequately deproteinized by any one of the foregoing methods. Pills of the same batch were divided into three groups. The first were ground and extracted with trichloroacetic acid, as described above. The second were treated with 1 ml. of 10 % sodium tungstate, 1 ml. of $2/3N$ H_2SO_4 and 8 ml. of water. After centrifuging, the supernatant

fluid was saved and the residues were extracted with three 5 ml. portions of the acid-tungstate mixture. The supernatant fluid and the washings were pooled, diluted to known volume and treated with copper sulphate and calcium hydroxide, after Van Slyke [1917]. The pills of the third group were extracted with four 5 ml. portions of 4 % mercuric sulphate in $N H_2SO_4$. The four extracts were pooled, treated with H_2S and centrifuged. The supernatant fluid was aerated until the H_2S was removed. Aliquots were made slightly alkaline with NaOH and treated with copper sulphate and calcium hydroxide. In each group, to certain pills lithium lactate in amounts comparable with the lactate already present was added. Exp. 2 shows that the estimated lactate was the same regardless of the type of protein precipitant, and also that recovery of added lactate was satisfactory. This has previously been shown for blood by Ronzoni & Wallen-Lawrence [1927], although they did not use trichloroacetic acid, by Edwards [1938] for blood and by Davenport & Davenport [1928] for muscle.

Table 2. *Comparison of results with and without preliminary aeration and treatment with copper-lime*

Results represent the averages of duplicate or triplicate estimations.

Species	No preliminary boiling and aeration		10 min. preliminary boiling and aeration	
	No Cu-lime mg./100 g.	Cu-lime mg./100 g.	No Cu-lime mg./100 g.	Cu-lime mg./100 g.
Frog	146	143	147	145
"	41	39	40	39
"	177	177	179	175
"	13	11	11	13
Guinea-pig	99	—	—	103
Man	30	—	—	29
Rabbit	95	—	—	92
Rat	143	—	—	138

It is well known that trichloroacetic acid may be used in wide concentrations and amounts without affecting the recovery of added lactate so long as there is enough to precipitate all the protein [Cori & Cori, 1932-33; Avery *et al.* 1935]. Exp. 3 (Table 1), containing data obtained from solutions of pure lithium lactate and from muscle, further demonstrates that over a range of 4 to 16 % concentration of the reagent, lactate may be accurately estimated and recovery of added lactate is satisfactory. It should be emphasized that it is necessary to have trichloroacetic acid with a small blank before such consistently satisfactory results are obtained. We have fixed on 4 % trichloroacetic acid only because of its convenience, and have made up 250 mg. of muscle to a volume of 25 ml. only to avoid the error inherent in pipetting small volumes.

The evidence that with muscle, as with blood, preliminary boiling and aeration and treatment with copper sulphate and calcium hydroxide are unnecessary is obtained in Table 2. Extracts of several frog muscles were prepared with 4 % trichloroacetic acid along with suitable reagent blanks. One-half of each extract was treated with 10 % copper sulphate and 20 % calcium hydroxide. The proportions were 25 volumes of extract, with enough 40 % NaOH to make it neutral, 10 volumes of copper sulphate, 10 volumes of calcium hydroxide and water to make 50 volumes. The mixture was allowed to stand at least one-half hour, with shaking every 5 min. It was centrifuged and aliquots of the supernatant fluid taken for analysis. An analysis of each type of extract was made with and without the preliminary boiling and aeration that are customary. The

same results were obtained with and without treatment with copper and lime and with and without preliminary boiling and aeration. The table includes also data derived from the muscles of several other animal species. These data do not include estimation of the untreated extract with preliminary boiling, or estimation of the treated extract without preliminary boiling, since the results led to the same conclusion as those from frog muscle. The suggestion that treatment with copper and lime may be dispensed with under certain circumstances has been made by Friedemann *et al.* [1927], Friedemann [1928], Friedemann & Kendall [1929], Boyland [1928], Scott & Berg [1936] and Edwards [1938]. One of the advantages claimed for methods such as those used by Gordon & Quastel [1939], Lehmann [1938], Miller & Muntz [1938] and Rappaport & Reifer [1937], which differ in principle from that of Friedemann *et al.* [1927], is that removal of carbohydrates before analysis is unnecessary. We consider that with muscle it is not necessary even for the method of Friedemann *et al.* [1927].

