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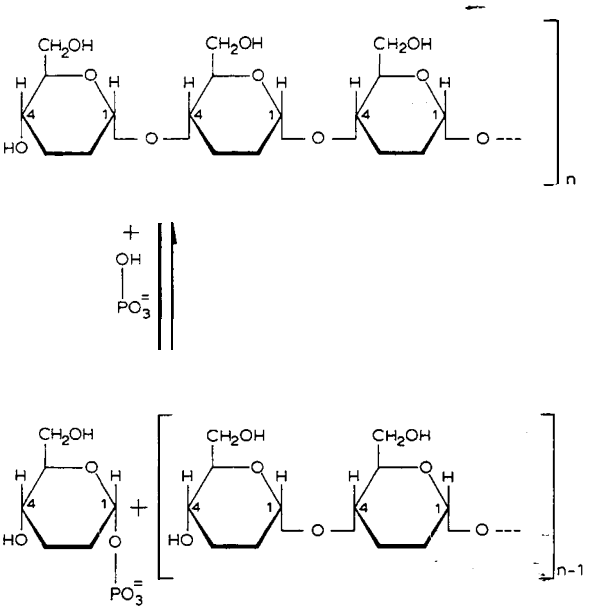
Polysaccharide phosphorylase

Nobel Lectures, December 11, 1947

Part I - by Carl F. Cori

Polysaccharide phosphorylase is characterized as an enzyme which can break or make an α-1-4-glucosidic bond at the termination (non-reducing end) of a glycogen or starch chain. The process is illustrated below:



The interaction of phosphate with the terminal glucosidic bond results in the formation of glucose-1-phosphate and the loss of a chain unit; in the reverse reaction the glucose part of glucose-1-phosphate is added as a new chain unit and phosphate is set free. This reversible enzymatic polymerization occurs with little change in free energy, as may be calculated from the equilibrium constant. The reaction which involves expenditure of energy in the conversion of glucose to glycogen is the hexokinase reaction, the formation of glucose-6-phosphate from glucose and adenosine triphosphate.

For the discussion which follows it is important to note that the phosphorylated hexoses can enter into the following enzymatic equilibria (Table 1)

Table 1. Enzymatic equilibria at pH 7 at 30°.

	glycogen + $PO_4^=$	*Concentration in resting muscle (mole per kg)*
Phosphorylase	23% ↓ ↑ 77%	
	glucose-1-phosphate	1×10^{-4}
Mutase	95% ↓ ↑ 5%	
	glucose-6-phosphate	
Isomerase	30% ↓ ↑ 70%	3×10^{-3}
	fructose-6-phosphate	

Resting mammalian muscle contains about 0.003 mole of hexose-6-phosphate ($\frac{2}{3}$ glucose-6- and $\frac{1}{3}$ fructosed-phosphate) per kilo. Assuming that the mutase reaction is also close to equilibrium in a resting muscle, we can calculate the concentration of glucose-1-phosphate to be expected as 0.0001 mole per kilo or less than 0.2 per cent of the total acid soluble phosphate content of muscle. It seems clear that the detection and isolation of this intermediate could not have been accomplished without a separation of phosphorylase activity from that of the other enzymes.

Formation of hexose-6-phosphate - The discovery of polysaccharide phosphorylase and glucose-1-phosphate can be traced to systematic work on the formation of hexosed-phosphate in muscle. Of particular importance was the fact that the method used for the determination of hexosed-phosphate consisted of two independent measurements, one based on the reducing power of the compound and the other on its phosphate content and that there was generally good agreement between these two measurements. In this manner it was found that a number of procedures led to an increase in the hexosed-phosphate content of muscle, among which may be listed, anaerobiosis, injection of epinephrine in intact animals, incubation of isolated frog muscle in Ringer's solution containing epinephrine, and electric stimulation of mammalian or frog muscle.

