LACTIC ACID FORMATION IN MUSCLE EXTRACT.

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Several attempts had been made to show that an enzyme or group of enzymes capable of converting carbohydrate into lactic acid could be extracted from muscle tissue before it was successfully demonstrated by Meyerhof in 1926. In 1902, Stoklasa (14) and coworkers reported the separation of a ferment from extracts of lung and muscle which was said to convert added glucose into lactic acid, and in 1910, Ransom (12) claimed to have demonstrated the production of lactic acid from glucose by muscle plasma of the frog and fowl. Fletcher (6) could not confirm the work of either, and ascribed their results to bacterial action.

In 1912, Embden, Kalberlah, and Engel (3) determined the lactic acid formed spontaneously in muscle press juice and found that there was a definite increase after 1 to 3 hours incubation at 40°C. even though the lactic acid content of the juice was relatively high by the time its preparation at 0°C. had been completed. The addition of glucose, glycogen, d-alanine, and inositol did not affect the total amount of lactic acid formed, hence they concluded that the precursor of lactic acid was none of these. Embden suggested that the unknown precursor be called lactacidogen, and later (4) obtained data which allowed the conclusion to be drawn that this substance was hexose phosphate and that it was similar if not identical to the hexose diphosphate formed by yeast juice during incomplete fermentation of sugar (9). The spontaneous formation of lactic acid in the muscle press juice was accompanied by an increase in inorganic phosphate which corresponded to a ratio of 1 molecule of lactic acid to 1 molecule of phosphoric acid. An osazone which had the same melting point as the osazone of yeast hexose phosphate was obtained from
fresh muscle, hence it seemed likely that the formation of lactic acid was due to the cleavage of hexose phosphate in the muscle juice.

Laquer and Griebel (10) in investigating the effect of adding carbohydrate to frog muscle found that glycogen, yeast hexose phosphate, and starch greatly increased the production of lactic acid during 3 to 5 hours incubation at 28°C. α-Glucose was found to be more effective than β-glucose, but both were less effective than glycogen. No phosphate determinations were made.

In 1924 Embden and Hayman (5) studied the behavior of the inorganic phosphate in freshly prepared dog and rabbit muscle press juice and found that in the unmodified juice the phosphate increased about 50 to 100 per cent during 2 hours incubation at 40 to 45°C. If however sodium fluoride were added, the inorganic phosphate decreased and if both fluoride and glycogen were added the inorganic phosphate decreased to a mere trace. An osazone which had a melting point of 150° (comparable to the osazone of yeast hexose phosphate), and which had a phosphorus content that agreed with the theoretical requirement, was isolated from the incubated juice to which sodium fluoride and glycogen had been added. No data were given for any of the other substances (glycogen, glucose, and lactic acid), which presumably participate in the reaction involving the synthesis of hexose phosphate, and since the amount of inorganic phosphate which disappeared was greatly increased by the addition of glycogen, but not affected by the addition of glucose or maltose, it seemed that the ferment action involved the esterification of the sugar at the moment of the splitting of the glycogen molecule. The function of the fluoride seemed to be that of an esterase inhibitor, thus allowing the synthetic action to become more apparent.

Beattie and Milroy (1) determined the changes in phosphate, glycogen, and lactic acid which occurred in hash made from the muscles of dogs, cats, and rabbits. This work appears to be the first attempt by means of simultaneous determinations to correlate the interrelated changes which occur among the three metabolites. They have shown that under the influence of sodium fluoride a much greater amount of glycogen is lost than is accounted for by the amount of lactic acid formed plus the amount
of inorganic phosphate decrease. Unfortunately their results cannot be interpreted as expressing any definite relationship between the loss of carbohydrate and the formation of lactic acid and hexose phosphate since they made no determinations of free or total sugar in the hash at the end of the incubation periods. The amylolytic action of the muscle hash on the glycogen would conceivably account for the greater part of the glycogen loss.

