CXL. SOME FACTORS INFLUENCING THE FORMATION OF ROBISON ESTER FROM GLYCOGEN AND INORGANIC PHOSPHATE IN MUSCLE EXTRACT

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PARNAS & BARANOWSKI [1935], Ostern & Guthke [1936], Ostern *et al.* [1936] and Parnas & Ostern [1936], have shown that inorganic phosphate can react with glycogen in presence of muscle extract to form Robison ester, i.e. a mixture of reducing hexosemonophosphates, discovered in yeast by Robison [1922] and first found in muscle, where the % of the individual components differs from that in yeast, by Embden & Zimmermann [1927]. The hexosemonophosphate formation from glycogen was claimed to be direct and not a secondary phosphorylation of glucose formed by hydrolysing enzymes. Balance experiments showed that the final glycogen plus hexosemonophosphate could account for all the initial carbohydrate. The authors called the formation of Robison ester a phosphorylysis of carbohydrate to distinguish it from the hydrolysis by amylase. Cori & Cori [1936; 1937], using washed muscle residue and well dialysed extract, detected the formation of a hexosemonophosphate other than Robison ester from glycogen and inorganic phosphate. The new ester was non-reducing yet Robison ester was readily formed from it after addition of Mg. It was identified as glucose-1-phosphate [Cori *et al.* 1937]. The formation of the Cori ester is increased by adenylic acid; an action not yet quite explained, but apparently not due to phosphate transport by the adenylic acid. (The Coris call the enzyme producing the 1-ester phosphorylase; the one which produces the 6-ester from the Cori ester phosphoglucomutase.)

To study the formation of Robison ester in muscle extracts is of interest as this step may be a key reaction of carbohydrate breakdown, both oxidative and anaerobic [Engelhardt & Barchash, 1938]. In the latter case Robison ester is phosphorylated to hexosediphosphate and broken down to lactic acid; in the first it may be a substrate of Warburg's coenzyme the triphosphodihydropyridine nucleotide and eventually be linked up with atmospheric O₂ to be burnt to phosphohexonic-acid, phosphoketohexonic acid and arabinose phosphate + CO₂ [Warburg *et al.* 1935; Lipmann, 1936; see also Dickens, 1938, 2].

The investigation of conditions for hexosemonophosphate formation can be arbitrarily divided into two parts: (a) the kinetics of the reaction, and (b) the influence of activators and inhibitors.

(a) There can be no doubt that the step glycogen → Robison ester is not a true reversible equilibrium, as Robison ester added to muscle extract does not yield glycogen and inorganic phosphate under the same conditions as it is formed from the two compounds. Nevertheless, it was possible to show that in

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dialysed yeast juice as well as in dialysed muscle extract only a certain part of the glycogen was phosphorylated giving a relation of about 1:20 of (phosphorylated carbohydrate) to (unphosphorylated carbohydrate times free phosphorus) [Lehmann, 1938, 1]. The relation prevailed regardless of how much glycogen or phosphate was added. Furthermore, the same weight of starch was only half as much phosphorylated as glycogen, and it is known to have about double the number of monosaccharide units per chemically determined chain [see Bell, 1937]. The question is still controversial, for instance, glycogens of different chain length show no corresponding difference in their phosphorylation [Lehmann, 1938, 2].

Also in the presence of sufficient Mg, extracts transform all the glycogen into Robison ester provided that enough phosphate is present. These facts and the observation that glucose and hexosemonophosphate inhibit phosphorylation of glycogen [Lehmann, 1938, 1] led Cori *et al.* [1939, 1] to suggest that inhibition of phosphorylation by glucose-1-phosphate may play a part in cases where incomplete phosphorylation is observed. They find the Cori ester a specially strong inhibitor as compared with Robison ester. Kiessling [1939] (see also Schöffner & Specht [1938], Schöffner [1939]) was able to show the existence of a reversible equilibrium, glycogen + phosphate $\rightleftharpoons$ Cori ester, by using purified yeast enzyme. This equilibrium can occur in muscle extract as well [Cori *et al.* 1939, 2] and may, under adverse conditions for the step Cori ester $\rightarrow$ Robison ester, determine the end-point of the reaction glycogen + phosphate $\rightarrow$ Robison ester.

(b) Several factors are known to influence lactic acid formation from carbohydrate in general. Phloridzin has been found [Lundsgaard, 1933] to act on the initial reaction, i.e. on phosphorylation. Others act on intermediate stages, while some have not yet been investigated as to the exact point where the inhibition or activation occurs.

It was felt that new knowledge of factors increasing or decreasing phosphorylation might be of interest for our understanding of carbohydrate breakdown in general, and might throw light on the conditions underlying the behaviour of weakly phosphorylating systems in particular. The following work was undertaken as an investigation of these influences in rabbit muscle extract; a subsequent publication will deal with the relation of these factors to phosphorylation in enzyme extracts from other species than rabbit and organs other than muscle.

METHODS

The tissue extracts used were all prepared according to Meyerhof [1926] by mixing two parts minced tissue with three parts water or 0.13% NaHCO_3 ; the suspension was filtered through muslin and inactivated for 1 hr. at room temperature. Most of the extracts were dialysed against water and KCl solutions at $+4^\circ$ for 48–60 hr. The degree of dialysis was tested by estimating the remaining inorganic phosphorus which was 0.5–5 mg./100 ml.

The glycogen used (British Drug Houses, Ltd.) was alkali-stable and did not contain reducing groups measurable under our conditions. The phosphate used was Sørensen buffer at pH 7. Mg was added as MgCl_2 , in concentrations of 0.05–0.1 mg. per ml. reaction mixture, while the adenylic acid used was the Na salt, in concentrations of 0.2–0.5 mg. per ml. of the reaction mixture. Except where otherwise stated the figures in this paper are calculated as mg. per ml. reaction mixture. The experiments were performed at 37° and were terminated by addition of trichloroacetic acid or strong KOH. The Robison ester formed was determined by measuring the loss of glycogen, the formation of new

difficultly hydrolysable phosphorus and reducing groups. The glycogen was estimated by Pflüger's method as modified by Good *et al.* [1933]. The phosphorus was determined after Fiske & Subbarow [1925] (see also Lohmann & Jendrassik [1926]) and hydrolysis of phosphorus esters was performed by Lohmann's principle [1928]. The reducing groups were measured according to Giragossintz *et al.* [1936] and Miller & Van Slyke [1936]. When this method is used the ratio of the reducing group of Robison ester measured as mg. glucose to the mg. P of the ester is 5.5 : 1. The Robison ester was isolated and identified after Ostern *et al.* [1936] on a large scale. The isolation on a micro scale after Cori & Cori [1931] was not performed in all experiments, but in all cases where a treatment led to an increase of Robison ester formation, the increase was confirmed by this method. The fraction isolated after Cori & Cori [1931] comprises both Cori and Robison esters. To obtain the true value for Robison ester it should be fractionated by hydrolysis. A necessary correction has to be made also for the P of adenylic acid present. Except where stated otherwise the amount of Cori ester present in our reaction-mixtures was negligible.

AMYLASES

Winfield & Hopkins [1915] observed that addition of pancreatic extracts to muscle "brei" caused an inhibition of lactic acid formation. Even a preparation of pancreatin, many years old, had the same inhibitory effect. Foster & Woodrow [1924] excluded both insulin and trypsin as the active principle. Ronzoni [1927] showed that lactic acid formation from glycogen was inhibited but not that from hexosephosphates. By using a cell-free enzyme solution from muscle prepared according to Meyerhof [1926] she found that the pancreatic extract acted by preventing the uptake of phosphate for esterification of glycogen. McCullagh [1928] confirmed her results and Case & McCullagh [1928] showed that the pancreatic factor was probably amylase. An inhibitory factor was found to be present in most tissues of the animal body, the strength of the inhibition corresponding to the amylase content and even plant amylases acted like pancreatic extracts.