Balance experiments during aerobic recovery ofpreviously stimulated and isolated frog muscle indicated that the hexose-6-phosphate which disappeared was in large part reconverted to glycogen; hence it was made probable that the reaction, glycogen → glucose-6-phosphate, was reversible. The next step was the finding that the increase in hexosed-phosphate in isolated frog muscle incubated anaerobically with epinephrine was accompanied by

a corresponding decrease in inorganic phosphate (average of 6 experiments per 100 g muscle, + 14 mg ester P, - 16 mg inorganic P). Phosphocreatine and adenosine triphosphate (ATP) remained unchanged, suggesting that they were not involved in the formation of hexose-6-phosphate, but since their regeneration through lactic acid formation was not excluded, the experiments were repeated with muscles poisoned with iodoacetate. The results were the same as with unpoisoned muscle and it was therefore concluded that hexosed-phosphate was formed from glycogen by ester&cation with inorganic phosphate:

$$\text{glycogen} + \text{phosphate} \rightarrow \text{hexose-6-phosphate}$$

These findings were presented in 1935 at the 15th International Physiological Congress and were discussed at that time with Professor Pamas who then stated that he had under consideration experiments with muscle extract. Prior to that time it has been assumed that glycogen reacted with ATP to form hexose diphosphate. Parnas and Baranowski found that a disappearance of inorganic phosphate could be demonstrated in a cell-free extract of muscle which did not contain phosphocreatine or ATP. This was ofimportance because it established beyond doubt the participation of inorganic phosphate in the splitting of glycogen, a process which has been aptly called "phosphorolysis" by Parnas. However, the mechanism of phosphorolysis remained unknown until glucose-r-phosphate had been isolated.

Formation of glucose-1-phosphate - The following experiments led to the detection and isolation of glucose-1-phosphate. Minced frog muscle was extracted three times with 20 volumes of cold distilled water, a procedure which removed most of the acid-soluble phosphates normally present in muscle, but did not remove glycogen. When the washed residue was incubated anaerobically at 20° in isotonic phosphate buffer at pH 7.2, some hexose monophosphate was formed. On addition of a catalytic amount of muscle adenylic acid, the formation of hexose monophosphate was very markedly increased. When phosphate was replaced by isotonic KCl, no ester formation occurred. The glucose part of the ester could have come only from glycogen, and the phosphate part only from the added inorganic phosphate, thus confirming the reaction postulated for intact muscle.

After short periods of incubation there was much more organic phosphate present in the hexose monophosphate fraction than corresponded to the reducing power of hexose-6-phosphate. Such a discrepancy had not been

encountered before in analyses of the hexose monophosphate fraction, and since the discrepancy became smaller or disappeared completely after longer periods of incubation, the formation of a precursor of glucosed-phosphate was suspected. Short hydrolysis in NH_2SO_4 at 100° (conditions under which hexosed-phosphate is not hydrolyzed) revealed the presence of a compound which yielded equivalent amounts of fermentable sugar and inorganic phosphate.

A representative experiment is shown in Table 2. Comparison of the second, third and the last columns shows that the additional organic phosphate present before hydrolysis is accounted for by this new compound. Furthermore, the disappearance of this compound in the third hour of incubation (-0.74 millimoles) is accounted for by a corresponding gain in reducing power ("hexose") before hydrolysis (+0.67 millimoles).

Table 2. Formation of glucose-1-phosphate in minced and washed frog muscle incubated in phosphate buffer plus adenylic acid.

The water-soluble, alcohol-insoluble barium salts (hexose monophosphate fraction) were isolated and analyzed for phosphate and reducing power before and after hydrolysis in *N* H_2SO_4 for 10 minutes at 100°.
AU-values are given in milimoles per 100 g muscle.

Hours of incubation	*Before hydrolysis*		*After hydrolysis*		*Difference (Org. P—Inorg. P)*
	Hexose	*Organic P*	*Fermentable sugar**	*Inorganic P*	
0	0.03	0.03			
1	0.44	1.68	1.22	1.26	0.42
2	0.73	2.22	1.42	1.45	0.77
3	1.40	2.16	0.68	0.65	1.51

* The sugar formed after hydrolysis was completely fermentable, while hexose-6 phosphate under the conditions chosen, was not fermented by the live yeast.