The most recent contributions to the knowledge of the enzymatic formation of lactic acid in muscle hash or muscle extract are those of Meyerhof (11). In 1926 he announced the demonstration of a ferment in extracts obtained from frog and rabbit muscle that was capable of producing lactic acid from carbohydrate, particularly from glycogen and starch. He pointed out that the production of lactic acid from hexose phosphate by this ferment was slower than the production of lactic acid from either glycogen or starch and considered this to be evidence that hexose phosphate could not be looked upon simply as an intermediary product between glycogen and lactic acid in the sense in which it had been considered by Embden. Later (August, 1926) he found that the extract maintained its ability to split hexose phosphate after it had lost its activity toward polysaccharides through deterioration. The ability of extract made from frozen rabbit muscle to convert glucose and fructose into lactic acid was lost very quickly but could be restored by a coferment prepared from autolyzed yeast. This coferment was obtained by precipitating yeast autolysate with 50 per cent alcohol and purifying the precipitate by resolution in water and reprecipitation with alcohol. When a small quantity of this coferment was added to muscle extract which had lost its activity toward hexoses the activity was not only restored but even increased over its original value. Fructose was much more readily attacked than glucose under these conditions. His later findings are therefore more in harmony with Embden's theory that hexose phosphate is an intermediary between glucose or glycogen and lactic acid. Meyerhof considers that in order to explain all his results it is necessary to assume the existence of a labile hexose monophosphate as well as a stable diphosphate and that the diphosphate accumulates during the first period of the reaction in approximately equimolecular ratio to the monophosphate hydrolyzed. During the second
period of the reaction, if sugar is absent, the diphosphate is slowly broken down to free phosphate and lactic acid.

The work reported in this paper represents an endeavor to obtain data which would permit assigning more definite stoichiometric relationships between carbohydrate, lactic acid, and inorganic phosphate when these substances undergo changes in muscle extract. In order to make a comparison between the carbohydrate loss and lactic acid gain and at the same time correlate the changes which were taking place in the inorganic phosphate we made simultaneous determinations of the free sugar, total carbohydrate after acid hydrolysis, lactic acid, and inorganic phosphate.

Some preliminary experiments made with muscle hash, with extracts, residues of tissue from which the extracts were made, and with mixtures of extracts and residues, by the same general procedure described below showed the following facts. The addition of glycogen greatly increased the amount of lactic acid formed while the addition of glucose had little if any effect. When sodium bicarbonate buffer was used the decrease in total sugar approximately corresponded with the increase of lactic acid, but with phosphate buffer there was a greater loss of total carbohydrate than could be accounted for by the lactic acid formed. This indicated that the phosphate was responsible for the disappearance of a part of the carbohydrate, and in subsequent experiments determinations of the inorganic phosphate were made. These later experiments were limited to muscle extracts because the extract represented a simpler system, and the unavoidable errors of sampling a tissue hash were eliminated. The results obtained from extract were much more uniform than those obtained from tissue.

The muscle extracts were prepared by mixing 3 volumes of distilled water with 1 part (by weight) of ground rabbit muscle. The muscle was taken immediately after the killing (by bleeding) of the animal which had been anesthetized with amytal administered intraperitoneally (75 mg. per kilo of body weight). When anesthesia was complete the hind legs were put into a mixture of ice and water and allowed to become cool (about 15°C.) before the animal was killed. The hind legs were skinned as quickly as possible after death, and the muscles removed and ground
in an ice-cold meat chopper. This ground tissue was weighed and put into a measured quantity of distilled water previously cooled to 0°C. At the end of \( \frac{1}{2} \) hour at 0°C. the mixture was strained through unbleached muslin and the tissue residue squeezed out by twisting the cloth around it.

The extract obtained in this manner contained a few blood cells, was pink-colored, and somewhat turbid. Further attempts to purify it by centrifuging were not made since any delay resulted in considerable loss of its lactic acid-forming ability.

One experiment was performed with tissue extract obtained from an animal which had been killed instantly by a blow on the head without anesthesia. The results obtained were similar to those obtained from anesthetized animals except that the initial lactic acid content of the muscle and extract was much higher. It seemed desirable therefore to use amytal anesthesia so that the nerve impulses sent to the muscles during the death struggles would be blocked and a low initial lactic acid content thereby obtained. The grinding of the muscle did not cause the marked lactic acid formation which was produced by the convulsive movements.

