CXLIV. THE OCCURRENCE OF PYRUVIC ACID AND METHYLGLYOXAL IN MUSCLE METABOLISM.

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(Received July 2nd, 1931.)

THAT the metabolic changes which occur in muscle are of profound complexity is evident. This work was undertaken with the object of studying some of the intermediary mechanisms of muscle carbohydrate metabolism by means of the use of reagents which would remove from the system some product which is only transitorily formed normally. The method is essentially the same as that employed by Neuberg in his study of the mechanisms involved in alcoholic fermentation. The fixative of which we have availed ourselves, namely sodium sulphite, is the same as that used by Neuberg in what constitutes his second form of fermentation, in which acetaldehyde is "fixed" as the bisulphite compound. It is well known that substances of an aldehydic or ketonic nature possess the property of forming addition compounds with sodium bisulphite. The conditions that determine the stability or otherwise of these compounds to further metabolic change have not, however, been worked out. In the conditions under which we have worked, pyruvic acid and methylglyoxal will form with bisulphite compounds that are relatively stable in this regard.

Neuberg and Reinfurth [1920] have shown that in the case of yeast the pyruvate-bisulphite complex is decarboxylated to acetaldehyde. Cook [1930] has confirmed this observation for yeast but has noted that *B. coli communis*, for example, is unable to bring about this decarboxylation. It was also noted that if oxidations of various substrates were carried out in the presence of sulphite, the sulphite served as a very effective fixative for any pyruvic acid that was formed. The method of sulphite fixation also furnishes evidence as to the occurrence of other aldehydic or ketonic compounds during the course of metabolism.

It may be, and often is, urged that the fixation method is liable to give a false impression of the chemical events taking place in that it tends to endow subsidiary reactions with a spurious prominence. It will be shown, however, that as regards the carbohydrate metabolism of muscle it is possible, if sufficient care be taken, to identify pyruvic acid in normal muscle in the absence of any fixatives, and in amounts commensurate with those found when a fixative is

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present. It is only to be expected, nevertheless, that under normal conditions the pyruvic acid, if of importance in metabolism, would quickly disappear. As will be shown later, this disappearance is indeed extremely rapid in the normal muscle.

Although there is no doubt that the presence of sulphite does affect the normal balance of reactions, we are of the opinion that this method of fixation affords useful indications concerning some of the chemical events associated with the metabolism of muscle.

The oxidation of lactate by muscle tissue.

It was postulated by Meyerhof, and the view commands general acceptance, that part of the lactic acid in muscle is oxidised in order to provide energy for the resynthesis of the remainder to carbohydrate. This, taken in conjunction with the fact that lactic acid is dehydrogenated to pyruvic acid by muscle tissue in the presence of methylene blue [Hahn, Fischbach and Haarman, 1928] possibly indicates that at least part of the lactic acid would, under aerobic conditions, with atmospheric oxygen as the hydrogen acceptor, be oxidised *via* pyruvic acid. In the instance of the oxidation of lactate by *B. coli*, pyruvate may be fixed by the use of sulphite, the oxidation (measured as oxygen uptake) corresponding to 72 % transformation of lactate to pyruvate [Cook, 1930]. It was hoped that by the employment of similar methods with muscle, any pyruvic acid which was formed would be made evident by the presence of the sulphite.

As a preliminary it was desirable to investigate the effect of the addition of lactate on the normal respiration of muscle. The respiration was measured as the oxygen uptake in the Barcroft differential manometer. The left-hand pot contained 1.5 cc. frog Ringer solution, 1.0 cc. phosphate buffer (p_H 7.2) and 0.5 cc. water. The right-hand pot contained 1.5 cc. Ringer solution, 1.0 cc. buffer and 0.5 cc. water or $M/10$ lactate solution, together with a weighed amount of frog muscle. Each of the small central pots contained a roll of filter-paper soaked in 6 % potassium hydroxide to absorb CO_2 .

It is evident that the metabolism of the muscle is affected by the physiological conditions of the tissue, *i.e.* is altered by circumstances such as chopping, mincing or washing. Some of the results obtained are shown in Table I.

It will be seen that there appears to be a slightly greater oxygen uptake with intact muscle on the addition of a solution of sodium lactate (Exp. 1). On the other hand the addition of lactate to unwashed chopped muscle has little effect on the oxygen uptake (Exp. 3). There is a slightly increased uptake upon the addition of lactate to washed chopped muscle (Exp. 5), but it is doubted if this is significant. The addition of sulphite causes a marked decrease in the oxygen uptake both with intact and chopped muscle (Exps. 2 and 4).

The initial velocities of oxidation of the intact gastrocnemii were the same in the two instances, but after 3 hours a small but appreciable difference was noted in the rate of oxygen uptake of the control as compared with the muscle

Table I.