The following experiments were performed to investigate whether this inhibition was due to the amylase itself or to its action on glycogen. In the latter case it had to be decided if the "amylase effect" was due either to a competition of phosphorylase and amylase for the substrate, or to the formation of an inhibitory compound formed from glycogen (it has been found, for instance, that glucose inhibited phosphorylysis [Lehmann, 1938, 1]).

Table I demonstrates that in the presence of different amylases the formation of Robison ester in muscle extracts was suppressed. The inhibition was less the more dilute the amylase solutions were.

In the experiments where amylase was present the glycogen did not remain unaffected, which showed that the amylase¹ was acting although phosphorylation was inhibited.

Trial experiments showed that a pH of 9 inhibited the rabbit liver amylase nearly completely but did not do the same to the phosphorylating enzymes of muscle extract. An experiment (Table II) at that pH was performed on the undialysed extract: the duration was 60 min., the initial glycogen 5.2 mg., and the reaction (pH 9) was obtained by $M/10$ Na_2CO_3 - NaHCO_3 buffer.

¹ The amylase content of rabbit and hen liver varied greatly and in an unpredictable manner. The findings in guinea-pig liver were more constant.

Table I

All the enzyme solutions were undialysed, except in the first experiment, where the muscle extract used was dialysed 60 hr. The saliva used was always fresh. The duration of experiments was 60 min. except in no. 2, where it was 120 min. and in no. 3, where it was 20 min. In this, and all experiments unless otherwise stated, the figures indicate mg. per ml. of reaction mixture.

No.	Enzyme solution	Age of enzyme in days	Enzyme solution : total volume	Initial glycogen	Initial P	Robison ester formed
1	Rabbit muscle extract	18	1 : 6	10.7	1.1	3.1
	Rabbit liver extract	0.5	1 : 6	10.7	1.1	0
	Rabbit muscle extract	—	1 : 6	10.7	1.1	1.1
	+ rabbit liver extract	—	1 : 6			
2	Rabbit muscle extract	22	1 : 4	4	1.0	1.7
	Hen liver extract	1	1 : 3	4	1.0	0
	Rabbit muscle extract	—	1 : 4	4	1.0	0.7
	+ hen liver extract	—	1 : 3			
3	Rabbit muscle extract	2	1 : 3	8	1.2	1.9
	Human saliva	—	1 : 7	8	1.1	0
	Rabbit muscle extract	—	1 : 3	8	—	0
	+ saliva	—	1 : 7			
4	Rabbit muscle extract	3	1 : 3	8	1.2	4.7
	Human saliva	—	1 : 7	8	1.2	0
	Rabbit muscle extract	—	1 : 3	8	—	1.2
	+ human saliva	—	1 : 7			
5	Rabbit muscle extract	8	1 : 3	8	1.2	4.8
	Human saliva	—	1 : 7	8	1.2	0
	Rabbit muscle extract	—	1 : 3	8	—	4.3
	+ human saliva	—	1 : 600			
	Rabbit muscle extract	—	1 : 3	8	—	3.5
	+ human saliva	—	1 : 300			
	Rabbit muscle extract	—	1 : 3	8	—	2.7
	+ human saliva	—	1 : 150			

Table II

Enzyme solution	Age of enzyme in days	Enzyme solution : total volume	Initial P	Robison ester formed
Rabbit muscle extract	48	1 : 6	0.8	1.3
Rabbit liver extract	4	1 : 6	0.7	0
Rabbit muscle extract	—	1 : 6	0.9	1.8
+ rabbit liver extract	—	1 : 6		

Table II shows that when the liver amylase was inhibited at pH 9 there was no inhibition of phosphorylation. It is not known to what the increase in the presence of liver extracts was due. It is also demonstrated that the amylase inhibition of phosphorylation in the previous experiments was due to the action of the hydrolysing enzyme on glycogen, rather than to the presence of the amylase itself. The next experiments were done to see whether amylase formed an inhibitory product from carbohydrate or if it used up the glycogen so quickly that no substrate for phosphorylation was left. Even amylatic breakdown products from glycogen of high molecular weight are not phosphorylated by muscle extracts [Euler & Bauer, 1938]. In the case of an inhibitory compound being formed, therefore, in a reaction mixture, in which amylase and phosphorylase had been incubated together with glycogen, and in which the formation of Robison ester had come to an end, no new Robison ester should be formed after addition of more glycogen. In the case of competition of the enzymes for the substrate the same amount of Robison ester should be formed from the second addition of glycogen as was formed from the first.

Table III

The extracts used were undialysed; fresh human saliva was used.

Enzyme solution	Age in days	Enzyme solution : total volume	Duration of exp. in min.	Initial glycogen	Initial P	Remarks	Robison ester formed
Rabbit muscle extract no. 1	12	1 : 3	60	8	1.2	—	3.8
Rabbit muscle extract + saliva	12	1 : 3 1 : 750	60	8	1.3	—	1.9
Rabbit muscle extract + saliva	12	1 : 3 1 : 750	60	8	1.2	+ 8 mg. glycogen after 30 min.	3.0
Rabbit muscle extract no. 2	29	1 : 3.6	45	8.9	1.4	—	3.6
Rabbit muscle extract	29	1 : 3.6	140	8.9	1.4	—	4.7
Rabbit muscle extract + saliva	29	1 : 3.6 1 : 180	45	8.9	1.4	—	2.6
Rabbit muscle extract + saliva	29	1 : 3.6 1 : 180	140	8.9	1.4	—	3.4
Rabbit muscle extract + saliva	29	1 : 3.6 1 : 180	140	8.9	1.4	+ 8.9 mg. glycogen after 45 min.	4.7

The addition of more glycogen after the inhibition had manifested itself (Table III) was followed by a rise in phosphorylation. This could not have occurred if the previous suppression of that process had been due to formation of an inhibitor, e.g. glucose. The amylatic inhibition of phosphorylation may therefore be conceived as due to lack of substrate for the phosphorylase due to the removal of the substrate by the hydrolysing enzyme.

OXIDATION

Another inhibitory factor for phosphorylating anaerobic breakdown of carbohydrate is the presence of O_2 or oxidation. Pasteur found that the fermentation of yeast was suppressed by O_2 and a similar inhibition of lactic acid formation in intact muscle has been described by Meyerhof. That muscle extract is able to glycolyse vigorously in the presence of air is due to the fact that the extract lacks the O_2 carriers and activators which are present in intact muscle tissue. Lipmann [1933] was able to show a reversible inhibition of glycolysis in extracts by dyes which linked up the oxidizable groups of the muscle extract with atmospheric O_2 . Lately, Rapkine [1938] has demonstrated that the oxidation of triosephosphate and hence the oxidoreduction between triosephosphate and pyruvic acid which occurs midway in the glycogen $\rightarrow$ lactic acid reaction, is inhibited by oxidizing agents such as oxidized glutathione and I_2 . Previously Lipmann [1934] had described an inhibition of glycolysis by $CuSO_4$ and Gemmill & Hellerman [1937] by Hg^{++} salts. The latter obtained indications that the inhibition occurred probably at the "hexose stage". The inhibitions could be removed by reducing agents such as reduced glutathione and cysteine [Wagner-Jauregg & Rzeppa, 1936, 1, 2; Gemmill & Hellerman, 1937].