Since a carbohydrate deficit appeared in the experiments performed with phosphate buffer, while all the carbohydrate was accounted for in those in which sodium bicarbonate was used, it seemed highly desirable to endeavor to determine the relationship of this phenomenon to the phosphate. If the carbohydrate were converted into hexose phosphate it would conceivably fail to reappear when the total carbohydrate is determined after acid hydrolysis. Fürth and Marian (8) have shown that when hexose diphosphate (yeast) is hydrolyzed by 23\% HCl for 3 hours only one-third of the theoretical amount of the hexose is obtained when it is determined by copper reduction. The carbohydrate which disappeared in our experiments, when an excess of phosphate was present, presumably could not be recovered completely as sugar but would be accounted for by a corresponding disappearance of inorganic phosphate.

EXPERIMENTAL.

50 cc. portions of extract were mixed with buffer and water or buffer and glycogen solution as follows:
Lactic Acid in Muscle Extract

<table>
<thead>
<tr>
<th>Flask No.</th>
<th>20 cc. buffer.</th>
<th>4 per cent glycogen solution.</th>
<th>Distilled water.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>B</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>&quot;</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>P</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>&quot;</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>6</td>
<td>&quot;</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

The buffer solutions were made as follows:

B. NaHCO₃........................................... 0.16
NaCl............................................. 0.20

P. NaHCO₃........................................... 0.12
K₂HPO₄........................................... 0.04
NaCl............................................. 0.20

F. Same as P plus NaF........................... 0.1

Immediately after mixing, a sample (15 cc.) was withdrawn from each and delivered into 20 cc. of 6 per cent HCl. This sample served as the control on the initial content of carbohydrate, lactic acid, and inorganic phosphate. The remainders of the mixtures were incubated in a water bath at 24.5°C. 15 cc. samples were withdrawn at 0.5, 1, 2, and 4 hour intervals, and treated in the same manner as the control samples.

Each sample was prepared for analysis as follows: The proteins were precipitated after acidification with HCl by the addition of 25 cc. of 5 per cent HgCl₂ according to the Schenck process. The tubes containing the precipitated protein mixture were allowed to stand overnight and then filtered. 10 cc. portions of each filtrate were diluted with an equal volume of 2 per cent HCl and hydrolyzed in a boiling water bath for 3 hours. The mercury was removed by H₂S and the H₂S removed by aeration. Total sugar determinations were made on the filtrates obtained from these hydrolyzed samples. The remainders of the original filtrates were freed from mercury in the same manner as the hydrolyzed portions, and determinations made for free sugar, lactic acid, and inorganic phosphate.

Sugar was determined by the Shaffer-Hartmann method (13),
lactic acid by the Friedemann, Cotonio, and Shaffer procedure (7), and phosphate by Briggs' modification of the Bell-Doisy method (2).

The effect of the presence of mercury in the hydrolyzing process for total sugar was ascertained by adding an amount of 5 per cent HgCl₂ solution comparable to that used in the experiments to a known solution of glycogen. This test was made for both pure glycogen and glycogen plus a small amount of protein material. The HgCl₂ had no effect on the results provided it were removed before sugar determinations were made.

All filtrates were neutralized with 55 per cent NaOH to approximately pH 5.0 before any of the determinations were made. The addition of the alkali increased the volume and introduced an error in the absolute values obtained which was in the opposite direction from the error caused by the concentration of the filtrates during aeration. No corrections have been applied for these errors because each sample was subject to the same treatment, hence the relative values are not affected. A consideration of other sources of error including the limitation of the analytical method indicates that they are too small (2 to 5 per cent) to affect the interpretation of the results obtained. The largest error is in the determination of free sugar in the samples which contained only a small amount of sugar. Mercuric chloride in acid solution does not remove all of the interfering reducing bodies, hence these values are high. This error does not materially affect the free sugar determinations where a relatively large quantity of sugar was present.

