LXXV. STUDIES IN GLYCOLYSIS
II. THE FIRST STAGES OF GLYCOLYSIS IN MUSCLE EXTRACTS

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In a previous communication [Kendal & Stickland, 1937, 1] it was shown that if rabbit muscle is minced and thoroughly washed with water, and then extracted several times with dilute Na_2HPO_4, the later extracts (usually the 4th) contain much of the glycolytic enzyme system but are freer from coenzymes than extracts prepared by earlier methods. This was shown by the fact that on addition of boiled muscle extract a rapid formation of lactic acid from polysaccharide took place, while on addition of Mg^{++}, adenosinetriphosphate, cozymase and hexosediphosphate practically no lactic acid was formed. It was also shown that if Mg^{++} and adenosinetriphosphate alone were added to such extracts considerable quantities of phosphate were esterified, although no lactic acid was formed. The present communication deals with the nature of the esters formed under these conditions.

The initial esterification process in the glycolytic enzyme system prepared by the method of Meyerhof [1925] has been studied by Parnas and his collaborators [Parnas & Baranowski, 1935; Ostern *et al.* 1936]. They found that a direct reaction between glycogen and inorganic phosphate takes place, giving rise to the Embden ester. Later Parnas & Mochnacka [1936] showed that by suitable treatment (electrodialysis at *p*H 8·2, or autolysis at 20° followed by long dialysis) extracts could be obtained which no longer phosphorylysed glycogen except on addition of adenylic acid or inosinic acid. Since the original extracts were thought to be free from adenosine phosphates it was supposed that they must have contained inosinic acid. As will be seen later esterification may be brought about by a concentration of adenosinetriphosphate or adenylic acid much too low to be detectable by chemical methods.

Cori & Cori [1936; 1937] discovered, as an intermediate step in the formation of the Embden ester, a new ester which they identified as an aldose-1-monophosphate, and later as phosphoric acid α-glucoside. This ester was formed under the action of washed muscle or pulp or ordinary dialysed (17 hr.) aqueous muscle extract, and was rapidly transformed by the same enzyme preparations into the ordinary Embden ester. The addition of adenylic acid increased the rate of formation of this new ester.

Our experiments with the new enzyme preparations confirm and extend the results of Parnas and of Cori & Cori.

Experimental

Preparation of extracts. The enzyme solutions were made as described in the previous paper [Kendal & Stickland, 1937, 1]. The 4th phosphate GP4 extract was used in nearly all the experiments, but for some purposes the 5th (GP5) and in one the 6th (GP6) proved more suitable.

Method of experiment. The solution incubated contained 2 ml. of the enzyme solution in a total volume of 5 ml. It also contained 0·5 % starch or glycogen, or other substrate, 0·25 % $NaHCO_3$, about 3·5 mg. inorganic P per 5 ml. and the necessary coenzyme solutions. All additions were brought to *p*H 7·5. The mixtures were incubated in an atmosphere of $N_2 + 5$ % CO_2 at 37° unless otherwise stated, Barcroft manometer vessels being used for convenience. At the end of the experiment the contents of each vessel were made up to 25 ml. with 5 % trichloroacetic acid and filtered.

In some cases, when the time course of the reaction was to be followed, a large portion of the reaction mixture (usually 30 ml.) was made up in a 100 ml. flask. Four such flasks could be incubated with shaking and continuous gassing on a special stand in the water bath: 5 ml. samples were withdrawn at the required times and made up to 25 ml. with 5 % trichloroacetic acid. As a check against possible evaporation from the solution, in several experiments total P estimations were done on all the samples, and it was found that no appreciable change occurred.

Determinations. All P determinations were done by the method of Fiske & Subbarow [1925]. Initial values were obtained either by determinations on the separate components of the mixtures, or by making up a blank experiment and treating it at once with trichloroacetic acid.

The total amount of ester formed was measured by the decrease in organic P, glucose-1-monophosphate[1] by the P liberated on hydrolysis for 10 min. in *N* HCl at 100°, and hexose-6-monophosphates by the difference between these two. This assumption that no ester but glucose-1-monophosphate and Embden ester was formed, was justified in two ways. First, hexosediphosphate was shown not to be formed by testing for alkali-labile P (triose-P), which was invariably absent. This test depends for its validity on the presence of sufficient cozymohexase to allow the equilibrium to be rapidly reached. That the extracts satisfy this condition was shown in our previous paper [Kendal & Stickland, 1937, 1] and it has since been confirmed. Secondly, the ester not hydrolysed in 10 min. was hydrolysed in 4 hr. at 100° by *N* HCl to the extent of 30 % (for the Embden ester Cori & Cori give 28 %, Ostern *et al.* 34·5 % and our own results 25 %). When a large amount of Embden ester was present, the results were corrected to allow for the fact that 3 % of this ester-P is hydrolysed in 10 min. in *N* HCl at 100°.