The following data are given to show the inhibiting effect of oxidizing compounds on Robison ester formation from glycogen and inorganic phosphate. As examples are given a biological oxidizing system (catechol + catechol oxidase) and oxidized glutathione. The catechol oxidase was prepared from mushrooms following advice from Dr K. Baghvat. We also obtained a highly purified sample from Dr T. Mann prepared by the method of Keilin & Mann [1938].

Table IV

25 days old, 72 hr. dialysed rabbit muscle extract. 1 : 4 of total volume.
45 min. duration of exp. Initial glycogen 6 mg. Initial P 0.75 mg.

Additions	Robison ester formed
—	2.5
+ Catechol oxidase	2.5
+ <i>M</i> /20 catechol	1.3
+ Catechol oxidase + <i>M</i> /20 catechol	0

20 days old undialysed rabbit muscle extract. 1 : 3 of total volume.
30 min. duration of exp. Initial glycogen 8 mg. Initial P 1.2 mg.

—	2.8
<i>M</i> /80 GSSG	2.3

2 days old, 60 hr. dialysed rabbit muscle extract. 1 : 3 of total volume.
120 min. duration of exp. Initial glycogen 6.25 mg. Initial P 0.4 mg.

—	1.65
<i>M</i> /24 GSSG	1.0
<i>M</i> /12 GSH	2.2

In the last experiment of Table IV the function of GSSG as inhibitor was controlled with a reduced sample of the same preparation. This was to exclude the action of any traces of Cu which might have been present in small amounts owing to its method of preparation. The reduced tripeptide not only had no inhibitory effect but actually increased the formation of Robison ester.

ADRENALINE

Instead of catechol, in the experiments when catechol oxidase was used, adrenaline may be added together with the oxidase to give an inhibitory effect. Adrenaline is even more suitable as it has no inhibitory effect by itself, unlike catechol which is never entirely free from oxidized forms and undergoes also a slight autoxidation under the conditions of our experiment. The only effect observed of adrenaline on Robison ester formation was this inhibitory one; high concentrations were necessary (0.2–1 %) and an oxidizing enzyme. At alkaline reaction the autoxidation of high concentrations of the hormone was sometimes sufficiently strong to effect an inhibition by itself. Smaller amounts of the hormone nearer physiological concentrations were never found by us to have an influence on the enzyme system forming Robison ester from glycogen. Our findings are in agreement with the results of Cori *et al.* [1939] on extracts.

REDUCING AGENTS

In Table IV an experiment was shown where reduced glutathione increased Robison ester formation. We were able to confirm that GSH in particular and reducing agents in general have an effect on Robison ester formation the reverse of that of oxidizing agents.

The estimation of reducing groups of the ester was, of course, not possible in the presence of other reducing compounds and had to be performed after isolation [Cori & Cori, 1931]. This was done in the presence of all individual reducing agents tested, but in most of the experiments reported in this section the glycogen loss and the formation of difficultly hydrolysable P were taken as a basis for the measurement of Robison ester.

Reduced glutathione

Table V

In both experiments 60 hr. dialysed, 9 and 10 days old rabbit muscle extract was used. Extract: total volume = 1:4. Initial glycogen 6 mg. Initial P 0.8 mg. pH 8.5 was obtained by the use of Na_2HPO_4 as a source of phosphate and was maintained by NaHCO_3 buffer.

Duration of exp. min.	pH	Addition	Robison ester formed
60	7	—	2.4
60	7	M/25 GSH	3.3
60	8.5	—	3.4
60	8.5	M/25 GSH	4.2
75	8.5	—	3.1
75	8.5	M/10 GSH	4.1

In all cases reduced glutathione increased Robison ester formation and it will be noted that whereas an alkaline reaction itself seems to have a favourable effect, the action of glutathione is evident at either neutral or alkaline reaction. Dyes inhibiting fermentation of yeast and oxidizing agents suppressing glycolysis of muscle extract might do so by oxidizing the enzyme necessary for the Robison ester formation, and reduced glutathione might be considered as an activator, reducing the inactive enzyme. It is known already that GSH can reduce proteins [Hopkins & Dixon, 1922; Hopkins, 1925] and enzymes (dehydrogenases) of carbohydrate metabolism [Rapkine, 1938; Hopkins & Morgan, 1938; Hopkins *et al.* 1938].

Fig. 1. % increase of Robison ester formation at different concentrations of reduced glutathione.

Fig. 1 shows that the % increase of Robison ester due to glutathione is in a logarithmic relationship to the concentration of the tripeptide. The graph is based on the last experiment in Table V. The Robison ester formation in absence of any glutathione was 3.1 mg. (= 100 %).

Cyanide

The action of reduced glutathione may be visualized as a reduction of SS groups of the muscle extract. The influence of KCN on phosphorylation was therefore investigated, since according to Mauthner [1907] it is a reducing agent in the sense that it transforms SS linkages into free SH groups.

Table VI

In all cases rabbit muscle extract was used with adenylic acid and Mg unless otherwise stated.

20 days old undialysed extract. Enzyme solution : total volume = 1 : 3.

Duration of exp. 20 min. Initial glycogen 8 mg. Initial P 1.1 mg.

Remarks	Robison ester formed
—	2.9
M/10 KCN	5.2

8 days old, 48 hr. dialysed extract. Enzyme solution : total volume = 1 : 3.

Duration of exp. 100 min. Initial glycogen 4 mg. Initial P 0.5 mg.

—	3.2
M/10 KCN	3.6

Fresh undialysed extract. Enzyme solution : total volume = 1 : 4.

Duration of exp. 60 min. Initial glycogen 6 mg. Initial P 0.9 mg.

—	4.9
M/7 KCN	5.7

15 days old, 60 hr. dialysed extract. Enzyme solution : total volume = 1 : 4.

Duration of exp. 180 min. Initial glycogen 6 mg. Initial P 0.8 mg.

No adenylic acid and Mg added	0.7
No adenylic acid and Mg added,	1.7
M/2 KCN	—
—	4.0
M/2 KCN	5.7

It will be noted that KCN increases Robison ester formation in old as well as fresh extracts. Its effect is evident both with and without addition of adenylic acid and Mg^{++} . The influence of K^+ of the KCN was checked with equivalent amounts of KCl. Mg^{++} may, in its effect on formation of Robison ester, be replaced by other positive ions [Cori *et al.* 1938] and Ohlmeyer & Ochoa [1937] working on yeast enzymes included K^+ in the list. The chloride was chosen because the KCN used by us was neutralized with HCl. KCl had no effect on the formation of Robison ester.

Thiolacetic acid

Thiolacetic acid added as the Na salt behaved similarly to the other reducing compounds investigated.

Table VII

28 days old undialysed rabbit muscle extract. 1 : 4 to total volume.

6 mg. initial glycogen, 0.8 mg. initial P.

Time of exp. min.	Robison ester formed	
	Control	N/10 thiolacetate
10	1.0	2.2
45	2.4	4.7
100	3.5	5.3

Denatured protein (boiled muscle extract)

Boiled protein may contain SH groups formed during the process of denaturation [Harris, 1923; Hopkins, 1925]. There are exceptions, but muscle extract is not one, as can be demonstrated by means of the nitroprusside test.