The experimental data obtained are given in Table I. Flasks 1 to 4 contained extract from one animal and Flasks 5 and 6 from another. A control on Flasks 5 and 6 containing glycogen but no fluoride was very similar to No. 4, and is not included because it furnishes no additional information.

**DISCUSSION.**

The interpretation of the data is concerned chiefly with the balance that may exist between the three metabolites studied, and is based on the assumption that the reaction involved proceeds in the following manner.

\[
\text{Glycogen or glucose } \rightarrow \text{ Hexose } \rightarrow \text{ Lactic acid } + \text{ Inorganic phosphate}
\]

\[
\text{Inorganic phosphate } \rightarrow \text{ Hexose phosphate } \rightarrow \text{ Lactic acid } + \text{ Inorganic phosphate}
\]
If glycogen (or glucose) passes through a phosphate complex in being converted into lactic acid by muscle enzymes, and if no other reactions involving carbohydrate, phosphate, and lactic acid...
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occur simultaneously, it should be possible to balance the molar equivalents of these substances according to the following equation.

\[
\text{Loss of total carbohydrate} - \text{Loss of inorganic phosphate by formation of hexose phosphate} + \text{Gain of lactic acid}
\]

Each mol of glucose lost is equivalent to 2 mols of lactic acid formed, or may require either 1 or 2 mols of phosphoric acid for esterification to give a monophosphate or a diphosphate. The values obtained for total carbohydrate must be used, since the free sugar is included in this figure. Furthermore, the free sugar represents merely that portion of the glycogen which was converted into reducing sugar (calculated as glucose) by the amylolytic action of the muscle extract and escaped conversion into lactic acid.

The total carbohydrate (calculated also as glucose) represents the copper reduction value obtained after hydrolysis by acid of all polysaccharides present. This value is accurate for the glycogen and free sugar present at the beginning of the incubation periods, but after the incubation is in progress the results become less certain because the amount of reducing sugar obtained from the hydrolysis of the carbohydrate involved in the phosphate complex is not accurately known. Since only one-third of the glucose in the phosphate complex of yeast is recovered by acid hydrolysis (8), it seems likely that hydrolysis of the phosphate complex of muscle may yield only a part of its carbohydrate as reducing sugar. This possible source of sugar has been ignored in the trial calculations of the relationship represented by the equation given above.

The values for the substances studied are expressed in millimols to facilitate comparisons. The data given in Table I show the total amounts found in the samples analyzed for the different time intervals, while Table II and Fig. 1 summarize the changes which occurred during the entire 4 hour period.

Flasks 1 and 2 contained only the phosphate which was present in the muscle extract, and Nos. 3 to 6 had an amount of phosphate added which approximately doubled that originally present.

The changes which occurred in Flasks 1, 3, and 5 are so small that it is doubtful whether the figures can be applied to the hy-
Lactic Acid in Muscle Extract

Theoretical equation. It is noteworthy that the increase of lactic acid, which occurred in Flasks 1 and 3, was prevented by the fluoride in No. 5. The changes which occurred in Flasks 2, 4, and 6, to which glycogen was added, have sufficient magnitude to justify an attempt to fit them into the equation. There was considerable disappearance of total carbohydrate in them, which is accounted for only in part by the lactic acid formed. This is particularly noticeable in Flask 6, in which the formation of lactic acid was almost completely inhibited by the fluoride. Here, the loss of carbohydrate occurred and the decrease in inorganic phosphate is more marked than in the absence of fluoride.

Table III gives a summary of the calculation. The gain of

\[
\begin{array}{|c|c|c|c|}
\hline
\text{Flask No.} & \text{Substances added} & \text{Lactic acid} & \text{H}_2\text{PO}_4 & \text{Free sugar} & \text{Total sugar} \\
\hline
1 & \text{None.} & +0.05 & +0.02 & 0 & -0.01 \\
2 & \text{Glycogen.} & +0.14 & -0.07 & +0.08 & -0.12 \\
3 & \text{Phosphate.} & +0.05 & +0.01 & 0 & -0.01 \\
4 & \text{Glycogen and phosphate.} & +0.15 & -0.11 & +0.11 & -0.18 \\
5 & \text{Phosphate and fluoride.} & -0.01 & -0.01 & 0 & -0.02 \\
6 & \text{Glycogen, phosphate, and fluoride.} & +0.01 & -0.22 & +0.09 & -0.15 \\
\hline
\end{array}
\]

+ gain, − loss.