In experiments with adenosinetriphosphate the results are worked out on the assumption that no ATP is hydrolysed in the course of the experiment. This is not a valid assumption, but the error introduced is not appreciable except in experiments with very high initial ATP concentration and long incubation time.

Results

The formation of CE from carbohydrate

The indispensability of coenzymes. The enzyme-substrate-buffer mixture was incubated with $MgCl_2$, with ATP and with the two together. The concentrations and the results are given in Table I.

[1] The following abbreviations will be used:

Glucose-1-monophosphate = Cori ester	= CE
Hexose-6-monophosphates = Embden ester	= EE
Hexosediphosphate	= HDP
Adenosine triphosphate	= ATP
Adenylic acid	= AA
Inosinic acid	= IA

Table I

Enzyme	Coenzymes	Ester formed in 1 hr. (mg. P/5 ml.) Total	CE
GP5	$MgCl_2$, M/250	0·01	0·00
	ATP, M/10,000	0·09	0·09
	Mg^{++} + ATP	0·54	0·54
GP4	$MgCl_2$, M/250	0·00	0·00
	ATP, M/20,000	0·10	0·07
	Mg^{++} + ATP	0·51	0·51

GP4 preparations appeared to contain variable amounts of Mg^{++}, sometimes enough for considerable activation by addition of ATP alone. On the average, addition of the optimal concentration of $MgCl_2$ caused a threefold increase in the rate of esterification.

Adenylic acid[1] at optimal concentration was found to be as active as adenosinetriphosphate.[2] Inosinic acid showed some slight coenzyme activity at high concentration (Table II).

Table II

Exp.	Coenzymes	Time of incubation min.	Esters formed (mg. P/5 ml.) Total	CE
1	$MgCl_2$, M/250	40	0·00	0·00
	,, + ATP, M/10,000	40	0·76	0·72
	,, + AA, M/1000	40	0·82	0·81
	,, + IA, M/1000	40	0·12	0·08
2	$MgCl_2$, M/250 + ATP, M/10,000	80	0·80	0·75
	,, + AA, M/2000	80	0·83	0·78
	,, + IA, M/1000	80	0·07	0·00
3	$MgCl_2$, M/250 + ATP, M/7500	120	0·85	—
	,, + AA, M/2000	120	0·84	—
	,, + IA, M/1000	120	0·06	—

The course of the reaction. When the amount of ester formed at different times was measured, it was found that the reaction proceeds very rapidly at first, but is approximately linear only for about 10 min., and after 1 hr. is very slow (see Fig. 1). That the reason for this slowing off is not the destruction of the coenzyme is readily shown in four ways. (1) The curve is of the same shape at all ATP concentrations (see Fig. 2). (2) ATP is dephosphorylated only slowly by the enzyme preparation, so that an adequate concentration for maximal esterification is still present even when the reaction has practically stopped. (3) If more ATP is added after 30 min., no increase in esterification is observed over a control (see Fig. 1). (4) If the mixture is incubated with no ATP for 30 min., and ATP then added, very little ester is formed (see Fig. 1). The only conclusion that can be reached is that the enzyme responsible for the conversion of starch into CE is rapidly destroyed during incubation at 37°.

On the other hand Kendal & Stickland [1937, 1] showed that if the enzyme is incubated with Mg^{++} + ATP, the addition of boiled muscle extract at any

[1] We wish to thank Prof. J. K. Parnas for samples of adenylic acid and very pure barium inosinate, and Dr D. M. Needham for another sample of adenylic acid.

[2] In a preliminary communication [Kendal & Stickland, 1937, 2] it was stated that adenylic acid could not replace ATP in this reaction. In arriving at this conclusion we were misled by statements in the literature to the effect that the products of acid hydrolysis of ATP are adenylic acid and orthophosphate. As no pure adenylic acid was available, such hydrolysates (7 min. in N HCl at 100°) of ATP were used. When we became aware of the contrary experience of Parnas & Mochnacka [1936] and Cori & Cori [1936] with adenylic acid, a reinvestigation showed us that the solutions we had previously used contained only adenine and pentosephosphate.

time up to 1 hr. caused rapid lactic acid formation. This reaction must be due initially to lactic acid formation from the preformed monophosphoric esters, though it may be supplemented later by further esterification coupled with the

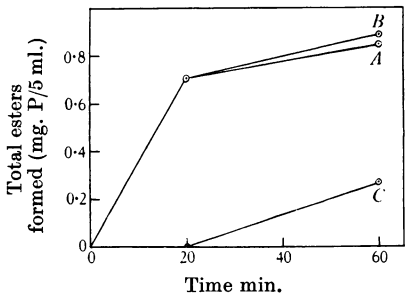

Fig. 1.