The increasing effect on lactic acid formation and fermentation of boiled muscle extract was recognized quite early as not necessarily due to protein [Meyerhof, 1918, 1, 2; Meyer, 1927] but to inorganic phosphate [Meyer, 1927; 1928], Mg and adenylypyrophosphate [Lohmann, 1931] and reduced codehydrogenase I [Harden-Euler's coenzyme] [Meyerhof & Ohlmeyer, 1936]. On the other hand, Kendal & Stickland [1937] observed an activation of diluted muscle extract with boiled dialysed muscle extract which was not shown by the dialysable compounds only. The point was made by Curtius & Ohlmeyer [1938] that some enzymes of muscle extract are remarkably resistant towards boiling and Kendal & Stickland [1937] may therefore have merely added active enzyme to the diluted extract. We are able to confirm both views. The Heidelberg investigators only observed the insensitiveness of the Parnas reaction enzyme of muscle to boiling; we found the same regarding the enzymes responsible for the formation of Robison ester from glycogen and inorganic phosphate.

Muscle extract boiled for 2 min. is capable of phosphorylating glycogen and forming Robison ester and the findings of Kendal & Stickland [1937] may thus find their explanation. The resistance of muscle extract to boiling is a phenomenon which we could not always repeat but the exceptions were few.

Table VIII. *Influence of muscle extract kept for 2 min. at 97°*

Total extract : total volume	Unboiled extract : total volume	Boiled extract : total volume	Robison ester formed
24 days old, 60 hr. dialysed rabbit muscle extract, boiled and unboiled.			
Duration of exp. 135 min. Initial glycogen 4 mg. Initial P 0.9 mg.			
1 : 4	1 : 4	—	1.4
1 : 4	—	1 : 4	0.7
1 : 2	1 : 4	1 : 4	2.3
27 days old, 100 hr. dialysed rabbit muscle extract, boiled and unboiled.			
Duration of exp. 100 min. Initial glycogen 6 mg. Initial P 0.7 mg.			
1 : 2	1 : 2	—	2.5
1 : 4	1 : 4	—	1.8
1 : 2	—	1 : 2	1.3
1 : 2	1 : 4	1 : 4	2.5

Table IX. *Influence of globulin x on phosphorylation in rabbit muscle extract*

Extracts to total volume 1 : 4; initial glycogen 6 mg.	
7 days old, 60 hr. dialysed extract. Duration of exp. 90 min. Initial P 0.8 mg.	
Additions	Robison ester formed in mg.
3% globulin x	4.0
3% denatured globulin x	4.4
8 days old, 60 hr. dialysed extract. Duration of exp. 90 min. Initial P 0.8 mg.	
—	3.3
3% globulin x	3.8
3% denatured globulin x	4.3
8 days old undialysed extract. Duration of exp. 90 min. Initial P 0.9 mg.	
3% globulin x	6.8
3% denatured globulin x	7.5
18 days old, 60 hr. dialysed extract. Duration of exp. 210 min. Initial P 0.7 mg.	
—	3.6
2% globulin x	3.6
2% denatured globulin x	4.4

Yet we were able to confirm Kendal & Stickland's view [1937] that boiled muscle extract may contain an activator not present in diluted muscle extract. This activator may actually be formed during boiling. A single muscle protein fraction "globulin *x*" though unable to phosphorylate glycogen, may sometimes slightly increase phosphorylation of muscle extracts and this influence is greatly increased after it has been denatured by boiling. At the same time a formation of SH groups after boiling was shown to have occurred by means of the nitroprusside test. The fact that the "globulin *x*" acts sometimes as an activator, even before being boiled, may well be due to some denaturation which has taken place already, as the purified protein denatures on standing. We are greatly obliged to Dr E. C. Smith of the Low Temperature Station, Cambridge, for repeated gifts of globulin *x*.

Sodium hydrosulphite

An inorganic reducing agent $\text{Na}_2\text{S}_2\text{O}_4$ was also shown to increase phosphorylation (Table X).

Table X

27 days old undialysed rabbit muscle extract, 1 : 3 total volume. Duration of exp. 65 min.
Initial glycogen 8 mg. Initial P 1.2 mg.

Additions	Robison ester formed
—	1.8
$M/45 \text{ Na}_2\text{S}_2\text{O}_4$	2.6
$M/135 \text{ Na}_2\text{S}_2\text{O}_4$	2.4

Evacuation

Physical deprivation of O_2 was chosen as another example of reduction of muscle extracts. To avoid any difference of the buffering of the extract by alteration of its CO_2 content all samples in the experiments were first thoroughly evacuated in Thunberg tubes. But whereas some of them were left so during the experiment, others were reopened and shaken up with air.

Table XI

Hen breast muscle extract, 11 days old, undialysed. 8 mg. initial glycogen,
0.8 mg. initial P.

Duration of exp. min.	Special conditions	Robison ester formed
60	In air	2.2
60	<i>In vacuo</i>	2.4
120	In air	3.1
120	<i>In vacuo</i>	3.7

Table XI shows that there is a very slight increase of Robison ester formation *in vacuo*.

METALS

The effect of SH groups on enzyme function has often been studied simultaneously with that of metallic ions. Work mentioned above [Lipmann, 1934; Gemmill & Hellerman, 1937 etc.] was done in connexion with the inhibition of fermentation and lactic acid formation by oxidizing agents showing that instead of dyes, I_2 , GSSG or quinone, the higher valent forms of metals which exist in different valency states may be used. It was to be expected, therefore, that Cu^{++} for instance, would inhibit Robison ester formation just as oxidized glutathione

does. Many authors, especially Hellerman & Perkins [1934; 1935], have shown that several enzymes involved in protein metabolism may be influenced by metals not only by oxidation of their SH groups or reduction of SS groups, but also by formation of metallic derivatives. We therefore investigated the effect of various metallic ions on the formation of Robison ester.

Again, it was not possible to estimate the reducing groups of the Robison ester even after the micro isolation of Cori & Cori [1931], as sufficient metal to upset the estimation remained in the isolated fraction. But the glycogen loss and formation of difficultly hydrolysable phosphate were in agreement and the organic phosphate formed was refound in the hexosemonophosphate fraction by the method of Cori & Cori [1931].

Table XII. *Influence of metals on Robison ester formation*

60 hr. dialysed rabbit muscle extract. Extract: total volume = 1:4. Initial glycogen 6 mg., initial P 0.8 mg. 0.5 mg. adenylic acid. 0.05 mg. Mg. Na_2SO_4 was used for control values.

Age of extract in days	13	16	13	17	18	19	23	23
Duration of exp. in hr.	2	2	2	2	2	2	5	5
Molarity of metal or Na_2SO_4	1×10^{-2}	5×10^{-3}	1×10^{-3}	5×10^{-4}	5×10^{-5}	1×10^{-5}	1×10^{-6}	1×10^{-7}
mg. Robison ester formed in presence of Na_2SO_4	2.1	2.1	2.1	2.5	2.2	2.1	4.4	4.4
$\pm$ % in presence of CuSO_4	-60	-78	-60	-95	-66	-34		
$\pm$ % in presence of $\text{Fe}_2(\text{SO}_4)_3$	-	-73	-	-15	-9	± 0		
$\pm$ % in presence of FeSO_4	-57	-29	-12	-13	-			
$\pm$ % in presence of ZnSO_4	-100	-81	-26	-30	-32	-14	± 0	+7
$\pm$ % in presence of NiSO_4	+40	+40	+51	+19	+6	-		
$\pm$ % in presence of $\text{Co}(\text{NO}_3)_2$	+20	+38	+51	+32	+26	+24	+10	± 0

In the experiments shown in Table XII no increase of phosphorylation was obtained with the ferrous salt, though the inhibition here was slightly less than in the case of the ferric salt. In low concentrations the ferrous salt is of course unstable.