Remarks	No. of exp.	Temp. (°)	Duration of exp. (hrs.)	Wt. of muscle (g.)	Controls			Ringer + phosphate		
					Ringer + phosphate p_H 7.2			+ $M/60$ Na_2SO_3 p_H 7.2		
					+ $M/60$ lactate			+ $M/60$ lactate		
					O_2 (mm. ³)	Wt. of muscle (g.)	O_2 (mm. ³)	Wt. of muscle (g.)	O_2 (mm. ³)	Wt. of muscle (g.)
Intact (gas- trocnemii)	1	24	4.75	0.79	208.5	0.76	230.8	—	—	—
	2	25	4	0.49	125	—	—	0.56	31.5	0.53
Chopped	3	23.5	2.5	0.5	336	0.5	340	—	—	—
unwashed	4	19.5	3.5	—	—	0.5	238	—	—	0.5
Chopped washed	5	23.5	4	0.5	61.6	0.5	73.2	—	—	—

to which lactate had been added. It is evident therefore that some oxidation of the added lactate has taken place.

It is realised that in the oxidation of lactate by muscle no increase in velocity would be expected in either intact or unwashed chopped muscles, as the enzymes dealing with the oxidation of lactate would be saturated with this substance; hence no difference in velocity could be expected as long as this amount remains in excess. None of our experiments was of more than 6 hours' duration and in no case was the oxidation in the presence of lactate very much greater than that in its absence. It is clear that under our conditions at least, the oxidation of lactate by muscle is a relatively slow process.

The muscles in Exps. 2 and 4 were removed from the pots after 4 hours, rapidly ground with sand and filtered through muslin. Tests for pyruvate were then carried out by the method of Simon and Piaux [1924]. (A description of this is given later.) Slight positive reactions were given by the intact muscle and unwashed chopped muscle in the presence of sulphite, the controls in which no sulphite was present being definitely negative.

Some estimations were carried out on the disappearance of lactic acid in the systems both with and without the addition of sulphite. The results obtained were however rather indefinite, but it appears that the oxidation of lactate constitutes only a small percentage of the total respiration. Under the conditions described the Friedemann, Cotonio and Shaffer [1927] method for the determination of lactic acid is rather unsatisfactory. It is hoped to give further details of the relation of pyruvic acid formation to lactic acid oxidation in a later paper.

Methods for the detection and estimation of compounds formed.

As an indication of the formation of pyruvic acid we have generally employed the test of Simon and Piaux [1924]. Considerable caution must, of course, always be exercised in using a colour reaction as the sole evidence of formation of any particular compound. As will appear later we have not relied exclusively upon colour tests, and we have invariably found that the indications afforded by other methods have run closely parallel with those obtained

by the Simon and Piaux reaction. As a qualitative indication of the presence of pyruvic acid this test is carried out as follows. About 3 cc. of the material to be examined are placed in a test-tube and ammonium sulphate is added in such quantity that a layer rests at the bottom of the tube. Crystals of sodium nitroprusside are sprinkled on this layer and an equal volume of ammonia (sp. gr. 0.880) is then added without shaking. In the presence of filtrates prepared from muscle a pink colour at first makes its appearance. This reaction is due to the .SH compounds present, but rapidly fades and does not interfere with the pyruvic acid reaction. This latter reaction appears about half an hour after the commencement of the test and consists in a blue or greenish-blue colour, which develops in the neighbourhood of the nitroprusside crystals, and if the concentration of pyruvate be sufficiently great spreads throughout the solution. The colour increases in intensity for about 2 hours, after which it begins to fade, and with higher concentrations of pyruvate a red colour makes its appearance after the lapse of about 12 hours. Although nitroprusside and alkali hydroxides give various colour reactions with numerous aldehydes and ketones and certain unsaturated sulphur derivatives, we are not aware of any substance of likely physiological importance, other than pyruvic acid, which gives with nitroprusside and ammonia the characteristic blue-green colour; and we are of the opinion that this test is a reliable criterion for the presence of pyruvic acid. We have tested the reaction with a solution of methylglyoxal but have not obtained a positive result. With nitroprusside and alkali hydroxide methylglyoxal gives a red colour which is however insufficiently specific to constitute a useful test.