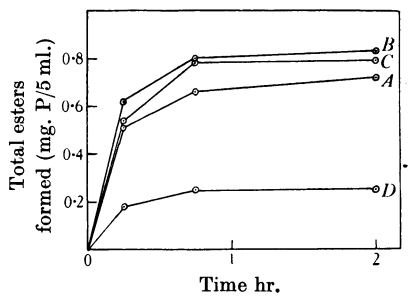

Fig. 2.

Fig. 1. Course of esterification. Curve *A*, ATP (10^{-4} *M*) present from the beginning. Curve *B*, ATP (10^{-4} *M*), further ATP (10^{-4} *M*) added after 20 min. Curve *C*, ATP (10^{-4} *M*) first added after 20 min. incubation.

Fig. 2. Course of esterification at different concentrations of ATP. Curve *A*, 5×10^{-4} *M*; Curve *B*, 10^{-4} *M*; Curve *C*, 2×10^{-5} *M*; Curve *D*, 4×10^{-6} *M*.

oxidoreduction reaction between triosephosphate and pyruvic acid [Meyerhof & Kiessling, 1935]. In either case it is clear that the whole enzyme system is stable at 37° except that part which brings about the nucleotide-catalysed phosphorylation of carbohydrate.

The importance of these experiments at the present juncture is merely to show that in kinetic experiments, when ATP is used as coenzyme, incubation periods up to 1 hr. may be used, even though the course of the reaction is not linear, i.e. total esterification in 60 min. is proportional to the initial velocity of esterification.

Concentration of ATP. The effect of variation of the concentration of ATP on the rate of esterification was tested in a number of preparations. The Mg^{++} concentration was *M*/250 in every case. All the results obtained with 11 out of 15 preparations tested are given in Fig. 3. It will be noted that 50 % of the maximum rate is obtained at a concentration of 10^{-5} *M*, and a measurable rate at 2×10^{-6} *M*. The other 4 preparations gave points lying to the left of this curve. The significance of this occasional variation in the ATP requirement is not known. It is not due to a more rapid decomposition of ATP, for one preparation which gave a discrepant concentration curve was found to hydrolyse ATP even more slowly than some of those which gave the more usual concentration curve.

Concentration of AA. Fig. 3 (curve *B*) shows the effect of varying the concentration of AA at constant Mg^{++} concentration (*M*/250). The amount required is fairly constant in all the preparations tested, and is about seven times that of ATP. This might be due to deamination of AA, for ATP is not deaminated till after dephosphorylation. It is impossible to measure the rate of deamination of AA at the concentrations concerned (*M*/10,000 or less), but indirect evidence suggests that this explanation is the correct one. At high concentrations of ATP or AA, and at low concentrations of ATP, the course of the esterification follows a similar curve, falling off at the same rate in each case. At low concentrations of AA, on the other hand, the esterification comes to a sudden stop

after a very short time (Fig. 4), suggesting that, apart from the inactivation of the enzyme, there has been decomposition of the coenzyme to an inactive product, in this case presumably inosinic acid. It is therefore impossible to say whether the different affinity curves of ATP and AA represent a genuine

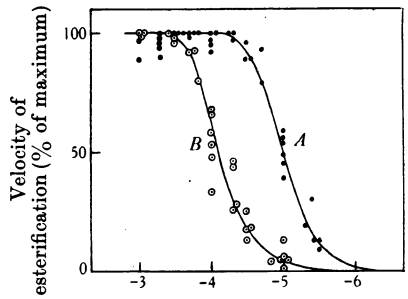

Fig. 3.

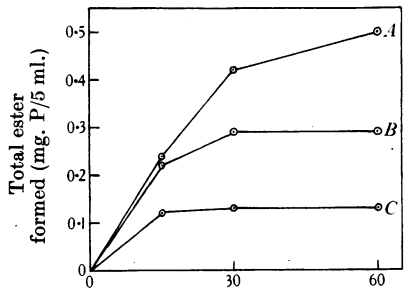

Fig. 4

Fig. 3. Relationship between concentration of coenzyme and velocity of esterification. Curve *A* adenosine triphosphate; Curve *B*, adenylic acid.