The new phosphate ester was isolated as the crystalline brucine salt in a large-scale experiment similar to that shown in Table 2 and identified as glucose-1-phosphate.

When glucose-1-phosphate was added to a cell-free frog or rabbit muscle extract, it was converted rapidly to glucose-6-phosphate by an enzyme which was named phosphoglucomutase. It was due to the leaking out of the mutase that glucose-1-phosphate accumulated in washed and minced

frog muscle. Mutase is greatly enhanced in its activity by magnesium ions. In order to demonstrate the formation of glucose-I-phosphate from glycogen and inorganic phosphate in muscle extract, it was necessary to remove magnesium ions by dialysis.

An experiment which shows the effect of magnesium ions as well as of adenylic acid is given in Table 3. Addition of magnesium ions to the dialyzed extract had no effect on the total amount of ester formed, but it prevented the accumulation of glucose-I-phosphate.

Experiments similar to those shown in Table 3 were performed with

Table 3. Formation of glucose-1-phosphate in dialyzed (17 hours) rabbit muscle extract.

Incubated for 60 minutes at 24° after addition of glycogen and inorganic phosphate. All values are given in micromoles per 10 cc. of extract:

Additions				
Adenylic acid	$MgCl_2$	*1-ester*	*6-ester*	*Total ester*
0	0	7.8	15.0	22.8
0	80	0.0	21.7	21.7
5	0	71.2	22.2	93.4
5	80	5.6	86.2	91.8

dialyzed extracts of other mammalian tissues (brain, heart, liver, kidney) and of yeast. In all of these the formation of glucose-I-phosphate could be demonstrated, pointing to a wide distribution of the enzyme phosphorylase. Hanes has described the occurrence of this enzyme in higher plants, particularly in tubers and seeds. In general, the enzyme is present in tissues and cells which contain glycogen or starch.

Properties and synthesis of glucose-l-phosphate - The ester, having no free reducing group, does not react with alkaline copper solutions or with hypoiodite and is resistant to the action of strong alkali. Complete hydrolysis occurs in 10 minutes at 100° in 0.1 *N* HCl or H_2SO_4 and equivalent amounts of free glucose and inorganic phosphate are formed. The quantitative determination of the ester is based on this property. The neutral barium and potassium salts of the ester are sparingly soluble in 66 per cent alcohol. A crystalline dipotassium salt, containing 2 H_2O, has been described by Kiessling.

The ester has been synthesized by a condensation of α–tetraacetyl glucose-

I-bromide with trisilver phosphate. An intermediate product, tri- (tetraacetyl glucose-I)-phosphate, is formed which yields glucose-I-phosphate on hydrolysis in 0.2 N HCl in methyl alcohol. The synthetic, like the natural product, is the α-isomer. The β-isomer is obtained by substituting dibenzyl phosphate or "monosilver" phosphate as the phosphorylating agent; it is not acted upon by phosphorylase. Neither are the synthetically obtained α-isomers of mannose-I- or galactose-I-phosphate.

Reversibility - The first clue for a possible reversibility of the reaction, glycogen + phosphate → glucose-I-phosphate, came from the observation that addition of glucose-I-phosphate to a reaction mixture containing enzyme, glycogen and phosphate was strongly inhibitory, while glucosed-phosphate had only a weak inhibitory effect on the formation of glucose-I-phosphate. Further investigation showed that conditions for reversibility were unfavorable because the concentration of glucose-I-phosphate could not be maintained, owing to the activity of phosphoglucomutase even in electrodialyzed extracts and at pH 6.5 (which is less favorable for its action than for the action of phosphorylase). It became clear that a separation of the two enzymes was necessary in order to investigate reversibility. A partial separation was first achieved by adsorption of phosphorylase on aluminium hydroxide, followed by elution with disodium phosphate and dialysis to remove inorganic phosphate. When glucose-I-phosphate was added to this enzyme preparation, inorganic phosphate was set free and a polysaccharide was formed in equivalent amounts, showing the reversibility of the reaction. Independently Kiessling had prepared a protein fraction from yeast juice by fractionation with 0.3 saturated ammonium sulfate which also catalyzed the reaction in a reversible manner, while some qualitative observations with yeast extract based on iodine colors had been made earlier by Schäffner and Specht.

