CCLIII. THE DETERMINATION OF LACTIC ACID IN MUSCLE.

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The method generally employed for the determination of lactic acid consists in the removal of protein by tungstic acid or trichloroacetic acid, the subsequent removal of sugar by copper sulphate and lime, the oxidation of the lactic acid by permanganate to acetaldehyde which is distilled off and trapped in sodium bisulphite solution and finally the titration with iodine of the bound bisulphite. It is evident that the "lactic acid" will include any substance which escapes removal by the preliminary treatment and which is either itself volatile and capable of binding bisulphite or is oxidised to such a substance.

The most obvious of possible interfering substances is methylglyoxal, which is volatile in steam, combines with bisulphite and has been found in muscle and other tissues by many workers. In fact, a simple experiment (Table I) shows

Table I.

All solutions contain 0·5 mg. lactic acid.

Substance added	mg. lactic acid found
1 mg. glucose	0·49
,, pyruvic acid	0·55
,, methylglyoxal	0·92
,, dihydroxyacetone	0·59
,, glyceric aldehyde	0·50
,, glycerophosphoric acid	0·53
,, phosphoglyceric acid	0·59
Nil	0·50

that methylglyoxal, alone among the probable precursors of lactic acid, interferes with the determination. Each of several possible interfering substances was added to a standard solution of lithium lactate, and the estimation was then made in the ordinary way. The results showed that methylglyoxal was the only one which increased the apparent amount of lactic acid to an appreciable extent, but that it did so to an extent greater than would be anticipated if it were distilling unchanged or were converted into acetaldehyde.

The problem therefore was to devise a means of removing methylglyoxal, and as a subsidiary point, to determine the changes undergone by methylglyoxal in the estimation of lactic acid.

The method which first suggests itself for the removal of methylglyoxal is a preliminary distillation after the removal of sugar but before the addition of

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permanganate. Such a process would involve the minimum of additional manipulation and no increase in the number of measurements. Unfortunately, it appears that while methylglyoxal undoubtedly distils and binds an equimolecular amount of sodium bisulphite, it undergoes some change in the presence of copper sulphate and lime which destroys its volatility but does not prevent its oxidation to a reducing substance by permanganate. Indeed, the bisulphite-binding power of methylglyoxal is increased about 40 % by treatment with copper sulphate and lime with subsequent oxidation. The substance bringing about this change is the lime, which can be replaced by other alkalis (Table II).

Table II.

Vol. of methylglyoxal (0·1 %) ml.	Treated with lime	$MnSO_4 + H_2SO_4$	$KMnO_4$	Titration ml. 1	Titration ml. 2
1 (undistilled)	−	−	−	1·35	1·31
1	−	+	−	1·10	—
1	−	−	+	0·25	0·23
1	+	−	−	0·0	0·0
1	+	+	−	0·0	0·0
1	+	+	+	1·79	1·80
1 (distilled 30 min.)	−	−	−	1·30	1·28

A second possibility is the removal of methylglyoxal (and incidentally other reducing substances), by means of 2 : 4-dinitrophenylhydrazine, either subsequent to glucose removal or in place of treatment with copper-lime (after treatment with alkali, methylglyoxal still reacts with 2 : 4-dinitrophenylhydrazine). In attempting to use this method it was found necessary to remove the excess of the 2 : 4-dinitrophenylhydrazine by repeated extraction with ethyl acetate and then to remove the dissolved ethyl acetate by a preliminary distillation before addition of permanganate. Under these circumstances, the results were invariably low, and further experiment showed some variable loss of lactic acid during the extraction with ethyl acetate. At this stage the solution was acid from addition of the HCl solution of 2 : 4-dinitrophenylhydrazine, but even from alkaline solution ethyl acetate still removed some lactic acid. It was also possible, of course, that a little lactic acid was oxidised by the hydrazine, and this may be suggested as a possible explanation of the isolation from tissues of small amounts of pyruvic acid 2 : 4-dinitrophenylhydrazone.

There remains the possibility of removing methylglyoxal by distillation prior to treatment with copper sulphate and lime. This method was eventually adopted, though it involves a long distillation—an hour—with continuous addition of water to replace that lost and an extra volume adjustment. In the mixtures whose analysis is shown in Table III, an aliquot of the filtrate from tungstic acid precipitation was distilled for 1 hour, the residual liquid was quantitatively transferred to a 50 ml. flask, copper sulphate and lime were added, and the volume was made up to 50 ml. with distilled water. The subse-

Table III.