Fig. 4. Course of esterification at low concentrations of adenylic acid. Curve *A*, ATP 5×10^{-5} *M*; Curve *B*, AA 10^{-4} *M*; Curve *C*, AA 5×10^{-5} *M*.

difference of affinity, or whether they are entirely accounted for by deamination of AA. Curve *B* of Fig. 3 is certainly to some extent misleading since the velocities recorded at the lowest concentrations of AA are *not* proportional to the initial velocities.

Concentration of IA. IA is only very slightly active even at *M*/1000, and at greater dilution of course shows no activity (Table III).

Table III

Enzyme	Coenzyme	Incubation time min.	Esterification total (mg. P/5 ml.)
GP4	Mg^{++} + IA, *M*/1000	60	0·07
	,, + IA, *M*/3000	60	0·01
	,, + IA, *M*/10,000	60	0·00
	,, + ATP, *M*/10,000	60	0·55

Mixtures of the three nucleotides show no additive effects (Table IV).

Table IV

Exp.	Coenzymes*	Incubation time min.	Esterification (mg. P/5 ml.) Total	CE
1	ATP, *M*/5000	20	0·55	0·54
	AA, *M*/5000	20	0·49	0·45
	ATP + AA	20	0·54	0·52
2	ATP, *M*/5000	60	0·74	0·66
	IA, *M*/1000	60	0·06	0·02
	ATP + IA	60	0·73	0·63

* $MgCl_2$, *M*/250 present in all.

Concentration of Mg. We have not succeeded in obtaining enzyme preparations which can be presumed to be completely free from Mg^{++} (see Table I).

The effect of increasing additions of Mg^{++} on the rate of esterification is shown in Table V.

Table V

ATP concentration M/10,000 in both experiments

Enzyme	Incubation time min.		Mg concentration (added) (M) 0	10^{-4}	3×10^{-4}	10^{-3}	3×10^{-3}	10^{-2}
GP6	30	Total	0·17	0·22	0·29	0·43	0·52	0·62
		CE	0·17	0·22	0·29	0·40	0·51	0·62
GP4	60	Total	0·04	0·10	0·13	0·18	0·30	0·39
		CE	0·04	0·10	0·11	0·16	0·27	0·37

Effect of temperature on rate of esterification. Experiments were carried out in the usual way at 37°, room temperature (20°) and at 0°. The rates of reaction at 37° and at 20° were identical, but at 0° very little esterification occurred (Table VI).

Table VI

Exp.	Incubation time min.		37°	20°	0°
1	60	Total ester	0·85	0·77	—
		CE	0·67	0·70	—
2	5	Total ester	0·10	0·11	—
	10	Total ester	0·21	0·20	—
	20	Total ester	0·33	0·35	—
		CE	0·29	0·32	—
3	60	Total ester	0·38	0·37	0·06
		CE	0·32	0·37	0·06

The action of inhibitors of glycolysis. The three most commonly used inhibitors of glycolysis are iodoacetic acid, fluoride and phloridzin. We have confirmed the findings of Lundsgaard [1933] and Ostern *et al.* [1936] that of these only phloridzin inhibits the initial phosphorylation of carbohydrate, and have further shown that this inhibition operates only on the very first stage, viz. the formation of CE from starch (Table VII).

Table VII

Enzyme GP4; ATP, M/10,000; Mg^{++}, M/250. Incubated 60 min.

Inhibitor	Ester formed (mg. P/5 ml.) Total	CE
—	0·75	0·55
Sodium fluoride M/50	0·70	0·53
Sodium iodoacetate M/400	0·72	0·55
Phloridzin M/100	0·07	0·00

The identification of CE. It can be seen in many of the experiments already quoted that with a short incubation period a good yield of almost pure CE is obtained. The quantity of ATP required is so small that the soluble Ba salts should not be contaminated with appreciable amounts of Ba adenylate.

