CX. LACTIC ACID FORMATION IN MUSCLE EXTRACTS.

II. THE EFFECT OF SODIUM HEXOSEDIPHOSPHATE ON THE RATE OF ESTER ACCUMULATION DURING THE INCUBATION OF GLYCOGEN IN CERTAIN TYPES OF EXTRACTS.

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IN the first paper of this series it was stated that by a modification of the conditions in an extract, the balance between ester formation from glycogen and the co-existing breakdown of that ester could be changed. One method of producing this is by adding sodium hexosediphosphate along with the glycogen, and this method is described in detail in this paper.

The idea that prompted these experiments was the following. Sodium hexosediphosphate when incubated alone in muscle extract shows a steady liberation of inorganic phosphate and lactic acid, as Embden [1914] first showed. Glycogen, in the type of extract used here, shows the same steady rate of lactic acid formation, but in this case the accompanying phosphate changes are of three types, depending on the glycogen concentration and also on the nature of the extract. This classification is based on the results of investigations with about a hundred different extracts. These three types are indicated in Fig. 1: the graphs show the changes in inorganic phosphate plotted against the time of incubation.

Type 1. In this type there is no ester accumulation even with concentrations of glycogen as great as 0.8 %. The phosphorus concentration remains practically the same until all the glycogen has been used up, after which the phosphate increases. This increase is from pre-existing lactacidogen in the extract.

Type 2. In this type, no ester accumulation takes place during the first 30 to 40 minutes of the incubation, but thereafter an extensive and rapid accumulation takes place between 40 and 90 minutes from the start. This type is often produced by concentrations of glycogen of the order of 1.0 % in extracts that give a "type 1" curve with about 0.5 % glycogen.

Type 3. In this type the ester accumulation is intense at the very start, is completed in most cases in the first 10 to 20 minutes of the incubation period and is followed by a second period of breakdown of the ester.

The inorganic phosphate liberated in an extract of the type used here, to which no carbohydrate has been added, ranges from 25 to 35 mg. per 100 cc. This process is completed in about 60 minutes. This would indicate a certain concentration of lactacidogen because the liberation of the phosphate is always accompanied by lactic acid formation and the two processes slow down and stop together. It seemed not at all unlikely that the difference between a "type 3" and a "type 1" extract, for example, lay in the fact that the latter

Fig. 1. Inorganic phosphorus changes.

Z. Plain sodium hexosediphosphate.

No. 1 type with glycogen.

No. 2 " "

No. 3 " "

type contained as a rule more lactacidogen than the former. The "phosphagen" content is likewise variable, but the present communication excludes investigations on the possible rôle of this labile phosphorus compound in this connection. This has been made the subject of investigation for a time in this laboratory and the results will be recorded in a subsequent communication. Considered here is solely the possible influence of the acid-stable lactacidogen and the related substance hexosediphosphate.

It appeared quite possible that if the lactacidogen were broken down at the same rate as the ester was being formed from the glycogen, the phos-

phorus liberated from the lactacidogen could be passed on to the glycogen (or to whatever breakdown product is converted into the ester) at a rate sufficient to keep the ester formation going at its maximum rate. This would spare, so to speak, the inorganic phosphate of the extract from taking part in this process, the concentration of inorganic phosphate would not change and the flat "type 1" curve would be produced. If the lactacidogen were not present to a great extent at the start, then the phosphorus liberated might not be set free at a rate great enough to keep the rate of ester formation at its maximum, and a call would then be made on the inorganic phosphate of the extract and thus a "type 3" curve might result. It was thought that, if, in the case of an extract yielding a "type 3" curve with glycogen, sodium hexosediphosphate were added along with the glycogen, the liberation of phosphate from this ester might be sufficiently rapid to balance the removal of phosphate by the esterification process, and thus a "type 3" curve might be converted into a flat "type 1" curve. The assumption made throughout is that lactacidogen or hexosediphosphate breakdown takes place in the presence of glycogen and simultaneously with the formation of ester from the latter. The phosphate curve is thus assumed to represent the balance between phosphoric ester breakdown and synthesis. As the experimental results will show, the production of a "type 1" curve is not conditioned by a high initial concentration of phosphoric ester; on the contrary the presence of a phosphoric ester along with glycogen either has no effect or leads to a decided accumulation of ester. Contrary to expectation a "type 1" curve is converted into a "type 3" curve.

EXPERIMENTAL PROCEDURE.

The extracts were prepared in exactly the same manner as those described in the first paper of this series, and when any special treatment is given it is stated. Two preparations of hexosediphosphate were used. One was prepared in this laboratory according to a private communication from Prof. Harden, using a dried yeast from the local brewery. The other preparation was a gift to the department from Bayer Products, Ltd. Both preparations yielded the same results.

The glycogen was added either as a 20 % or a 10 % suspension made up in boiling water. The sodium hexosediphosphate was added in the case of the Bayer product as a 20 % solution; in the case of the author's preparation, the strength of the stock solution, concentrated by distillation under reduced pressure, was determined from the difference between the inorganic phosphorus and the total phosphorus as estimated by the nitric acid method of wet ashing. In the case of the plain glycogen controls, water was added equivalent to the volume of the zymophosphate. The addition of these solutions and the method of sampling were as described in the previous paper.

EXPERIMENTAL RESULTS.

In Exp. 217, a typical case, the phosphorus changes are represented in Fig. 2. All inorganic phosphorus concentrations are expressed as mg. per 100 cc. extract. Lactic acid estimations were not made in several of these preliminary experiments.

In Exp. 218 (Fig. 3) the presence of the zymophosphate shifted the period of maximum ester accumulation to an earlier point in the incubation. The higher concentration of zymophosphate is more effective than the lower.