Needless to say, any satisfactory proof of the formation of a compound must involve either the isolation of the actual compound or the isolation of some convenient derivative. The first alternative is in the present case obviously rather difficult to undertake. For the purposes of identification we have used *p*-nitrophenylhydrazine and 2 : 4-dinitrophenylhydrazine. The method used in the earlier experiments was to precipitate the proteins with trichloroacetic acid and then to add drop by drop a freshly prepared saturated solution of *p*-nitrophenylhydrazine in glacial acetic acid. This has the disadvantages that in the first place considerable concentration of the material under test is necessary, and that in the second place precipitation of the reagent itself is apt to occur when the acid solution is added even to water alone. Hence the precipitates were frequently contaminated with considerable amounts of unchanged reagent, which tended to interfere with the subsequent colour reactions with alcoholic potassium hydroxide. Nevertheless, since we at first used this substance, we have prepared the derivatives of pyruvic acid and methylglyoxal from these compounds themselves, and have for convenience incorporated some of their properties in Table II. For the separation of these derivatives we have followed the method described fully by Dakin and Dudley [1913].

It was found that far better results were obtained by using 2 : 4-dinitro-

phenylhydrazine, which is very easily prepared by the method given by Allen [1930]. This substance is used in the form of a solution of 0.5 g. in 30 cc. 2*N* hydrochloric acid. No precipitation of the reagent occurs on adding to water. The method employed is essentially the same as that described by Neuberg and Kobel [1930]. The characteristics of the derivatives of this compound are also given in Table II. The acetaldehyde hydrazones are included in addition, in view of the fact that Neuberg and Gottschalk [1925] have reported the occurrence of acetaldehyde in the course of muscle metabolism in the presence of sulphite. As will be mentioned later, we have not been able to demonstrate the presence of acetaldehyde in any amount comparable with that of pyruvic acid or methylglyoxal in any of our experiments. We have employed Rimini's [1904] test for acetaldehyde, but the reaction obtained would correspond to the presence of only extremely small amounts of this substance.

Table II.

Reagent	Pyruvic acid hydrazone			Methylglyoxal osazone			Acetaldehyde hydrazone		
	Colour of pre- cipitate	Reaction with alcoholic potash (6 %)	M.P.	Colour of pre- cipitate	Reaction with alcoholic potash	M.P.	Colour of pre- cipitate	Reaction with alcoholic potash	M.P.
<i>p</i> -Nitrophenyl- hydrazine	Yellow	Cherry- red	223– 225°	Red*	Deep blue	302– 304°	Yellow	Brown- red	128°
2 : 4-Dinitro- phenylhydrazine	Lemon- yellow	Deep red	214°	Light red†	Blue- violet	298°	Yellow	Deep red	147°

* Recrystallised from nitrobenzene and heated for some time at 140°.

† Recrystallised once from pyridine and once from nitrobenzene.

A useful confirmatory test, though in itself anything but conclusive, for the presence of pyruvic acid and methylglyoxal is the iodoform reaction. Iodoform is rapidly produced in the cold by both methylglyoxal and pyruvic acid on the addition of alkali and iodine.

We have also employed the capacity of these compounds for binding sulphite as a method of estimating roughly the amounts formed. The procedure is described by Cook [1930]. It is possible by this method to estimate both the pyruvic acid and the methylglyoxal in a single sample. The total bound sulphite is estimated, the solution then being made acid and the methylglyoxal distilled off into sulphite and titrated in the distillate.

Experiments on rabbit muscle.

When working with frog muscle one is necessarily limited with respect to the amount of muscle it is possible to employ and hence the quantity of metabolic products which it is possible to detect or isolate. In order to overcome this limitation it was thought desirable to attempt to use rabbit muscle. The use of mammalian muscle for experiments of this nature is attended with several disadvantages, prominent among which are its comparatively rapid

deterioration when excised, either at the temperature of the animal or at lower temperatures, and the impossibility of ensuring that the tissue shall be permeated by any constituent of the fluid in which it may be immersed. We therefore decided to try minced rabbit muscle, although from the outset we realised fully the unphysiological condition of the tissue, and the fact that mincing or chopping destroys the power of the muscle to resynthesise carbohydrate.

The muscle was taken from the hind limbs and back of a rabbit killed by a blow on the head and bled from the heart. It was immediately minced, weighed, and apportioned out into beakers containing the various solutions prepared to receive it. After thorough mixing and disintegration of the chopped mass, the contents of the beakers were thoroughly aerated with oxygen, bubbled through for the duration of the experiment, which as a rule lasted for several hours at room temperature. At intervals nitroprusside tests were performed on about 3 cc. of the mixed tissue and fluid.

The details of the first of these experiments are as follows. Beaker A (control), 250 cc. phosphate buffer (p_H 7.2), 100 g. minced muscle; Beaker B (with sulphite), 242.5 cc. phosphate buffer, 7.5 cc. $M Na_2SO_3$, 100 g. minced muscle. These were oxygenated at room temperature for $3\frac{1}{2}$ hours. At the end of this time the contents of both beakers were strained through muslin and deproteinised by the addition in each case of 9 cc. of 50 % trichloroacetic acid. After filtering, the fluids were concentrated on a water-bath to about 20 cc. When cool, a few drops of an acetic acid solution of *p*-nitrophenylhydrazine were added to each, and the precipitates obtained were filtered off and washed. It was noted that the yield of solid from B was considerably the greater of the two.