For the isolation of the ester 400 ml. of GP4 were made up to 1 l., containing 0·5 % starch, 0·25 % $NaHCO_3$, M/250 $MgCl_2$, M/10,000 or M/20,000 ATP and about 700 mg. P as inorganic phosphate mixture at pH 7·5. Preliminary experiments showed that variation of the phosphate concentration from this figure in either direction reduced the yield of ester, while variation of the starch concentration between 1·5 and 0·25 % had little effect. The mixture, with N_2 + 5 %

CO_2 bubbling through it, was rapidly heated to 37° and maintained at that temperature for usually 30 min. It was then treated with an equal volume of alcohol and enough trichloroacetic acid to make the whole 1 %, cooled thoroughly, allowed to stand for $\frac{1}{2}$ hr. and filtered. The filtrate was neutralized and filtered from a further precipitate of starch, evaporated *in vacuo* to a small volume to remove the alcohol and brought to about 200 ml. with water. Sufficient Ba acetate was added to give a small excess over the total phosphate present, the *p*H adjusted to 9 and the precipitate of Ba phosphate removed by centrifuging. The precipitate was redissolved in HCl and the Ba phosphate reprecipitated at *p*H 9. The two solutions were mixed, treated with alcohol to 60 % and left at 0° overnight, the Ba salt of CE being then centrifuged off and dried *in vacuo*.

This product contained no esters but CE and a trace of EE, but was still contaminated with P-free material of high M.W., its total P content being usually about 6 %. The simplest method for removal of this impurity was found to be by dialysis through collodion. The product, moistened with a little water, was placed in a small collodion bag and dialysed against 100 ml. of water overnight. The Ba salts in the dialysate were then precipitated by the addition of alcohol to 60 %.

The salt could not be dried satisfactorily even over P_2O_5 *in vacuo*. It was extremely hygroscopic, and its total P content never rose above 7·4 % (theoretical 7·84 %). When left in a moist atmosphere till constant weight was reached, total P was 6·65 % ($4H_2O$ requires 6·65 %; Cori & Cori [1936] found after drying $3H_2O$). The data for one sample of the Ba salt are given in Table VIII.

Table VIII

	%
Total P (dried over P_2O_5)	7·35
Total P (exposed to water vapour)	6·65
P hydrolysed in 10 min. in *N* HCl at 100°	6·75 (92 % of total)
Reducing power (as glucose):	
Initial	2·5
Increase on hydrolysis for 10 min.:	
Hagedorn and Jensen	41·0
Hypoiodite [McLeod & Robinson, 1929]	40·0
Equivalent of P hydrolysed	39·2

Hydrolysis at 70° in *N*/100 HCl:

Time min.	P %	Sugar %	Cori & Cori (%)
60	42	45	38
120	65	67	62

Rotation calc. for the anhydrous barium salt, $[\alpha]_D$ ($c = 0{\cdot}3$) = 79°
(Cori & Cori [1936] $[\alpha]_D = 75{\cdot}5°$)

The formation of hexose-6-monophosphate (EE) from carbohydrate

In some of the results already given it can be seen that, especially after longer incubation (60 min.), not all the ester formed is hydrolysable in 10 min. If the incubation is further prolonged, an appreciable amount of difficultly hydrolysable ester is found to appear (see Fig. 5), and this ester was readily identified as hexose-6-monophosphate (Embden ester). In our earlier experiments on the action of HDP as a coenzyme in glycolysis it was found that HDP at a concentration of *M*/2000 greatly increases the formation of EE (see Fig. 5). Fig. 5 also shows that the action of HDP is to accelerate the transformation of CE to EE, and not to superimpose the latter on the former. This is more clearly

shown in Fig. 6, where the HDP was added after the formation of CE had been proceeding for 20 min.

The majority of the experiments with hexosediphosphate were carried out with a solution of the rather impure Na salt prepared directly from the commercial Ca salt. A purified sample of the Ba salt, made by taking advantage of its lower solubility in water at higher temperatures, gave a sodium salt which had exactly the same coenzyme properties as that made from the less pure Ca salt.

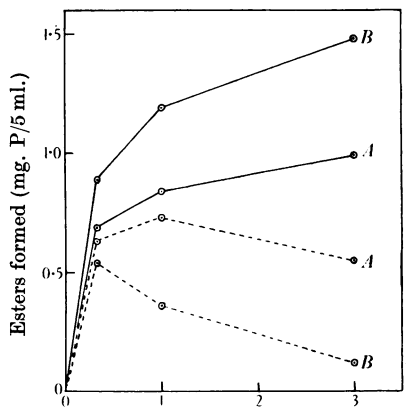

Fig. 5.

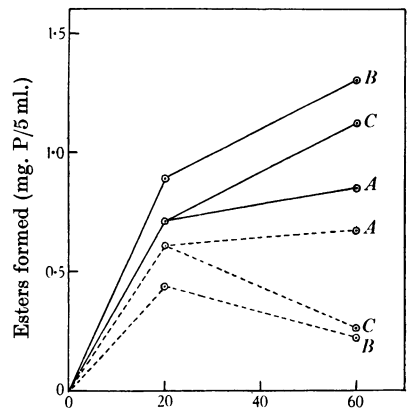

Fig. 6.