An original experiment with a partially purified preparation of muscle phosphorylase is reproduced here (Fig. 1) because it is instructive in relation to later developments. The curve in Fig. 1 shows a definite lag period; the polysaccharide which was formed gave a blue color with iodine and the reaction did not attain a true equilibrium owing to incomplete separation of phosphorylase from phosphoglucomutase. Reversibility could also be demonstrated with phosphorylase preparations of heart and brain. The iodine color of the newly formed polysaccharide was brown to reddish purple rather than blue as with muscle phosphorylase. Preparations of liver phosphorylase formed a polysaccharide which could not be distinguished from glycogen in iodine color and other properties.

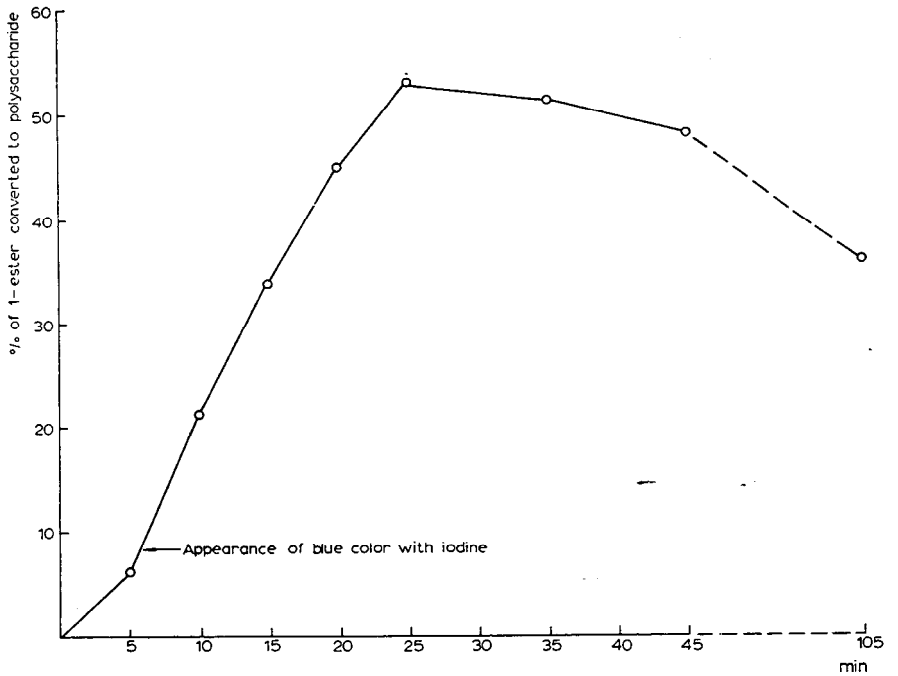


Fig. 1. Synthesis of polysaccharide with a partially purified phosphorylase preparation of muscle. (Experiment of March 11, 1939.)

After these observations had been made, it became clear that further progress depended on the isolation of phosphorylase. This is described in Part 2 which is inserted here in order to avoid an interruption in the sequence of exposition.

Part 2 – by Gerty T. Cori

For a detailed study of the action of phosphorylase and for an understanding of its mechanism, it appeared necessary to work with highly purified enzyme preparations. Muscle was chosen as starting material since in it the concentration of the enzyme is much higher than that found in other tissues. The rapid rate of glycogen breakdown connected with muscular contraction may explain the relatively high concentration in skeletal muscle of phosphorylase as well as of the other enzymes which are concerned with lactic acid formation.