The results are embodied in Table III.

Table III.

<i>Nitroprusside reactions.</i>		
Time after commencement of exp. (mins.)	A (control)	B (with sulphite)
15	+++	++
40	++	++++
65	++	++++
210	+	++++
<i>Colour reactions of hydrazones with alcoholic potassium hydroxide.</i>		
A	B	
Dirty bluish brown, similar to the colour obtained with excess of reagent	Strong deep red. Indicative of pyruvic acid	

It is obvious that although towards the beginning of the experiment pyruvic acid was present to about an equal extent in both mixtures, it rapidly disappeared in the control, but on the other hand increased in concentration in the presence of the fixative.

Although the occurrence of pyruvic acid in minced rabbit muscle was thus

demonstrated, there was of course nothing to indicate that its presence was in any way dependent upon any oxidative process, in spite of the strongly aerobic conditions. The next step was to repeat the experiment under both aerobic and anaerobic conditions. To this end mixtures were set up as follows:

A. 50 g. minced muscle 200 cc. buffer (p_H 7.2)	} Aerobic	C. As A }	} Anaerobic
B. 50 g. minced muscle 194 cc. buffer 6 cc. $M Na_2SO_3$		D. As B }	

A and B were oxygenated as before in beakers, while C and D were placed, together with 1 cc. of $M/10$ potassium cyanide solution, in Büchner flasks, which were evacuated. Nitroprusside tests were done from time to time over a period of about 4 hours. At the end of this time, 5 cc. of each mixture were ground with sand and the filtrates used for iodine titrations for the purpose of getting a rough idea of the relative amounts of bound sulphite. (In the cases of A and C a little sulphite was added to the liquid before titration.) The main bulk of the contents of the vessels was filtered through muslin and made up to 250 cc., including the addition of 20 cc. of 50 % trichloroacetic acid. The filtrates from this were concentrated to about 25 cc. and after cooling, 4 cc. of 2 : 4-dinitrophenylhydrazine solution in HCl added to each.

Table IV gives the complete results of this experiment.

Table IV.

<i>Nitroprusside reactions.</i>				
Time after commencement of exp. (mins.)	A	B	C	D
40	-	-	-	+
175	+ -	+	-	++
240	-	++	-	++ -
300	-	++	+ -	++
<i>Iodine titrations for bound sulphite.</i>				
(5 cc. filtrate before deproteinisation was used; strength of iodine was 0.008 N.)				
	A	B	C	D
	0.15 cc.	1.05 cc.	0.10 cc.	1.10 cc.
<i>Extent to which precipitation occurred on addition of reagent.</i>				
	A	B	C	D
10 mins. after adding reagent	-	++	+ -	++
90 mins. after adding reagent	+	+++	++	+++
<i>Colour reactions of hydrazones with alcoholic potash.</i>				
A	B	C	D	
Blue-violet.	Deep red.	Blue-violet.	Deep red.	
Indicative of methylglyoxal	Indicative of pyruvic acid	Indicative of methylglyoxal	Indicative of pyruvic acid	

Here again it is seen that, in the presence of sulphite, pyruvic acid accumulates in quantities much greater than are found in the absence of the fixative. The colours given by the precipitates with 2 : 4-dinitrophenylhydrazine correspond exactly with the distribution of pyruvic acid as evinced by the nitroprusside tests. It is interesting also to note that in the absence of sulphite

methylglyoxal seems to preponderate over pyruvic acid. But the most important fact brought to light by this experiment is that in the presence of sulphite, the imposition of either aerobic or anaerobic conditions seems to make no difference to the appearance of pyruvic acid. This is of great significance, indicating as it does that the occurrence of pyruvic acid is not necessarily associated with oxidative processes, since these are excluded in C and D.

One explanation that suggests itself is that pyruvic acid (and methylglyoxal) may be intermediaries in the formation of lactic acid; indeed it seems difficult to place any other interpretation upon the results. If this is so, it should be possible to detect pyruvic acid among the substances present in an aqueous muscle extract during glycolysis. The next experiment to be done was then the following.

A. 100 cc. muscle extract 5 cc. phosphate buffer (p_H 7.2) (ten times strength) 3 cc. M Na_2SO_3 15 cc. 2 % glycogen 5 cc. water	}	Oxygenated	B. 100 cc. muscle extract 5 cc. buffer ($\times 10$) 3 cc. M Na_2SO_3 15 cc. water 5 cc. $M/30$ KCN	}	Anaerobic
C. 100 cc. muscle extract 5 cc. buffer ($\times 10$) 3 cc. M Na_2SO_3 15 cc. 2 % glycogen 5 cc. $M/30$ KCN	}			}	

The muscle extract was prepared from rabbit muscle in the manner described in a former paper [Case, 1929]. Nitroprusside tests were performed as usual at intervals, and at the conclusion all the fluids were precipitated with 10 cc. of 50 % trichloroacetic acid, filtered, concentrated and treated with *p*-nitrophenylhydrazine. Table V sets forth the results.