Solution contained (mg.) Lactic acid	Glucose	Methylglyoxal	Lactic acid found
8·0	—	—	8·0
8·0	5·0	5·0	8·0
8·0	5·0	10·0	7·8
8·0	10·0	5·0	8·2
16·0	5·0	5·0	15·5

quent treatment then followed the usual course. When, however, this method was applied to tungstic acid filtrates of muscle, it was found that the result was invariably higher than by the standard method. The reason for this is not clear, but filtration of the muscle extract through kieselguhr removes the interfering substance (or substances). With this treatment the modified method gives reproducible results lower than those obtained by the standard method (Table IV).

Table IV. *Apparent lactic acid per g. of muscle.*

Muscles 1–4 were completely fatigued; 5 was partially fatigued; and 6 was fresh (no special precautions in isolating).

	Weight of muscle g.	Old method mg.	New method mg.	Difference mg.
1	1·00	3·00	2·50	0·5
2	1·52	2·90	2·55	0·35
3	1·42	3·20	2·78	0·42
4	1·41	3·35	2·93	0·42
5	1·20	1·96	1·56	0·40
6	1·70	1·19	0·74	0·45

The kieselguhr filtration does not affect the accuracy of the results in analysis of "synthetic" mixtures. Since the interfering substance which is present after simple paper filtration appears only in the modified method involving the boiling of an acid solution for 1 hour, and since it is not derived from glucose, it may conceivably be glycerol and other products of the hydrolysis and oxidation of lipoid matter.

In the case of one muscle extract the distillate during the one-hour's distillation was trapped in 2 : 4-dinitrophenylhydrazine, and the solution, on evaporation to a small volume, gave approximately 2 mg. of a red hydrazone. This was identified as that of methylglyoxal. The total amount of osazone to be expected, on the basis of lactic acid estimation, was about 3 mg. Since the mother-liquor contained a little osazone in solution it is evident that the difference between the results yielded by the two methods is mainly due to methylglyoxal.

The problem of the changes undergone by methylglyoxal during treatment with copper sulphate and lime with subsequent permanganate oxidation has not yet been completely solved. When an alkaline solution of methylglyoxal stands for half an hour, distillation of the solution, even after acidification, yields no more than traces of methylglyoxal (as determined by the bisulphite-binding power of the distillate or by its ability to form a 2 : 4-dinitrophenylhydrazone in HCl solution). The solution, however, gives an amorphous, yellowish brown, low-melting osazone, completely different from the red crystalline osazone of methylglyoxal itself. This substance has not yet been obtained in crystalline form suitable for analysis or melting-point determination, but one may suggest that it is probably a hydrazone of some polymeride of methylglyoxal.

If the alkaline solution of methylglyoxal, containing this hypothetical polymeride, is boiled with acid permanganate, and the distillate is passed into a HCl solution of 2 : 4-dinitrophenylhydrazine, a golden yellow crystalline hydrazone is produced. This, after several recrystallisations from methyl alcohol or from methyl alcohol and water, forms long needles, orange-yellow from the former solvent, pure yellow from the latter. These melt, in either case, at 161° and give no depression of the melting-point when mixed with the 2 : 4-dinitrophenylhydrazone of acetaldehyde. The melting-point of the latter compound is recorded in the literature as 148° [Case and Cook, 1931] but a sample

which we prepared and crystallised from methyl alcohol melted unchanged at 161°. Case and Cook crystallised their preparation, apparently, from nitrobenzene or pyridine, but we found that a sample of acetaldehyde 2 : 4-dinitrophenylhydrazone, recrystallised from pyridine, washed with benzene and light petroleum and dried in air, melted at 161°. The crude hydrazone we obtained appeared, on microscopic examination, to contain no other substance except a small amount of the red hydrazone of methylglyoxal itself. Acetaldehyde is probably therefore the sole product capable of forming a hydrazone. The simultaneous production of formic acid (which does not react with 2 : 4-dinitrophenylhydrazine) would account for the increased bisulphite-binding power already referred to. The conversion of methylglyoxal into acetaldehyde and formic acid, however, involves no oxidation, and as Table I showed, the participation of permanganate is essential for the changes we have observed. We must therefore regard the problem as being only partly solved.

Summary.

1. The ordinary method for determination of lactic acid in muscle *etc.* appears to give results which are too high owing to interference by methylglyoxal. This substance can be removed by distillation prior to treatment with copper-lime.

2. Copper sulphate and lime do not remove methylglyoxal but convert it into some substance (? a polymeride) which is oxidised by acid permanganate to acetaldehyde. No other substance has been detected among the oxidation products of the hypothetical polymeride, but the amount of bisulphite bound corresponds to about 1½ molecules acetaldehyde per molecule of methylglyoxal.

3. The modified method gives results in muscle about 10 % lower than the standard method.

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REFERENCE.

Case and Cook (1931). *Biochem. J.* **25**, 1319.