The primer action of glycogen - The protein fraction of a muscle extract, precipitated by less than 0.5 saturation with $(NH_4)_2SO_4$, showed a marked rise in phosphorylase activity per unit of protein over the unfractionated starting material. This was however the case only when the enzyme was catalyzing the reaction toward the right:

$$\text{glycogen} + \text{inorganic phosphate} \rightleftharpoons \text{glucose-1-phosphate} \qquad (1)$$

When enzyme activity was tested in the opposite direction a puzzling difficulty was encountered. Activity set in only after a lag period; refractionation of the enzyme increased this lag period from minutes to hours and in some preparations completely abolished the activity toward polysaccharide formation. A similar observation was made by Kiessling with yeast phosphorylase and led him to conclude that he had separated two enzymes, one concerned with glycogen synthesis, one with its breakdown.

Liver phosphorylase, upon salt fractionation, was found to retain activity toward polysaccharide synthesis. Such preparations always contained traces of glycogen, while the purified muscle enzyme was free of glycogen. This observation offered a clue. Addition of glycogen to the reaction mixture in as low a concentration as 10 mg per cent led to immediate activity of muscle phosphorylase preparations, seemingly inactive when tested without gly-

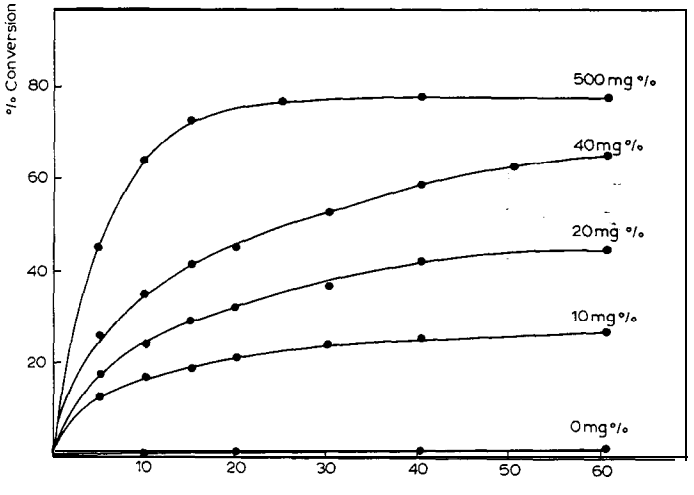


Fig. 2. Rate of conversion of glucose-1-phosphate to polysaccharide in the presence of crystalline muscle phosphorylase and increasing amounts of glycogen.

cogen addition. Fig. 2 illustrates these findings as well as the effect of increasing concentrations of glycogen on the rate of the reaction.

From these observations it followed that glycogen was needed for the activity of the enzyme in both directions. Since the equilibrium of the reaction is in favor of polysaccharide formation, it was of considerable advantage that the enzyme could now be tested when acting in that direction.

Enzyme test - Under certain conditions, Reaction (1) is of the unimolecular type; toward the left, it can be followed conveniently by determination of the amount of inorganic phosphate which is set free. Polysaccharide is formed in equivalent amounts. Since the reverse reaction occurs simultaneously, an equation for a reversible first-order reaction is applicable: $k = I/t \log x_e/(x_e - x)$; x_e represents the per cent of glucose-I-phosphate converted to inorganic phosphate (and polysaccharide) at equilibrium, 79 per cent at pH 6.7, while x represents the per cent converted at time t (in minutes). The position of the equilibrium changes with pH, hence a different value for x_e must be used at other pH values. Hanes has shown, however, that at equilibrium the ratio of the ionic forms of orthophosphoric and glucose-I-phosphoric acid remains constant over the pH range investigated. The mean ratio of the divalent ions of the two acids was found to be 2.2 for potato and 2.0 for muscle phosphorylase between pH 5 and 7.5.

In the standard test the enzyme is diluted with 0.03 M cysteine at pH 6.7. Unless a reducing agent is present the enzyme has low activity and the reaction is not of the first order. To a solution of glycogen, glucose-r-phosphate and adenylic acid (5-phosphoriboside), adjusted to pH 6.7, an equal volume of dilute enzyme solution is added to start the reaction. The order of addition of the different components of the system makes an appreciable difference in the initial rate of the reaction. When enzyme or glucose-1-phosphate is added last, the initial rate is the same, but when glycogen is added last, the initial rate is less and the shape of the rate curve is different.