Table V.

Nitroprusside reactions.			
Time from commencement of exp. (mins.)	A	B	C
3	-	-	-
60	-	-	-
120	-	+	+
345	+	+	+++
Colour reactions of <i>p</i> -nitrophenylhydrazones (-osazones) with alcoholic potassium hydroxide.			
A	B	C	
Purplish-blue. Indicative of methylglyoxal with slight admixture of pyruvic acid	Deep blue. Indicative of methylglyoxal	Deep red, with very slight purplish tinge. Indicative of pyruvic acid, slightly contaminated with methylglyoxal	

In this experiment, we find once more an appearance of pyruvic acid under anaerobic conditions, though not so marked quantitatively as in the case where the minced tissue was present in the system. Moreover, the addition of glycogen (in C) has caused a rise in the amount of pyruvic acid formed. As up to the present no attempt has been made to repeat this, we hesitate to

place much reliance on the result; but the fact, if confirmed, that added glycogen does have this effect seems to support the possibility that pyruvic acid is concerned in the process of glycolysis.

An experiment was now carried out with the object of ascertaining whether the presence of added lactate in the solution would have any similar effect. If pyruvic acid is being formed during the oxidation of lactic acid it might be expected that this would be the case.

A. 70 g. minced muscle 12.5 cc. buffer ($\times 10$) 3.25 cc. $M Na_2SO_3$ Water to 125 cc.	}	Aerobic, at room temperature	C. As A	} Aerobic, in incubator
			D. As B	
B. 70 g. minced muscle 12.5 cc. buffer ($\times 10$) 3.25 cc. $M Na_2SO_3$ 10 cc. $M/10$ sodium lactate Water to 125 cc.	}			

Table VI.

Nitroprusside reactions.

Time after commencement of exp. (mins.)	A	B	C	D
0	++	++	++	++
110	+++	+++	+++	+++
180	++	++	++	++
240	++	+	+	+

No hydrazones were prepared in this case, as the filtrates were used for other purposes, but it is obvious that the addition of lactate has not brought about any increase in amount of pyruvic acid, such as was observed when glycogen was added.

Up to this point attention has been concentrated mainly upon the detection of pyruvic acid. The demonstration of the presence of methylglyoxal is a more difficult matter, apart from the isolation of the osazone, since no rapid test exists for its identification in solution, corresponding to the Simon and Piaux reaction for pyruvic acid. In order to effect a convenient separation of methylglyoxal from pyruvic acid we resorted in several experiments to the procedure of which the ensuing is a typical example.

A flask was set up containing 25 cc. buffer ($\times 10$), 6.5 cc. $M Na_2SO_3$, water to 250 cc. and 100 g. of minced muscle. This was allowed to incubate at room temperature for 5 hours. The nitroprusside reaction was then positive for pyruvic acid. The mixture was filtered through muslin, precipitated with trichloroacetic acid and again filtered. 20 cc. of 10 % sulphuric acid were added, and the whole of the fluid was distilled for an hour, the distillate being

Table VII.

	Product of interaction of 2 : 4-dinitrophenylhydrazine with	
	Residue in flask	Distillate
Colour of precipitate	Light yellow	Orange-red
Colour reaction with alcoholic potash	Deep red	Blue-violet

received in 10 cc. of 1 % sodium bisulphite cooled in ice. After cooling, both the residue in the flask and the distillate were treated with 2 : 4-dinitrophenylhydrazine (Table VII).

This shows that methylglyoxal has been distilled over, leaving pyruvic acid behind.

Attempts to demonstrate the presence of acetaldehyde in the distillate, where if formed it would be found, met with no success. The Rimini test was negative, and the characteristics of the hydrazones were not consistent with the presence of acetaldehyde (see Table II). If present at all, it can be only in very small amounts as compared with the amounts of methylglyoxal and pyruvic acid. Nitroprusside tests were strongly positive for the residue in the distilling flask, and negative for the distillate. Both fractions gave strongly positive iodoform reactions. In the case of a control experiment in which no sulphite was added, practically no nitroprusside test was given, and the iodoform reaction was only faintly positive.