Fig. 5. The formation of Embden ester from Cori ester. Curves *A*, HDP absent. Curves *B*, HDP 5×10^{-4} *M*. Solid lines, total ester formed; dotted lines, CE formed.

Fig. 6. The conversion of CE into EE. Curves *A*, no HDP. Curves *B*, 5×10^{-4} *M* HDP present from the beginning. Curves *C*, 5×10^{-4} *M* HDP added after 20 min. Solid lines, total ester formed; dotted lines, CE formed.

The further reaction to EE proceeds of course equally whether the primary esterification was produced by ATP or AA. There is, however, one constant difference between the two cases, viz. the total amount of ester formed with ATP+HDP is considerably greater than with ATP alone, whereas with AA as coenzyme the addition of HDP causes no increase of the total yield of ester (see Table IX). We can offer no explanation of these facts.

Table IX

Exp.	Coenzymes	(mg. P/5 ml.) Total ester	CE	EE
1	ATP, *M*/4000	0·74	0·71	0·03
	,, +HDP, *M*/2000	0·98	0·49	0·49
	AA, *M*/4000	0·70	0·69	0·01
	,, +HDP, *M*/2000	0·73	0·23	0·50
2	ATP, *M*/10,000	0·60	0·60	0·00
	,, +HDP, *M*/2000	0·85	0·45	0·40
	AA, *M*/10,000	0·52	0·51	0·01
	,, +HDP, *M*/2000	0·42	0·10	0·32
3	ATP, *M*/3000	0·62	0·57	0·05
	,, +HDP, *M*/2000	0·85	0·31	0·54
	AA, *M*/3000	0·58	0·43	0·15
	,, +HDP, *M*/2000	0·56	0·07	0·49

The effect of temperature on the rate of the reaction CE → EE

We have shown that the rate of formation of CE is the same at 20° as at 37°. On the other hand, the rate of conversion of CE into EE is very much greater at 37° than at 20° (Table X). It will be seen that the amount of EE formed from starch at 37° is much greater than that formed at 20°.

Table X

Exp.	Coenzyme	Temp. °C.	Time of incubation min.	Esters formed Total	CE	EE
1	ATP, *M*/20,000	37	60	0·38	0·32	0·06
	,, +HDP, *M*/2000			0·48	0·27	0·21
		20	60	0·37	0·37	0·00
				0·45	0·44	0·01
2	ATP, *M*/10,000	37	60	0·85	0·67	0·21
	,, +HDP, *M*/2000			1·30	0·30	1·00
		20	60	0·77	0·70	0·07
				0·89	0·66	0·23

In the experiments of Parnas & Mochnacka [1936] in which inosinic acid was active as the coenzyme of phosphorolysis, the ester formed was entirely EE. As we find that HDP is necessary for the rapid formation of this ester from CE, there was a possibility that IA+HDP might catalyse the formation of EE in our enzyme preparations, even though IA alone is inactive in the formation of CE. This proved however not to be the case (Table XI).

Table XI

	Time of incubation (min.)					
	40		80		160	
	Esters formed (mg. P/5 ml.)					
Coenzymes*	Total	CE	Total	CE	Total	CE
ATP, *M*/10,000	0·67	0·62	0·80	0·75	0·96	0·62
AA, *M*/2000	0·72	0·72	0·83	0·78	0·93	0·65
IA, *M*/1000	0·02	—	0·07	—	0·12	—
IA, *M*/1000 + HDP, *M*/2000	0·08	—	0·09	—	0·09	—

* $MgCl_2$, *M*/250 present in all.

The identification of Embden ester

As was stated earlier, the identity of the difficultly hydrolysable portion of the mixture of esters formed was checked in many experiments by carrying out a 4 hr. hydrolysis in *N* HCl at 100°. The degree of hydrolysis found always agreed with that required for EE (about 30 % in 4 hr.).

To obtain the pure ester, large-scale experiments were set up as described for the preparation of CE, except that HDP was added to a concentration of *M*/2000 and the incubation was prolonged to 2–3 hr. The Ba salt was isolated by the same procedure, except that after the first alcohol precipitation of the ester Ba salts the solution was treated with an equal volume of 2*N* HCl and heated at 100° for 10 min. to hydrolyse the little CE present. The inorganic phosphate liberated by this treatment was removed in the usual way, and the preparation finished as before. The properties of a sample of this salt are given in Table XII.