As the cobalt salt used was $\text{Co}(\text{NO}_3)_2$ controls were performed with NaNO_3 which, however, had no influence on Robison ester formation. (It will be noted that an optimal concentration of Mg^{++} is present and the influence of Co^{++} can therefore not be explained as a replacement of Mg^{++} . Co^{++} can take the place of Mg^{++} in well dialysed enzyme preparations [Cori *et al.* 1938; 1939, 1].)

The influence of a metal ion is generally the same in the range of concentrations even although Co^{++} may sometimes inhibit at 10^{-2} M and Zn^{++} may activate rather than inhibit at very low concentrations. But whereas a qualitative consistency of the influence of the various metals on different muscle extracts can be observed, there is a great inconsistency in the quantitative effect. 10^{-3} M Co^{++} may increase the Robison ester formation in one extract 200 %, in another only 30 %. Co^{++} and Zn^{++} in equal molecular concentrations counteract each other, as shown in Table XIII.

The action of Co^{++} was further elucidated (in experiments which will appear fully in a subsequent publication) as being more than a mere replacement of Mg^{++} , for Co^{++} increased the phosphorylating power of weakly phosphorylating

Table XIII

50 hr. dialysed, 21 days old rabbit muscle extract. 0.05 mg. Mg, 0.5 mg. adenylic acid. Initial glycogen 6 mg. Initial P 0.8 mg. Robison ester formed 1.6 mg. in 4 hr.

Molarity of metal	± % Robison ester formation in presence of metals				
	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷
Zn	-25	-13	± 0	± 0	+6
Co	+ 5	+ 5	+ 5	+5	± 0
Co	± 0*	± 0*	-13*	—	—

* 10⁻⁴ M Zn present.

enzyme systems, although Mg⁺⁺ had no influence on them [see also Gill & Lehmann, 1939]. It may act either as a complex former in the sense of Hellerman & Perkins' [1935] suggestion of enzyme-metal complex formation, or as a reducing agent in the sense of Michaelis and his collaborators. They observed that Co⁺⁺ combines with cysteine with subsequent uptake of O₂ [Michaelis *et al.* 1929; Michaelis & Yamaguchi, 1929]. Rosenheim & Davidsohn [1904] showed formation of a complex of thiolacetic acid with Co⁺⁺ and with Zn⁺⁺. Yet we find a difference in the interactions between Zn⁺⁺ and Co⁺⁺ with thiolacetic acid.

When Zn⁺⁺ or Co⁺⁺ are added to thiolacetic acid at neutral reaction, a loss of power of the thiolacetic acid to consume I₂ can be demonstrated in the case of Co⁺⁺ but not in the case of Zn⁺⁺. This is shown in Table XIV.

Table XIV. *Effect of Co on I₂ consumption of thiolacetic acid*

Reaction mixture	Consumption of N/200 N I ₂ (ml.)
1 ml. M/20 thiolacetic acid 5 ml. M/10 phosphate (pH 7) 3 ml. 25% KI	4.3
Ditto + 0.5 ml. M/10 Co(NO ₃) ₂	1.5
Ditto + 0.2 ml. M CO(NO ₃) ₂	1.3
5 ml. M/10 phosphate (pH 7) 3 ml. 25% KI 0.2 ml. M Co(NO ₃) ₂	0.01

The most plausible explanation perhaps is a combination of the views of the Baltimore and New York workers in suggesting a specific and powerful reduction of the enzyme through complex formation with the metal.

ASCORBIC ACID

Ascorbic acid is a reducing compound and might be expected as such to increase phosphorylation. But, whereas we had one experiment when ascorbic acid acted in this way, we were never able to repeat it and we found rather that ascorbic acid in large amounts inhibited phosphorylation. Its inhibitory effect occurred in concentrations similar to those required for glucose to exert an inhibitory effect, but its inhibition was only one-quarter of that of glucose. It may join, therefore, the other similarly acting unphosphorylated carbohydrates such as mannose and dimeric glyceraldehyde [Lehmann & Needham, 1938].

Yet the influence of ascorbic acid is dependent on pH whereas that of glucose is not. Ascorbic acid is more inhibiting at pH 8 than at pH 7. A destruction of the compound at neutral pH could be excluded: we are obliged to Mr Crook

Table XV

7 days old, 60 hr. dialysed rabbit muscle extract. Extract:total volume = 1:4. Initial glycogen 6 mg. Initial P 0.8 mg. Duration of exp. 130 min. Robison ester formed 3.3 mg.

% Glucose	Inhibition in %	% Ascorbic acid	Inhibition in %
1	-55	1	-15
0.8	-53	0.8	-17
0.6	-46	0.6	-15
0.4	-40	0.4	-5
0.2	-15	0.2	-4
0.8% glucose + 0.2% ascorbic acid	-54	0.8% ascorbic acid + 0.2% glucose	-36

who measured ascorbic acid at the beginning and end of several of our experiments. The reason for a stronger inhibition at pH 8 may be the formation of oxidation products from ascorbic acid.

INSULIN

Insulin as a SS compound would be expected to act as an inhibitor. This it actually does [Lehmann, 1938, 2], but at a concentration too small for its effects to be comparable with that of SS glutathione. Cori *et al.* [1938] suggested that the Zn content of commercial preparations may have been the reason for Lehmann's [1938, 3] findings. They were able to repeat them with a commercial preparation but not with a specially purified sample. The insulin used was a British Drug Houses sample of the crystalline hormone which contained insufficient Zn to inhibit after the insulin was destroyed by reduction. It might be questioned also if the combined Zn of crystalline insulin can act at all like the free ion. Furthermore, insulin hydrochloride acts as an inhibitor as well and the following experiment shows a comparison of the effect of different samples. Insulin which contained only 0.018% Zn was very kindly provided by Messrs Boots Drug Co. Ltd. The final concentration in the experiment was therefore 0.07 μ g./ml. or 10^{-6} M.

Table XVI. *Different samples of insulin*

5 hr. dialysed rabbit muscle extract, fresh. Extract to total volume = 1:4. Initial glycogen 2.5 mg./ml. Initial P 1.2 mg./ml. 0.25 mg. adenylic acid. No extra Mg added.

Addition of 0.4 mg. Insulin = 1.3×10^{-6} M	Robison ester formed	Inhibition
—	1.1	—
Crystalline insulin (Zn insulinate)	0.7	35%
Insulin hydrochloride (Zn-poor)	0.7	
Commercial insulin hydrochloride	0.7	

There is no difference in the inhibitory action of the different samples of insulin, whether they are Zn insulinate or not. The analysis showed that glycogen

Table XVII. *Accumulation of Cori ester in the presence of insulin*

4 days old undialysed rabbit muscle extract. No adenylic or Mg added. Initial glycogen 8 mg. Initial P 0.8 mg. Duration of exp. 20 min.

Addition	Total hexosemono- phosphate	Cori ester	Inhibition of Robison ester formation
—	3.3	0	—
0.07% crystalline insulin	1.9	0.4	50%

and Robison ester could not account for all the carbohydrate in our experiments and an accumulation of Cori ester was observed. Insulin inhibited the formation of Robison ester but allowed an accumulation of Cori ester.

The inhibition by insulin can be counteracted by increasing the Mg in the reaction mixture [Lehmann, 1938, 4]. On the other hand in well dialysed muscle extracts to which Mg is not added it inhibits little of the small phosphorylation going on, but after addition of Mg the increase due to the ion can be suppressed by insulin.