It was considered desirable to perform a large-scale experiment with a view to isolating the derivatives of the products in sufficient quantity to carry out not only colour reactions but also melting-point determinations, in order to place their identification beyond question. The mixture employed consisted of 50 cc. phosphate buffer ($\times 10$), 25 cc. M Na_2SO_3 , water to 1 litre, 463 g. minced muscle. This was incubated at room temperature for 5 hours, at the end of which period a very strong nitroprusside test for pyruvic acid was given. The mixture was filtered through linen, using a press. Just over 1000 cc. of filtrate was obtained, and this was deproteinised with 100 cc. of 50 % trichloroacetic acid and filtered. The clear filtrate was again tested with nitroprusside and still gave a strongly positive reaction. It was then concentrated at about 40° *in vacuo* to a volume of 150 cc., more sulphite being added to ensure that the methylglyoxal and pyruvic acid should remain bound. After cooling, 40 cc. of 2 : 4-dinitrophenylhydrazine in HCl were added, the mixture was heated for a few minutes on a boiling water-bath and allowed to stand for some hours¹. A fairly bulky orange precipitate was obtained, which was filtered off and washed with a little 0.5 N HCl. The precipitate was treated with warm 25 % sodium carbonate, which dissolved the pyruvic acid derivative giving a dark-brown solution [Neuberg and Kobel, 1930]. On acidifying this solution with HCl, the hydrazone of pyruvic acid separated out as a lemon-yellow solid, which was further purified by re-solution in sodium carbonate and re-precipitation with acid, and finally by crystallisation from ethyl acetate.

Meanwhile the residue from the first sodium carbonate extraction, containing the methylglyoxal derivative, was washed once more with hot sodium carbonate to remove traces of the pyruvic compound, then with hot 2 N HCl, and lastly with boiling water. It was then dried with a small quantity of

¹ When sulphite is present, this heating is frequently essential in order that hydrolysis of the sulphite-complex may be effected. If it be omitted, precipitation may be delayed or even suspended altogether.

alcohol and ether and recrystallised from pyridine; a dark red crystalline mass was obtained which on further crystallisation from nitrobenzene appeared as light red glistening crystals. These were washed with alcohol and ether. Both the pyruvic acid hydrazone and the methylglyoxal osazone were dried for 24 hours in a vacuum desiccator.

Previously to this, specimens of the hydrazone and osazone had been prepared from actual pyruvic acid and from a solution of methylglyoxal (made according to Neuberg and Hofmann [1930]), and had been purified in the same way.

Melting-points:

(a) Pyruvic acid 2 : 4-dinitrophenylhydrazone (from pyruvic acid)	214°	(uncorr.)
Neuberg and Kobel [1929] give 216°, Allen [1930] gives 213°		
(b) Pyruvic acid 2 : 4-dinitrophenylhydrazone (from muscle) ...	214°	„
(c) Mixed melting-point	214–215°	„
(d) Methylglyoxal 2 : 4-dinitrophenylosazone (from methylglyoxal)	297.5°	„
Neuberg and Kobel [1928] give 298°		
(e) Methylglyoxal 2 : 4-dinitrophenylosazone (from muscle) ...	297°	„
(f) Mixed melting-point	296°	„

From a study of the foregoing Tables, notably III and VI, it will be observed that pyruvate, as demonstrated by the nitroprusside test, is sometimes to be met with in the solutions during the very early periods of the incubation. Where sulphite is present the quantity of pyruvate tends to increase as the incubation proceeds, but in the absence of sulphite the pyruvate progressively disappears. If this compound is indeed a transitory phase of lactic acid production, it is to be expected that immediately after a treatment such as mincing, which is strongly provocative of rapid glycolysis, the system will contain relatively large quantities of pyruvic acid. It is also apparent from the Tables that in the absence of a fixative this pyruvate very quickly disappears. It was therefore deemed of interest to try the effect of the addition of pyruvate to minced muscle both in presence and absence of sulphite, and the following experiment was accordingly performed.

A. 120 g. minced muscle	B. 120 g. minced muscle
25 cc. buffer (× 10)	25 cc. buffer (× 10)
5 cc. 1 % potassium pyruvate	5 cc. 1 % potassium pyruvate
Water to 250 cc.	6.5 cc. <i>M</i> Na ₂ SO ₃
	Water to 250 cc.

The experiment was aerobic. Nitroprusside tests were carried out as usual at intervals, the first test being made as soon as possible after setting up the mixtures. This time an attempt was made to render the tests to some extent quantitative.

For each test 5 cc. of the muslin filtrate were precipitated with 1 cc. of 50 % trichloroacetic acid. After filtering, 1 cc. of the clear solution was taken for estimation. The reagents employed were:

- (a) 40 % acetic acid;
- (b) freshly prepared 1 % aqueous sodium nitroprusside;

Although at the inception of this work frog muscles were exclusively used, and rabbit muscle was not employed till later on, it has been found more

convenient for the purposes of this paper to describe these latter experiments before those which are about to be reported. The methods used for detecting and identifying pyruvic acid and methylglyoxal are identical in the two series, and hence need not be further described in detail.