In Exp. 219 the following were tried. Glycogen 0.33 % alone and with 1.2 % zymophosphate; glycogen 0.66 % alone and with 0.6 % and 1.2 % zymophosphate; glycogen 1.3 % alone and with these same two concentrations of zymophosphate. In those cases where the glycogen concentration

Fig. 2. Exp. 217.

- A. Zymophosphate 1.0 % alone.
- B. Glycogen 0.65 % alone.
- C. Glycogen 0.65 % with zymophosphate 1.0 %.

was 0.33 % or 0.66 %, none of the zymophosphate concentrations was effective. In the case of 0.33 % glycogen with 1.2 % zymophosphate, there was more phosphate liberated in the later stages of the incubation than with glycogen alone, indicating at this stage that, after all the glycogen had been broken down, the zymophosphate was hydrolysed. The results with the 1.3 % glycogen are shown in Fig. 4.

In Exp. 224, one-half of the extract was acidified to about p_H 6.5 by HCl, the molar equivalent of NaCl being added to the other half which was adjusted to p_H 7.5. The results are shown in Fig. 5. The acid extract gave the better result; the glycogen curve is a typical "type 1" curve and the effect of the zymophosphate is very decided.

In the next set of experiments the lactic acid formation was followed in addition to the changes in inorganic phosphorus. The lactic acid was estimated by the method mentioned in the preceding paper. The results of Exp. 238 are not given in detail. The phosphorus changes in a mixture of 1.5 % zymophosphate and 0.7 % glycogen were very similar to those in the 0.7 % glycogen alone. Nevertheless it was noticed that the total lactic acid formed with glycogen alone in 90 minutes was 167 mg. per 100 cc. extract and in the glycogen-zymophosphate mixture only 130 mg., a decrease of 22 %.

Fig. 3. Exp. 218.

- A. Glycogen 0.6 % alone.
 B. " 0.6 % with zymophosphate 0.6 %.
 C. " 0.6 % " 1.2 %.

In Exp. 240 glycogen 0.9 % alone and in conjunction with 1.0 % zymophosphate was investigated. There was a slight "extra ester accumulation" in this case associated with a 30 % decrease in the lactic acid production. The lactic acid produced in the plain glycogen was 130 mg. and in the glycogen plus zymophosphate mixture only 90 mg. on incubation for 90 minutes.

Exp. 241 was, however, a good case. Two concentrations of glycogen were tried with 1.0 % zymophosphate. In the case of 0.4 % glycogen, the zymophosphate did produce extra ester equivalent to 26 mg. of phosphorus. At the end of 60 minutes, the lactic acid increase in the plain 0.4 % glycogen was 226 mg. and in the corresponding glycogen-zymophosphate mixture only 167 mg., a decrease of 26 %.

Fig. 4. Exp. 219.

- A. Glycogen alone 1.3 %.
 B. " 1.3 % with zymophosphate 0.6 %.
 C. " 1.3 % " 1.2 %.

Fig. 5. Exp. 224.

- { A. Glycogen 0.9 %, p_H 7.5.
 { B. " 0.9 % with zymophosphate 1.5 %, p_H 7.5.
 { C. " 0.9 %, p_H 6.5.
 { D. " 0.9 % with zymophosphate 1.5 %, p_H 6.5.

The results with 1.0 % glycogen alone and with 1.0 % zymophosphate with the extract of Exp. 241, are shown in the graphs of Figs. 6 and 7. It will be seen that the inhibition of the lactic acid formation by the zymophosphate comes decidedly into evidence after the first 10 minutes of the incubation and when the "extra ester accumulation" effect begins to appear. In this case the 1.0 % glycogen alone showed at the end of 90 minutes a production of 439 mg. lactic acid, the glycogen-zymophosphate mixture only 171 mg., a decrease in this case of 61 %. Although, in this case, the

Fig. 6. Exp. 241. Lactic acid.

A. Glycogen 1.0 % alone.

B. Glycogen 1.0 % with zymophosphate 1.0 %.

inhibition of the lactic acid formation is related to the "extra ester accumulation," inhibition of the lactic acid production is not apparently always accompanied by any decided effect of the zymophosphate on the phosphorus changes in the controls.

In addition to the above observations the following was noted. Using 3 cc. of the trichloroacetic filtrates which were used for the inorganic phosphate estimations and adding 0.3 cc. of $N/50$ iodine, the reddish brown colour of the glycogen iodine compound is produced. By comparing in a comparator the colour of the corresponding samples of plain glycogen and of glycogen-zymophosphate mixture, a decided diminution in the strength of the colour

is readily detected in those cases where "extra ester accumulation" is produced to a decided extent by the zymophosphate. In addition, therefore, to the inhibition of the lactic acid formation, there occurs this phenomenon of more rapid loss of glycogen.

Fig. 7. Exp. 241. Inorganic phosphorus.
 A. Glycogen 1.0 % alone.
 B. Glycogen 1.0 % with zymophosphate 1.0 %.

SUMMARY.

With the type of extract used the following has been noted. In a mixture of zymophosphate and glycogen in certain concentrations, there is less lactic acid produced than in the corresponding glycogen concentration in the absence of zymophosphate. This is associated, not invariably but very frequently, with an increased accumulation of phosphoric ester in the glycogen-zymophosphate mixture as compared with the corresponding glycogen experiment. This "extra ester accumulation," due to the presence of zymophosphate, is also associated with a more rapid loss of glycogen in addition to the above-mentioned inhibition of the lactic acid formation.

In concluding the author wishes to express his indebtedness to Bayer Products, Ltd., for a gift of 100 g. of pure sodium hexosediphosphate.

REFERENCE.

Embden (1914). *Z. physiol. Chem.* **93**, 1.