Table XVIII

48 hr. dialysed, 12 days old rabbit muscle extract. Extract: total volume = 1:4. 6.5 mg. adenylic acid added. Initial glycogen 6 mg. Initial P 1.2 mg. Duration of exp. 30 min. The insulin added was neutralized with NaOH.

mg. Mg added	mg. insulin hydrochloride added	Total hexosemono- phosphate formed	Robison ester formed
—	—	2.6	1.1
—	4.5	2.3	1.2
0.1	—	4.0	3.6
0.1	4.5	3.0	1.9

There seems, therefore, to be an interaction between insulin and Mg^{++} taking part in the insulin inhibition. The effect cannot be demonstrated either in the presence of too much or of too little Mg^{++} at a given concentration of insulin (0.3–1 %). This interaction can be observed in model experiments. At a neutral to slightly alkaline reaction Mg^{++} forms a precipitate with insulin. The precipitate cannot be observed in a 0.3 % insulin solution with Mg^{++} concentration below 0.01 mg./ml. If more is added an effect takes place which may be described as follows. The Mg^{++} becomes attached to the negatively charged protein molecule. If enough Mg^{++} is present the negative charges are neutralized and the protein molecule now forms an isoelectric precipitate. Much Mg^{++} leads to resolution due to the zwitterion effect.

Thus in tissue extracts a given amount of insulin at about neutral pH will precipitate Mg^{++} . No precipitation occurs if the concentration of Mg^{++} is diminished by dialysis or if a further addition of Mg^{++} has caused resolution of a previously formed precipitate. A similar effect of phosphate on the solubility of haemoglobin has been described and its theory has been explained by Adair & Adair [1934].

The insulin effect observed is not specific; instead of Mg^{++} any other divalent positive ion can be chosen and insulin may be replaced by a protein of a similar isoelectric point.

Insulin reduced by cyanide neither inhibits phosphorylation, nor does it at a neutral pH show the same effect on a Mg^{++} solution. This may be an explanation of the fact that the insulin inhibition wears off with the duration of the experiment [Lehmann, 1938, 2]. It is possible to demonstrate that insulin is reduced during incubation with muscle extract [Lehmann & Schlossmann, 1938], by separating the insulin from the muscle extract by short heating with subsequent centrifuging. The insulin stays in solution while the muscle proteins are precipitated. The reduction can be shown furthermore by using a washed suspension of minced muscle according to the technique of Hopkins & Dixon [1922] for showing reduction of glutathione by muscle proteins. In this method no boiling is needed before centrifuging. It may be mentioned that reduced insulin is precipitable with trichloroacetic acid and can therefore be separated

from cysteine and glutathione which may have been liberated from muscle proteins during incubation under the influence of insulin; and which compounds might show a positive SH test in the insulin fraction.

SUGARS

The inhibition of Robison ester formation from glycogen by glucose seems to be an indirect one. We found that glucose does not inhibit the formation of Robison ester from Cori ester. (We are grateful to Dr B. E. Holmes for providing us with a sample of the K salt of the Cori ester prepared after Kiessling [1938].) Table XX shows the effects of various sugars on phosphorylation. The inhibition with mannose is only demonstrable with a freshly dissolved sample, a few days standing in the ice chest causing the inhibitory power to disappear. The effect of glucose is in agreement with Haarmann & Stratmann's [1932] finding that 0.5 % glucose inhibits the lactic acid formation from glycogen in skeletal muscle. They observed a great individual variety in the degrees of inhibition. We too have to report that the inhibition of phosphorylation varies at constant glucose concentrations. Most extracts are inhibited from 50 to 80 % at 1 % glucose concentration, but we had several extracts which were inhibited only 20 % and higher concentrations of hexose did not increase the inhibition further.

Table XIX. *Phosphate uptake in presence of glycogen and various sugars*

All values expressed as mg. in total reaction mixture.

1 ml. 60 hr dialysed, 21 days old rabbit muscle extract. 2 ml. additions containing: 0.5 *M*/15 phosphate, 20 mg. glycogen, 1 mg. adenylic acid (Na salt). 0.2 mg. Mg as MgCl_2 . Initial P 1.15 mg. Duration of exp. 120 min. Total volume of reaction mixture: 3 ml.

Addition	Inorganic P found (mg.)	P uptake mg.
—	0.68	0.47
1% Fructose	0.71	0.44
1% Glucose	0.99	0.16
1% Mannose	0.95	0.20
1% Galactose	0.83	0.32
1% Lactose	0.76	0.39
1% Arabinose	0.71	0.44
2% Maltose	0.79	0.36
2% Sucrose	0.77	0.38
3% Raffinose	0.66	0.48

Table XX. *Phosphate uptake inhibited by Robison ester and glucose*

1 ml. 11 days old, 60 hr. dialysed rabbit muscle extract. 2 ml. additions containing 0.5 ml. *M*/15 phosphate (*pH* 7), 10 mg. glycogen, 1 mg. adenylic acid (Na salt). 0.3 mg. Mg as MgCl_2 . Duration of exp. 120 min. Initial P 1.15 mg.

Additions	Inorganic P found (mg.)	P uptake mg.
—	0.37	0.78
0.6% Glucose	0.81	0.34
1.0% Robison ester*	0.75	0.40

* The Robison ester was free from inorganic P.

Hexosediphosphate differs from other sugars inasmuch as at $3-1 \times 10^{-3}$ *M* it stimulates the phosphorylation of glycogen. The increase in Robison ester formation is not inhibited by iodoacetate or arsenate. The latter especially would poison specifically all indirect phosphorylation due to oxido-reduction of triosephosphate [Needham & Pillai, 1937].

Table XXI. *Increase of Robison ester formation in the presence of hexosediphosphate*

2 days old undialysed rabbit muscle extract. Extract to total volume = 1 : 2. Initial glycogen 6 mg. Initial P 1.9 mg. Duration of exp. 40 min.

Addition	Robison ester formed (mg.)	
	<i>M</i> /400 iodoacetate	<i>M</i> /100 sodium arsenate
—	2.8	2.2
<i>M</i> /200 Hexosediphosphate	7.2	5.6

Both iodoacetate and arsenate have at high concentrations a diminishing effect on Robison ester formation, which should be distinguished from a specific inhibition. The inhibition of Robison ester formation by iodoacetate (Table XXI) was 18 % in absence, 20 % in presence of hexosediphosphate; arsenate inhibited in both cases by 40 %.

Kendal & Stickland [1938] claimed that it was necessary to have traces of hexosediphosphate present for the action of the enzyme converting Cori ester into Robison ester. This was not confirmed by Cori *et al.* [1938]. The effect observed by us is not identical with that of Kendal & Stickland. Hexosediphosphate seems to act on the initial phosphorylation of glycogen. Its action results first in an accumulation of Cori ester which subsequently is slowly transformed into Robison ester as is shown in Table XXII.

Table XXII

6 days old undialysed rabbit muscle extract. Extract : total volume = 1.5 : 2. Initial glycogen 6 mg. Initial P 0.8 mg.