In all the experiments reported below, when muscles are referred to as "stimulated," the technique consisted in immersing the limbs in the appropriate solutions in small beakers, and, by means of a small induction coil, stimulating indirectly *via* the nerves. It has since been found more effective to apply direct stimulation, as in the presence of compounds such as sulphite the nerve seems to lose its irritability much sooner than does the muscle. Those portions of wire actually in contact with the muscle were always of silver. Where muscle is described as "chopped" it was dissected from the bone and chopped fairly finely with scissors. In aerobic experiments oxygen was bubbled through the solutions bathing the muscles, while in anaerobic ones the beakers containing the muscles were enclosed in a large vacuum desiccator on the bottom of which was a layer of alkaline pyrogallol, while the desiccator itself was filled with nitrogen by repeated evacuation and replenishment from a nitrogen cylinder. The wires for stimulation in such cases were led out through the rubber stopper.

It will be best, for the sake of brevity, to present the whole series of experiments in a consecutive manner and then discuss the significance of the results.

Exp. I.

- A. 20 cc. frog Ringer solution ($\times 10$)
 20 cc. phosphate buffer, p_H 7.2 ($\times 10$)
 Water to 200 cc.
 B. As A, with the addition of 5 cc. $M Na_2SO_3$

Two limb-pairs, severed at the girdle, were placed in each and were stimulated as long as irritability lasted. The experiment was aerobic and continued for 5 hours. The muscles were then ground with sand and water (15 cc.). Nitroprusside tests were done on the muslin filtrates.

	A	B
	-	+ + + +

Exp. II.

- A. 5 cc. frog Ringer solution ($\times 10$)
 5 cc. buffer ($\times 10$)
 5 cc. $M/10$ sodium lactate
 Water to 100 cc.
 B. As A, with the addition of 3 cc. $M Na_2SO_3$

In each beaker were placed the muscles from three limb-pairs, dissected carefully from the bones, but not chopped. Oxygen was bubbled for 4 hours, after which the muscles were ground with sand and trichloroacetic acid.

The nitroprusside reactions were negative for pyruvate in both cases.

The *p*-nitrophenylhydrazine derivatives were prepared; B yielded about twice as much as A. After washing, these both gave a strong pure blue colour

with alcoholic potassium hydroxide, indicating that the product was in each case methylglyoxal *p*-nitrophenylosazone.

Exp. III.

A and B. 10 cc. buffer ($\times 10$)
 10 cc. frog Ringer solution ($\times 10$)
 5 cc. *M*/10 sodium lactate
 2.5 cc. *M* Na_2SO_3
 Water to 100 cc.

One limb-pair was placed in each, the only difference being that the muscles in A were stimulated, while those in B were not. The experiment was aerobic and lasted for 5 hours. The muscles were then ground with sand and 15 cc. of their respective fluids.

Nitroprusside reactions:

A	B
+ + + +	+

The remainder of the filtrates were deproteinised with trichloroacetic acid, filtered, concentrated, and treated with *p*-nitrophenylhydrazine.

Colours with alcoholic potassium hydroxide:

A	B
Deep red. Indicative of pyruvic acid	"Mixed" colour, with preponderance of blue. Indicative of methylglyoxal, slightly con- taminated with pyruvic acid

Exp. IV.

A. 10 cc. buffer ($\times 10$) 3 cc. <i>M</i> Na_2SO_3 Water to 100 cc. Chopped muscle from one limb-pair	B. 10 cc. frog Ringer solution ($\times 10$) 3 cc. <i>M</i> Na_2SO_3 Water to 100 cc. One intact limb-pair, not stimulated
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The experiment was aerobic and lasted for 6 hours. Both lots of muscle were then ground with sand and water.

Nitroprusside reactions:

A	B
-	+ + + +

The remainder of the filtrates were deproteinised and treated with *p*-nitrophenylhydrazine.

Colours with alcoholic potassium hydroxide:

A	B
Deep blue. Indicative of methylglyoxal	Deep red. Indicative of pyruvic acid

Exp. V.

50 cc. frog Ringer solution ($\times 10$)
 12.5 cc. *M* Na_2SO_3
 0.25 g. NaHCO_3
 Water to 500 cc.

A. Two limb-pairs unstimulated in 100 cc. B. Two limb-pairs stimulated in 100 cc. C. Muscle removed from 4 legs (not chopped) in 50 cc.	}	Aerobic
D. Two limb-pairs unstimulated in 100 cc. E. Two limb-pairs stimulated in 100 cc. F. Muscle removed from 3 legs (not chopped) in 50 cc.	}	Anaerobic

The limbs were chosen so that the weight of muscle in each vessel was approximately the same (about 8 g.). The duration of the experiment was 2 hours; the muscles were then ground with sand and 15 cc. of water.