The final composition of the reaction mixture in micromoles per cc. is : 15 cysteine, 16 glucose-I-phosphate, 0.5 adenylic acid and glycogen (10 mg per cc.). Inorganic phosphate is determined in an aliquot after 5 and 10 minutes of incubation at 30°, and the data are used for a calculation of the first-order velocity constant.

The number of enzyme units present are expressed as k multiplied by 1,000, for convenience. In order to compare different preparations, the activity of the enzyme is expressed as units per mg protein and is calculated for the amount of protein which is present in 1 cc. of reaction mixture.

The pH optimum of the reaction is between 6.5 and 6.8 and the temperature optimum is close to 38°. The conversion of glucose-r-phosphate (pK_2 6.1) to orthophosphate (pK_2 6.8) results in a shift in pH. This change is small and has a neglible effect on the rate during the early course of the reaction. The pH can be kept constant by the addition of various buffers but they all decrease the activity of the enzyme.

Crystalline muscle phosphorylase has an activity of about 3,000 units per mg protein at pH 6.7 and 30°. Calculated for the initial rate of the reaction, this corresponds to a turnover number of 40,000 molecules of glucose-I phosphate per molecule of enzyme (mol. wt. 400,000) per minute.

Phosphorylase a and b - It had been observed in experiments with dialyzed and aged muscle extracts that the rate of phosphorylase activity was increased 10 times or more by the addition of adenylic acid in low concentrations. Later it was found that phosphorylase preparations obtained by precipitation of a fresh-water extract of rabbit muscle at 0.41 saturation with $(NH_4)_2S0_4$, when freshly dissolved, had in the absence of added adenylic acid as high as 66 per cent of the full activity (i.e. activity in the presence of added adenylic acid). When the enzyme solution was kept for 1 hour at 25° its activity without adenylic acid dropped to zero; at this point addition of adenylic acid to the reaction mixture brought back the original activity.

These observations led to the conclusion that there exist two forms of phosphorylase, one active (phosphorylase *a)* and one inactive (phosphorylase *b)* without adenylic acid addition and that muscle tissue contains a factor, soon shown to be an enzyme, which converts the *a* into the *b* form. The assumption that this enzyme removes adenylic acid from the a form seemed justified at this point and the enzyme was designated as PR (prosthetic group removing enzyme). Later work, however, failed to establish the presence of adenylic acid in phosphorylase *a* and the nature of the prosthetic group which is removed by the PR enzyme remains obscure. There is evidence that an organic phosphate which is dialyzable and difficult to hydrolyse in hot acid is split off from phosphorylase a by PR.

In order to preserve the a form, it is necessary to prevent action of the PR enzyme. *In vivo* this is accomplished by avoiding as much as possible stimulation of the muscles before or during excision; *in vitro,* by the earliest separation of phosphorylase from the PR enzyme.

Preparation of crystalline phosphorylase a - The procedure described below is based on experience gained in a large number of preparations. Information

concerning the various steps is given which has not been included in the original description of the method.

A rabbit is killed by injection of amytal and the muscles of the hind legs and back are rapidly excised and weighed. The following steps are carried out in a cold room at 4°. The muscles are passed through an ordinary meat grinder and extracted with one volume of water for about 10 minutes (extraction in a blendor is to be avoided since it leads to conversion of the a to the *b* form). After the residue is separated by straining it through gauze it is re-extracted with another volume of water. The combined extracts are filtered through cotton and coarse filter paper; this should be accomplished in 1-2 hours. It is unnecessary to obtain a completely clear extract. The pH of the extract is measured with a glass electrode and if it is above 6.2, it is adjusted to that pH by adding 0.05 *N* HCl with stirring. The extract is then dialyzed in cellophane tubes (diameter 2.3 cm) against running tap water of 4-10° for 3-4 hours. The turbid extract is collected in a beaker and 0.05 N HCl is added to bring the pH to 5.7. A flocculent precipitate forms which contains most of the PR enzyme and only a small amount of phosphorylase. This is the case only in rabbit muscle. In other species this separation is less complete and so far the enzyme has only been crystallized from rabbit muscle. The isoelectric precipitate is removed by centrifugation followed by filtration. Table 4 shows that no purification is achieved in this step, which nevertheless, because of the separation from the PR enzyme, is essential for the success of the preparation.