Time of exp. min.	Total hexosemono-phosphate formed mg.	Robison ester formed mg.	Cori ester formed mg.	Addition
10	1.1	1.0	0.1	
	2.8	1.2	1.6	<i>M</i> /300 Hexosediphosphate
30	1.2	1.2	—	
	2.8	1.9	0.7	<i>M</i> /300 Hexosediphosphate
120	2.2	2.2	—	
	2.9	2.9	0	<i>M</i> /300 Hexosediphosphate

Negelein & Brömel [1939] observed that glyceraldehyde phosphate in presence of a purified yeast enzyme combines with inorganic phosphate to form a triosediphosphate. This can transfer one phosphate molecule to glucose. Hexosediphosphate added to muscle extract yields glyceraldehyde phosphate [Meyerhof & Lohmann, 1934] and a reaction in muscle extract may take place like the one described in yeast. Under the conditions of our experiments neither hexosediphosphate nor triosephosphate were themselves phosphorylated. Yet ordinary yeast juice does not show this process either and a purification of the enzymes concerned may be necessary for isolating this phosphorylation.

DISCUSSION

The fact that amylases compete with phosphorylating enzymes for the substrate glycogen supports the view that there are at least two possible paths of anaerobic glycogen breakdown (see especially Parnas [1937]). The question

arises as to whether these two systems can act in muscle side by side. Whereas Parnas [1937] denies the occurrence of amylases in muscle, they have been described repeatedly by other authors [see Lohmann, 1926; Willstätter & Rohdewald, 1938]. Lately, Augustin [1938] reported from Parnas' laboratory the existence of amylase and phosphorylase side by side in heart muscle of several mammals. The contradictory statements for skeletal muscle may find an explanation in the individual variation of the amylase content of skeletal muscle in the same species of animals, a fact which we observed specially in frog muscle.

In a subsequent publication we will deal with the biochemical background for the poor phosphorylating power of certain tissues [see also Gill & Lehmann, 1939]. We observed that some frog muscle extracts contain very little amylase, others very much. The phosphorylation in these extracts was in inverse proportion to their amylase activity. Case [1931] observed a loss of I_2 coloration after incubation of glycogen with boiled muscle tissue. No lactic acid was formed. He concluded that there were amylases acting on the glycogen. Our findings regarding the heat stability of the enzymes involved in the formation of Robison ester suggest that a formation of Robison ester might also have occurred. Yet we ourselves found a loss of I_2 staining power in glycogen after a short incubation with rabbit muscle extract followed by isolation using Pflüger's method. The loss of I_2 staining power did not correspond to the formation of hexosemonophosphate or the loss of glycogen, that is to say, the same amount of glycogen, isolated according to Pflüger, had less I_2 staining power after incubation with rabbit muscle extract than it had before. For starch, the I_2 coloration has been found by Hanes & Cattle [1938] to be a reliable index of amylatic degradation.

The experimental data of this paper seem to indicate that Robison ester may not be an intermediate of the oxidative breakdown of carbohydrate.

The fact that oxidation inhibits its formation and that reduction increases it has only been established for extracts. The possibility cannot be excluded that very fresh not inactivated extracts and especially the enzyme of the living cell never attain a state of oxidation sufficient to prohibit the Robison ester formation. Rapkine [1938] excluded the formation of phosphoglyceric acid from triosephosphate in oxidized muscle extract. We suggest now that Robison ester is one of the compounds probably not formed from glycogen and inorganic phosphate in oxidized muscle. Whether or not phosphorylation plays any part in oxidative carbohydrate breakdown cannot be decided before more is known of the enzymic oxidation of the Cori ester. The phosphorylation of glycogen by inorganic phosphate through adenylypyrophosphate is a possibility which should not be excluded [Lehmann & Needham, 1937]. Yet it is improbable that it occurs in the process of oxidation, as the source of phosphorylation of adenylic acid is just that oxidoreduction which Rapkine [1938] has shown to be inhibited by O_2 [Meyerhof *et al.* 1937; Needham & Pillai, 1937]. Nevertheless, rapid changes between the reduced and oxidized state may permit Robison ester formation in the former and oxidative breakdown of the compound in the latter state (cf. the correlation of oxidation and phosphorylation in haemolysed blood [Runnström & Michaelis, 1935]). There is also the possibility of phosphorylation of glycogen by creatine phosphate via adenylypyrophosphate [Lehmann & Needham, 1937]. Sacks & Sacks [1933] found that "hexosephosphate" was formed in intact muscle only at the expense of creatine phosphate breakdown. Yet Sacks [1933; 1938] doubts whether hexosephosphate is formed at all in the "steady state" in aerobic muscle.

An increase of aerobic fermentation of yeast by cysteine and SH glutathione has been described [Quastel & Wheatley, 1932; Runnström & Sperber, 1937; 1938], although it has not yet been shown that this effect was due to an increase in Robison ester formation from glucose.

The action of reduced compounds on Robison ester formation throws new light on the work of Gaddie & Stewart [1935]. They observed an increased lactic acid formation from glycogen in muscle extracts after addition of SH glutathione. That other authors could not find the same for glycogen breakdown [Geiger, 1935] or phosphorylated glucose breakdown (muscle extract plus yeast hexokinase [Meyerhof & Kiessling, 1935]) may find its explanation in the possibility that Gaddie & Stewart were working with an oxidized extract, the others with sufficiently reduced enzyme solutions. Our finding may be thought to have a bearing on the question whether glutathione is a coenzyme for glycolysis in embryo. Needham & Nowinski [1937] and Needham & Lehmann [1937] suggest that the glucolytic breakdown in embryo may be different from that of glucose in yeast or glycogen in muscle. It has been pointed out that their arguments are negative and as such unconvincing in themselves [MacFarlane, 1939]. One of the positive arguments was that dialysed embryo enzyme needed glutathione for reactivation. This reactivation cannot be a revival of phosphorylation like the one described in this paper for rabbit muscle extract, as it is specific for glutathione and cannot be shown by using another SH compound like cysteine [Needham & Lehmann, 1937].

SUMMARY

1. Amylases inhibit the formation of Robison ester in muscle extracts. The hydrolytic enzyme acts simply as a competitor with the phosphorylating enzyme for the substrate, glycogen.

2. Oxidizing agents inhibit Robison ester formation: the agents used were catechol plus catechol oxidase, and SS-glutathione. Adrenaline in large amounts can be used instead of catechol. Small amounts of adrenaline had no effect on Robison ester formation.

3. Reducing compounds and agents increasing the content of SH-groups in the medium augment Robison ester formation. Such were SH-glutathione, cyanide, thiolacetic acid, denatured protein, sodium hydrosulphite and evacuation.

4. Muscle extract boiled for 2 min. is still able to phosphorylate glycogen and to form Robison ester.

5. Cu^{++} , Fe^{+++} , Fe^{++} and Zn^{++} inhibit Robison ester formation; Ni^{++} and Co^{++} increase it. Co^{++} increased Robison ester formation at optimum Mg^{++} concentration.

6. Ascorbic acid inhibits Robison ester formation. It acts in concentrations similar to those of glucose.

7. Insulin inhibits Robison ester formation but allows accumulation of Cori ester to go on. Its action seems largely to be due to the removal of Mg^{++} from solution. Insulin, the SS groups of which have been reduced either by cyanide or by muscle extract, does not inhibit Robison ester formation.

8. Data are given regarding the inhibition of phosphorylysis by sugars. Of these the most active are glucose, mannose and Robison ester. They inhibit, like amylases, the phosphorylation of glycogen and consequently the formation of Robison ester. Hexosediphosphate increases phosphorylation of glycogen and consequently the formation of Robison ester. Its effect is not inhibited by iodoacetate or arsenate.