We have tried repeatedly without success to detect in trichloroacetic acid extracts of frog muscles any appreciable amount of preformed volatile substances that will bind bisulphite.

Gordon & Quastel [1939] introduced the use of ceric sulphate instead of potassium permanganate as oxidant, claiming the advantage that treatment of muscle extracts with copper sulphate and calcium hydroxide is thereby rendered unnecessary. Exp. 4 compares their method with ours for the same muscle extract. The present method requires one-fourth of the time, employs simpler apparatus and gives the same results as that of Gordon & Quastel [1939].

SUMMARY

1. The simplifications which Edwards [1938] introduced into the method and apparatus of Friedemann *et al.* [1927] for estimating lactate in normal human blood are applicable also to muscle. Treatment of muscle extracts with copper sulphate and calcium hydroxide, as well as preliminary boiling and aeration, may be dispensed with. From five to ten estimations can be made without cleaning out the apparatus or adding fresh reagents.

2. A rapid, accurate method for preparing protein-free extracts of muscle, which uses the tissue grinder of Potter & Elvehjem [1936], is described.

3. No difference in the estimated preformed lactate or in recovery of added lactate is found when either trichloroacetic acid, mercuric sulphate or acid sodium tungstate is used for deproteinizing.

4. The use of ceric sulphate as oxidant is no more precise or convenient than the use of potassium permanganate and requires more time and more complicated apparatus.

REFERENCES

- Avery, Kerr & Ghantus (1935). *J. biol. Chem.* **110**, 637.
Boyland (1928). *Biochem. J.* **22**, 236.
Cori & Cori (1932-33). *J. biol. Chem.* **99**, 493.
Davenport & Davenport (1928). *J. biol. Chem.* **76**, 651.
Edwards (1938). *J. biol. Chem.* **125**, 571.
Flock, Ingle & Bollmann (1939). *J. biol. Chem.* **129**, 99.
Friedemann (1928). *J. biol. Chem.* **76**, 75.
— Cotonio & Shaffer (1927). *J. biol. Chem.* **73**, 335.
— & Graesser (1933). *J. biol. Chem.* **100**, 291.
— & Kendall (1929). *J. biol. Chem.* **82**, 23.

- Gordon & Quastel (1939). *Biochem. J.* **33**, 1332.
Kerly & Ronzoni (1937). *J. biol. Chem.* **103**, 161.
Lehmann (1938). *Skand. Arch. Physiol.* **80**, 237.
Lehnartz (1928). *Hoppe-Seyl. Z.* **179**, 1.
Long & Grant (1930). *J. biol. Chem.* **89**, 553.
—— & Horsfall (1932). *J. biol. Chem.* **95**, 715.
Lundsgaard (1933). *Biochem. Z.* **264**, 209.
Miller & Muntz (1938). *J. biol. Chem.* **126**, 413.
Newman (1938). *Amer. J. Physiol.* **122**, 359.
Potter & Elvehjem (1936). *J. biol. Chem.* **114**, 495.
Rappaport & Reifer (1937). *Microchim. Acta*, **2**, 62.
Ronzoni, Glaser & Barr (1928). *J. biol. Chem.* **80**, 309.
—— & Wallen-Lawrence (1927). *J. biol. Chem.* **74**, 363.
Scott & Berg (1936). *J. biol. Chem.* **115**, 163.
Van Slyke (1917). *J. biol. Chem.* **32**, 455.
Wendel (1931–32). *J. biol. Chem.* **94**, 717.