To the filtrate which is red and must be perfectly clear, sodium glycerophosphate or $KHCO_3$ is added in substance until the pH is 6.8. Then the extract is precipitated by bringing it to 0.41 saturation with a $(NH_4)_2S0_4$

Table 4. Preparation of crystalline muscle phosphorylase.

	Total protein (*mg*)	*Phosphorylase protein*	
		(*mg*)	(*per cent*)
1. Muscle (210 g) extracted twice with 210 cc. H_2O	4,420	86	2
2. Dialyzed 3 hours, pH adjusted to 5.75, ppt. removed	3,910	71	2
3. Precipitated with 41% saturated $(NH_4)_2SO_4$	147	58	39
4. Dialyzed against cysteine-buffer mixture pH 6.8; crystals centrifuged off	58	52	90
5. Mother liquor of crystals	85	5	6

solution saturated at room temperature and neutralized to pH 6.8. Overnight the flocculent precipitate settles to the bottom and most of the supernatant fluid can be decanted. Finally the precipitate, which contains the enzyme is separated by centrifugation, preferably at high speed (10,000 r.p.m.). The well-drained precipitate is dissolved in water, about 3 to 4 ml per 100 g muscle used originally. Table 4 shows that with a small loss the enzyme has now been purified about 20 times and in this particular experiment was 39 per cent pure.

The solution, which is slightly turbid, is transferred to a cellophane tube (diameter 1.3 cm) and after a short dialysis (1/2 to 1 hour) against cold running tap water, the tube is transferred to a cysteine solution (0.005 M) brought to pH 6.6 to 6.8 with 0.03 M sodium glycerophosphate. Buffers other than glycerophosphate have been used successfully, while no crystals were obtained when glutathione was substituted for cysteine or when cysteine was omitted. Dialysis is continued at 0°, against several changes of the same cysteine-buffer solution, until most of the sulfate has been eliminated from the enzyme solution. Precipitation of the enzyme sets in before this is the case; the precipitate is sometimes, but not always, crystalline. It is separated by centrifugation, the supernatant fluid is carefully drained off and

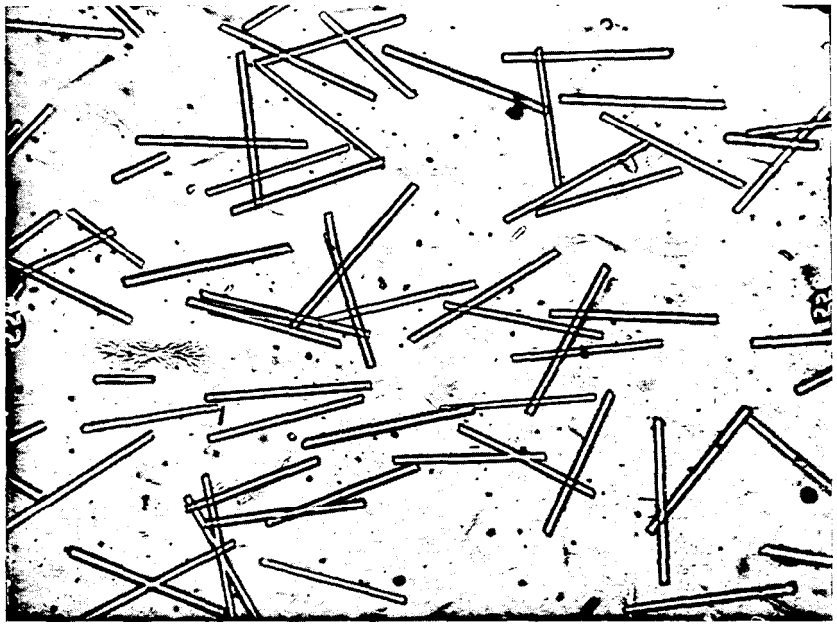


Fig. 3. Crystalline phosphorylase prepared from rabbit muscle (x 130).