Table XII

Total P 7·6% (theoretical 7·84%)

Hydrolysis in *N* HCl at 100°:

Time min.	% hydrolysed	Ostern *et al.* [1936]
10	3	—
60	11	18
120	17	25·5
240	25	34·5

Reducing power:

Alkaline ferricyanide (Hagedorn-Jensen) 32·4% (as glucose)

Hypoiodite [McLeod & Robinson, 1929] 30·1%

Rotation calc. for anhydrous Ba salt ($c = 1·6$), $\alpha_D = 11°$.

The rate of hydrolysis of this ester is, in our experiments, smaller than that reported by previous workers (e.g. Ostern *et al.* [1936]; see Table XII). We have hydrolysed several preparations with constant results (25·3, 25·0 and 25·6% hydrolysis in 4 hr. in *N* HCl at 100°) and think it likely that this lower figure is correct, as earlier workers did not suspect the possibility of contamination with a more easily hydrolysable monophosphate.

The formation of EE from CE

Some experiments have been carried out with isolated CE as substrate. A weighed quantity of the Ba salt was dissolved in water, treated with a slight excess of Na_2SO_4 and filtered. The amount of conversion was determined by measuring the decrease in the quantity of phosphate hydrolysable in 10 min. in *N* HCl at 100°, and this was checked by carrying out a 4 hr. hydrolysis in *N* HCl at 100° to confirm that the product was EE.

Indispensability of Mg^{++} and HDP as coenzymes. Mg^{++} and HDP are both essential for the conversion of CE into EE (Table XIII A), but adenosine phosphates play no part (Table XIII B). A number of other substances, including α-glycerophosphate, phosphoglycerate, EE and glutathione, have been tried in place of HDP, but no other has been found to have any effect on the rate of this reaction. The HDP added in these experiments appears to remain unchanged even after 2 hr. incubation, except for the reaction to the equilibrium mixture with triosephosphate. All the results are corrected for the known degree of hydrolysis of HDP and triosephosphate.

It will be noted that this reaction differs from the formation of CE in that it follows an almost linear course until the conversion is practically complete.

Table XIII

Coenzymes	Amount of CE converted into EE (mg. P/5 ml.) 15 min.	30 min.	60 min.
A. Initial CE, 0·92 mg. P/5 ml.			
—	—	0·02	0·03
$MgCl_2$, *M*/250	—	0·12	0·10
HDP, *M*/2000	—	0·07	0·12
$MgCl_2$ + HDP	0·23	0·42	0·79
B. Initial CE, 0·96 mg. P/5 ml.			
—	—	—	0·11
$MgCl_2$, *M*/250 + HDP, *M*/2000	—	—	0·73
,, + HDP, *M*/2000 + ATP, *M*/10,000	—	—	0·77

Concentration of Mg^{++} and HDP. The effect of varying the concentrations of Mg^{++} and of HDP is shown in Table XIV. It is clear that $M/2000$ HDP is insufficient to give the maximal rate of reaction for between $M/4000$ and $M/2000$ the rate is proportional to the concentration, but higher concentrations were not used as this would have made the coenzyme concentration of the same order as the substrate concentration ($M/1000$ HDP = 0·31 mg. P/5 ml.). The effect of changing the Mg^{++} concentration will be mentioned again later.

Table XIV

Initial CE, 1·09 mg. P/5 ml.

HDP, $M/2000$. Incubated 60 min.

Conc. of Mg (M)	0	5×10^{-4}	10^{-3}	2×10^{-3}	4×10^{-3}	10^{-2}
CE converted (mg. P)	0·11	0·40	0·51	0·73	0·85	0·73

$MgCl_2$, $M/250$. Incubated 60 min.

Conc. of HDP (M)	0	$2·5 \times 10^{-5}$	5×10^{-5}	10^{-4}	$2·5 \times 10^{-4}$	5×10^{-4}
CE converted (mg. P)	0·22	0·22	0·26	0·33	0·44	0·85

Phosphoric acid β-glucoside

Cori & Cori [1937] have shown that their ester is the α-glucoside of phosphoric acid. We attempted to make glucose-1-monophosphate synthetically by the method of Komatsu & Nodzu [1924], and obtained a product which differed in several ways from the natural ester. It was a glucose-1-monophosphate (total P 7·4%, no reducing power before hydrolysis, phosphate and reducing sugar liberated simultaneously during hydrolysis), but it was more difficult to hydrolyse (62% in 10 min., 84% in 60 min. in N HCl at 100°) and the rotation of its Ba salt was +22°. There is little doubt that this product was phosphoric acid β-glucoside, and the enzyme system in the presence of Mg^{++} and HDP had no action on it.