9. Points 1, 2 and 3 are discussed in their possible significance for the biochemistry of muscle.

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REFERENCES

- Adair & Adair (1934). *Biochem. J.* **28**, 1230.
Augustin (1938). *Hoppe-Seyl. Z.* **255**, 61.
Bell (1937). *Perspectives in Biochemistry*. Cambridge.
Case (1931). *Biochem. J.* **25**, 561.
—— & McCullagh (1928). *Biochem. J.* **22**, 1060.
Cori & Cori (1931). *J. biol. Chem.* **94**, 561.
—— (1936). *Proc. Soc. exp. Biol., N.Y.*, **34**, 702.
—— (1937). *Proc. Soc. exp. Biol., N.Y.*, **36**, 119.
—— Colowick & Cori (1937). *J. biol. Chem.* **121**, 465.
—— (1938). *J. biol. Chem.* **124**, 543.
—— (1939, 1). *J. biol. Chem.* **127**, 771.
—— Schmidt & Cori (1939, 2). *Science*, **89**, 2316.
Curtius & Ohlmeyer (1938). *Biochem. Z.* **298**, 412.
Dickens (1938, 1). *Biochem. J.* **32**, 1626.
—— (1938, 2). *Biochem. J.* **32**, 1645.
Embden & Zimmermann (1927). *Hoppe-Seyl. Z.* **167**, 114.
Engelhardt & Barchash (1938). *Biochimia*, **3**, 501.
Euler & Bauer (1938). *Naturwissenschaften*, **26**, 431.
Fiske & Subbarow (1925). *J. biol. Chem.* **66**, 375.
Foster & Woodrow (1924). *Biochem. J.* **18**, 562.
Gaddie & Stewart (1935). *Biochem. J.* **29**, 2101.
Geiger (1935). *Biochem. J.* **29**, 811.
Gemmill & Hellerman (1937). *Amer. J. Physiol.* **120**, 522.
Gill & Lehmann (1939). *Chem. Ind.* **58**, 254.
Giragossintz, Davidsohn & Kirk (1936). *Mikrochemie*, **21**, 21.
Good, Kramer & Somogyi (1933). *J. biol. Chem.* **100**, 485.
Haarmann & Stratmann (1932). *Biochem. Z.* **256**, 361.
Hanes & Cattle (1938). *Proc. roy. Soc. B*, **125**, 387.
Harris (1923). *Proc. roy. Soc. B*, **94**, 425.
Hellerman & Perkins (1934). *J. biol. Chem.* **107**, 241.
—— (1935). *J. biol. Chem.* **112**, 175.
Hopkins (1925). *Biochem. J.* **29**, 5.
—— & Dixon (1922). *J. biol. Chem.* **54**, 529.
—— and Morgan (1938). *Biochem. J.* **32**, 611.
—— & Lutwak-Mann (1938). *Biochem. J.* **32**, 1829.
Keilin & Mann (1938). *Proc. roy. Soc. B*, **125**, 187.
Kendal & Stickland (1937). *Biochem. J.* **31**, 1758.
—— (1938). *Biochem. J.* **32**, 578.
Kiessling (1938). *Biochem. Z.* **298**, 421.
—— (1939). *Naturwissenschaften*, **27**, 129.
Lehmann (1938, 1). *Nature, Lond.*, **141**, 470.
—— (1938, 2). *Nature, Lond.*, **141**, 690.
—— (1938, 3). Ph.D. Thesis, Cambridge University.
—— (1938, 4). *Chem. Ind.* **57**, 976.

- Lehmann & Needham (1937). *Biochem. J.* **31**, 329.
 — (1938). *Enzymologia*, **5**, 95.
 — & Schlossmann (1938). *J. Physiol.* **94**, 2P.
 Lipmann (1933). *Biochem. Z.* **265**, 133.
 — (1934). *Biochem. Z.* **268**, 314.
 — (1936). *Nature, Lond.*, **138**, 588.
 Lohmann (1926). *Biochem. Z.* **178**, 444.
 — (1928). *Biochem. Z.* **194**, 306.
 — (1931). *Biochem. Z.* **237**, 445.
 — & Jendrassik (1926). *Biochem. Z.* **178**, 419.
 Lundsgaard (1933). *Biochem. Z.* **264**, 209.
 MacFarlane (1939). *Biochem. J.* **33**, 565.
 Mauthner (1907). *Hoppe-Seyl. Z.* **78**, 29.
 McCullagh (1928). *Biochem. J.* **22**, 1060.
 Meyer (1927). *Biochem. Z.* **183**, 216.
 — (1928). *Biochem. Z.* **193**, 139.
 Meyerhof (1918, 1). *Hoppe-Seyl. Z.* **101**, 165.
 — (1918, 2). *Hoppe-Seyl. Z.* **102**, 1.
 — (1926). *Biochem. Z.* **178**, 395.
 — & Kiessling (1935). *Biochem. Z.* **283**, 83.
 — & Lohmann (1934). *Naturwissenschaften*, **22**, 134.
 — & Ohlmeyer (1936). *Naturwissenschaften*, **24**, 741.
 — Schulz & Schuster (1937). *Biochem. Z.* **293**, 309.
 Michaelis, Barron & Guzman (1929). *J. biol. Chem.* **83**, 191.
 — & Yamaguchi (1929). *J. biol. Chem.* **83**, 367.
 Miller & Van Slyke (1936). *J. biol. Chem.* **114**, 583.
 Needham & Lehmann (1937). *Biochem. J.* **31**, 1210.
 — & Nowinski (1937). *Biochem. J.* **31**, 1165.
 — & Pillai (1937). *Biochem. J.* **31**, 1837.
 Negelein & Brömel (1939). *Biochem. Z.* **301**, 135.
 Ohlmeyer & Ochoa (1937). *Biochem. Z.* **293**, 338.
 Ostern & Guthke (1936). *C.R. Soc. Biol., Paris*, **121**, 282.
 — — & Terzakowec (1936). *C.R. Soc. Biol., Paris*, **121**, 1133.
 — — (1936). *Hoppe-Seyl. Z.* **243**, 9.
 Parnas (1937). *Ergebn. Enzymforsch.* **6**, 57.
 — & Baranowski (1935). *C.R. Soc. Biol., Paris*, **120**, 307.
 — & Ostern (1936). *Bull. Soc. Chim. Biol.* **18**, 1471.
 Quastel & Wheatley (1932). *Biochem. J.* **26**, 2169.
 Rapkine (1938). *Biochem. J.* **32**, 1729.
 Ronzoni (1927). *Proc. Soc. exp. Biol., N.Y.*, **25**, 178.
 Robison (1922). *Biochem. J.* **16**, 809.
 Rosenheim & Davidsohn (1904). *Z. anorg. Chem.* **41**, 231.
 Runnström & Michaelis (1935). *J. gen. Physiol.* **18**, 717.
 — & Sperber (1937). *Naturwissenschaften*, **25**, 540.
 — (1938). *Nature, Lond.*, **141**, 689.
 Sacks (1938). *Amer. J. Physiol.* **122**, 215.
 — & Sacks (1933). *Amer. J. Physiol.* **105**, 151.
 Schäffner (1939). *Naturwissenschaften*, **27**, 195.
 — & Specht (1938). *Naturwissenschaften*, **26**, 494.
 Wagner-Jauregg & Rzeppa (1936, 1). *Hoppe-Seyl. Z.* **240**, 1.
 — (1936, 2). *Hoppe-Seyl. Z.* **243**, 166.
 Walker (1925). *Biochem. J.* **19**, 1082.
 Warburg, Christian & Griesse (1935). *Biochem. Z.* **282**, 157.
 Willstätter & Rohdewald (1938). *C.R. Lab. Carlsberg*, **22**, 555.
 Winfield & Hopkins (1915). *J. Physiol.* **50**, 5P.