Lactic acid expressed as equivalent millimols of glucose (\( \text{mm lactic acid} \div 2 \)) is subtracted from the loss of total sugar and gives the amount of carbohydrate presumably present as a phosphate complex. This value can be compared with the decrease of inorganic phosphate.

The deficit of sugar is in fairly close molecular agreement with the loss of phosphate, and the calculation is therefore consistent with the idea that the carbohydrate may have been present in a complex, equivalent to hexose monophosphate. In order to justify this agreement it is necessary to assume that sugar was not liberated from this complex during acid hydrolysis, for if such had occurred, and the liberation had been quantitative there would have been no carbohydrate deficit. The loss of phosphate,
however, would have occurred since phosphate determinations were made on the unhydrolyzed samples. Satisfactory phosphate determinations were not obtained on hydrolyzed material because so much phosphate was liberated by hydrolysis that it seemed likely that there was some source of phosphate other than that associated with the carbohydrate changes.

It is not possible to decide the question involved with the data available at present, but we can tentatively interpret the results

as supporting the hypothesis that glycogen is converted into a phosphate complex, the composition of which corresponds to hexose mono- or diphosphate. The enzymatic hydrolysis of the complex yields 2 molecules of lactic acid and 1 or 2 of inorganic phosphate. The rather remarkable effect of fluoride in preventing the hydrolysis of the complex by the enzyme should be the means of producing an accumulation which may lead to its isolation. Efforts to do this are being undertaken.
Lactic Acid in Muscle Extract

SUMMARY.

1. Simultaneous determinations of lactic acid, inorganic phosphate, free sugar, and total carbohydrate were made on incubating mixtures of fresh muscle extract in a buffered solution.

2. The addition of glycogen to the extract caused a marked increase in lactic acid formation, and usually a decrease in the inorganic phosphate. The amount of free sugar increased as a result of hydrolysis of the glycogen by the amylolytic ferment of the muscle.

3. The loss of carbohydrate from mixtures to which glycogen was added was about twice as great as the amount of lactic acid formed.

4. The addition of phosphate in sufficient quantity to double the amount naturally present in the extract caused only a slight increase in the amount of lactic acid formed and carbohydrate used, provided glycogen also were added. If no glycogen were added no noteworthy changes took place.

5. The addition of fluoride prevented the formation of lactic acid and augmented the decrease in inorganic phosphate, but did not prevent the decrease in total carbohydrate.

6. The difference between the carbohydrate loss and lactic acid formed was approximately equal to the loss of phosphate. This relationship suggests the formation of a carbohydrate-phosphate complex.

7. Our results confirm those obtained by Embden and by Meyerhof, and show in addition that there is a simultaneous decrease in carbohydrate when the free phosphate decreases and lactic acid is formed.

<table>
<thead>
<tr>
<th>Flask No.</th>
<th>2</th>
<th>4</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substances added</td>
<td>Glycogen</td>
<td>Glycogen and PO₄</td>
<td>Glycogen, PO₄, and NaF</td>
</tr>
<tr>
<td>Decrease of total sugar, mmo</td>
<td>0.12</td>
<td>0.18</td>
<td>0.15</td>
</tr>
<tr>
<td>Gain of lactic acid ÷ 2</td>
<td>0.07</td>
<td>0.075</td>
<td>0.005</td>
</tr>
<tr>
<td>Differences</td>
<td>0.05</td>
<td>0.105</td>
<td>0.145</td>
</tr>
<tr>
<td>Decrease of H₂PO₄</td>
<td>0.07</td>
<td>0.11</td>
<td>0.22</td>
</tr>
</tbody>
</table>

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