Experiments with glycogen

In all the experiments with carbohydrate substrate described so far the substrate was starch. The essential results have been checked with glycogen in place of starch, and no qualitative difference has been found. The only quantitative difference consisted of a slightly greater rate of reaction with glycogen (Table XV).

Table XV

Enzyme	Substrate	Coenzymes	Esters formed (mg. P/5 ml.) Total	CE	EE
GP3	Starch	ATP, $M/2000$	0·70	0·59	0·11
		,, +HDP, $M/2000$	1·24	0·39	0·85
	Glycogen	ATP, $M/2000$	0·78	0·65	0·13
		,, +HDP, $M/2000$	1·37	0·48	0·89
GP4	Starch	ATP, $M/2000$	0·61	0·57	0·04
		,, +HDP, $M/2000$	0·68	0·41	0·45
	Glycogen	ATP, $M/2000$	0·69	0·65	0·04
		,, +HDP, $M/2000$	0·87	0·56	0·31

Discussion

The position of inosinic acid as a coenzyme in the formation of CE is still obscure. Parnas & Mochnacka [1936] stated that IA is rather less active than AA, Cori & Cori [1937] that IA is one-half as active, and in our experiments IA

is only about one-tenth as active. (Parnas & Mochnacka do not state the concentration employed; Cori & Cori used AA at 0·1 mg. P/10 ml. (about $M/3000$) and presumably used IA at the same concentration; and we have used concentrations up to $M/1000$.) Our results might theoretically be explained in several ways. (1) In our preparations inosinic acid may be rapidly decomposed. This is extremely improbable on several grounds. (2) The resynthesis of AA from IA in the preparations of Parnas and Cori & Cori cannot be excluded *a priori*. On the other hand this reaction has never been observed in muscle preparations, and is again extremely improbable. (3) Yet another coenzyme may be required to enable IA to function in this reaction, and this substance may be present in the enzyme preparations of Parnas & Mochnacka, present at a lower concentration in the enzymes of Cori & Cori and almost absent from ours. This is a possible hypothesis, but we have no evidence as to the nature of this activator except that it is not HDP.

In our earlier experiments [Kendal & Stickland, 1937, 1] we showed that HDP is an essential coenzyme for glycolysis in our muscle extracts, and the present work shows one stage at which it is required (viz. CE → EE). The question arises as to the way in which the conversion of CE into EE is brought about in these muscle extracts which rapidly form EE with no coenzyme addition other than ATP or AA. In the experiments of Ostern *et al.* [1936] or Parnas & Mochnacka [1936] after 2–3 hr. incubation no ester but EE is found, and to achieve this result with our enzyme preparations the addition of HDP at a concentration of at least $M/2000$ would be required. It seems most unlikely that after very prolonged dialysis, autolysis followed by dialysis, or electrodialysis, the enzyme solutions used by these workers could contain HDP at a concentration of the order of $M/1000$, and this presents a difficult problem for which we can at present offer no solution. Moreover, the experiments of these workers were carried out at room temperature and under this condition we have found the conversion of CE into EE to take place much more slowly than at 37°.

The effect of the addition of Mg on the rates of the two reactions (starch → CE and CE → EE) also presents difficult problems. Cori & Cori [1937] found that with their dialysed enzyme preparations addition of Mg^{++} had no effect on the former reaction, but a considerable accelerating effect on the latter, and conclude that, if Mg^{++} is needed at all for the formation of CE, then a very low concentration is sufficient. Our experiments show clearly that Mg^{++} is necessary in both reactions (Tables I and XIII), but the position is complicated by the following observations. (1) If the enzyme preparation is not free from Mg^{++}, so that esterification to CE takes place on addition of ATP alone, Mg^{++} still has to be added to enable the second reaction to proceed. This is exactly in accordance with the results of Cori & Cori. (2) If the enzyme preparation is practically free from Mg^{++}, then the concentrations of Mg^{++} required for activation are of the same order for each reaction (see Tables V and XIV). At present we can see no way of reconciling these two facts.

Summary

A quantitative study has been made of what recent work suggests are the first two known steps in the formation of lactic acid from carbohydrate in muscle extracts, viz. (1) carbohydrate to glucose-1-monophosphate (Cori ester) and (2) Cori ester to hexose-6-monophosphate (Embden ester).

In (1) Mg^{++} and adenosinetriphosphate or adenylic acid are essential, and inosinic acid is only slightly active as coenzyme. In (2) Mg^{++} and hexose-

diphosphate are essential coenzymes, and the nucleotides play no part. In each case the effects of various factors, including the concentration of each of the coenzymes, on the rate of the reaction have been investigated.

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