Nitroprusside reactions:

A	B	C	D	E	F
+	+++	+	++	+++++	+

Iodine titrations for bound sulphite (on 2 cc. of filtrate from ground tissue, and 5 cc. of fluids in vessels). Strength of iodine 0.008 *N*.

	A	B	C	D	E	F
Fluid	0.10	0.15	0.91	0.15	0.20	0.55
Tissue	3.60	3.60	2.80	4.30	4.60	2.50

These iodine figures bear out the results of the nitroprusside tests in that in D and E (the anaerobic whole limbs) the bound sulphite is greater in amount than in the corresponding aerobic limbs A and B.

They show also that in the case of intact muscles diffusion of the metabolic products into the surrounding fluid is almost negligible.

The main facts emerging from this series of experiments, which is of necessity as yet far from complete, are as follows.

(i) Stimulation always leads to an increased amount of pyruvic acid in the tissue (Exps. I, III, V).

(ii) Pyruvic acid can be demonstrated to occur even in the absence of sulphite (Exp. I).

(iii) Aerobic conditions are not essential for the production of pyruvic acid, which indeed on the other hand appears to be favoured by anaerobiosis (Exp. V).

(iv) As with rabbit muscle, methylglyoxal can also be detected (Exps. II, III, IV), and in presence of sulphite seems to be fixed to a greater extent (Exp. II). This compound appears to be produced under conditions which are not yet determined, but it is noteworthy that in cases where the muscle has been removed from the limbs, as in Exp. II, methylglyoxal always seems to occur rather than pyruvic acid. This has frequently been found to be the case in other similar experiments not reported here, but at present we have no idea of what the significance of this circumstance may be.

At all events, on the whole the experiments with frog muscle confirm strikingly the results obtained with rabbit muscle. The favourable influence of stimulation upon pyruvic acid formation is evidence for the intermediary occurrence of this substance during lactic acid production, as is also the absence of any sign of inhibition of its formation under anaerobic conditions.

DISCUSSION.

The results recorded in this paper are to be regarded as being of a preliminary nature only. Much more work will have to be accomplished before

the great technical difficulties associated with a problem of this kind can be surmounted to such an extent as to render the results quantitative.

We believe that we have fairly conclusively shown that both pyruvic acid and methylglyoxal do participate at any rate in some process or other occurring in muscle. With regard to the factors conditioning their appearance or non-appearance there is practically nothing that can at present be said with any degree of certainty. There can be no doubt that these substances in their relation to one another constitute part of a very delicately balanced system, the equilibrium of which is likely to be disturbed by seemingly slight variations in the surrounding medium and conditions. This relation, it is intended, will form the subject of further research.

That pyruvic acid is of importance in the metabolism of muscle is strongly suggested by the recent work of Toeniessen and Brinkmann [1930], who demonstrated a rapid removal of this substance when perfused through the musculature of a rabbit. Our own results with minced muscle confirm this rapid disappearance.

It has long been a widely-held view that methylglyoxal occupies an important position in the series of changes resulting in lactic acid production in muscle, and our results are in accord with this also.

Very many fresh experiments suggest themselves, which we propose to make the subject-matter of a future communication. Among these may be mentioned a study of pyruvic acid formation in muscle poisoned with iodoacetic acid, with fluoride and with amylase. The bearing of such experiments upon the question as to whether pyruvic acid and methylglyoxal are indeed being formed during the change carbohydrate $\rightarrow$ lactic acid is obvious.

The possibility must not be overlooked that lactic acid may be oxidised to pyruvic acid anaerobically in the presence of hydrogen acceptors in the muscle, or even that the source of the pyruvic acid may not be carbohydrate in nature at all, but that for instance this compound may be an intermediary in fat metabolism. On the whole, however, we are not inclined to regard either of these hypotheses as very probable.

SUMMARY.

1. The technique of the use of sodium sulphite as a fixative has been employed for investigating some of the substances concerned in the intermediary metabolism of muscle.

2. Pyruvic acid and methylglyoxal have been shown to occur in frog and rabbit muscle, both in the presence and absence of sulphite, and under both aerobic and anaerobic conditions. These substances have been isolated and identified as the 2 : 4-dinitrophenyl-hydrazone and -osazone respectively.

3. In view of the conditions, in so far as they have been established, under which these compounds make their appearance, it is suggested that they may both be intermediary between carbohydrate and lactic acid.

It is with much pleasure that we express our thanks to Sir F. G. Hopkins and Prof. J. B. S. Haldane for their interest in this work.

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