the precipitate is stirred into a 0.01 to 0.03 M cysteine-glycerophosphate solution of pH 6.6 to 6.8 at 30 to 35°. The volume should be about the same as in the first crystallization. Solution of most of the protein should be rapidly achieved by vigorous stirring with a glass rod, however foaming should be avoided: the material is then centrifuged at room temperature (25°) at 10,000 r.p.m. for about 5 minutes. If a considerable amount of protein remains undissolved, it can be re-extracted. The small insoluble residue consists mostly of cystine crystals. The supernatant fluid should be perfectly clear and almost colorless. It is immediately transferred to an ice bath. Crystals appear rapidly, the rate depending, among other factors, on the concentration of enzyme in the solution. In order to obtain the large crystals shown in Fig. 3 it is necessary to use a dilute solution and slow cooling.

Table 4 shows that only 2 per cent of the protein in the crude extract was phosphorylase; 60 per cent of the enzyme was recoveredin the first crystals. Recrystallization causes a slight rise in specific activity. Eventually the mother liquor has almost the same specific acitivity as the crystals.

During summer, when the temperature in St. Louis is well over 30° on many days, the phosphorylase content of the muscles drops to such a low level that crystallization becomes impossible. When the rabbits are kept for about 1 week at 13° the level rises sufficiently to obtain crystals.

Table 5. Comparison of properties of phosphorylase *a* and *b*.

	Phosphorylase a	*Phosphorylase* b
Molecular weight calculated from diffusion and ultracentrifugation	4×10^5	
Diffusion constant, $D_{20}(w \times 10^7)$	3.3	3.3
Electrophoretic mobility (sq. cm per volt per sec $\times 10^5$, phosphate buffer pH 7.15, μ 0.1; temperature 2°)	− 3.25	− 2.75
Isoelectric point	5.5−5.6	5.8
Solubility (pH 7.2, water)	insoluble	soluble
0.1 *M* KCl (24°)	poorly soluble	»
0.08 *M* KCl + 0.02 *M* cysteine (24°)	soluble	»
0.08 *M* KCl + 0.02 *M* cysteine (0°)	crystallizes	»
Crystal form	long needles	rhomboid plates
Activity, without adenylic acid (%)	65	none
With adenylic acid (%)	100	80
Dissociation constant for combination with adenylic acid (pH 6.7, 25°)	1.5×10^{-6}	5×10^{-5}
Phosphorus content (%)	0.08	0.02

Phosphorylase *b* was prepared by letting purified PR enzyme act on twice crystallized phosphorylase *a.* The solution was then brought slowly to 0.28 saturation with $(NH_4)_2S0_4$. The enzyme crystallized in the form of rhomboid plates. Table 5 gives a comparison of the properties of the two forms of the enzyme.

Determination of phosphorylase *a* and *b* in mixtures is based on two parallel activity determinations, one without and one with the addition of adenylic acid in 10^{-3} M concentration. In the former case the a form has 66 per cent of its full activity, while the *b* form is inactive; in the latter case both forms are fully active.

Conversion of phosphorylase a to b, in vitro - The PR enzyme originally obtained by isoelectric precipitation of dialyzed muscle extract, was considerably purified by fractionation with $(NH_4)_2SO_4$. It was shown that its activity was greatly increased in the presence of cysteine and that Mn^{++} ions had an activating effect. The conversion of the *a* to the *b* form follows the first-order reaction rate equation. PR enzyme units could thus be expressed in terms of the first-order velocity constant.

A conversion of phosphorylase *a* to *b* could also be effected by crystalline trypsin at pH 6 to 6.2, at which pH the proteolytic acitivity of trypsin is kept at aminimum. This conversion is not a first-order reaction and is not accelerated by Mn^{++} ions. Work by E. Krebs (unpublished) indicates that phosphorylases *b* obtained by PR and trypsin may not be identical.

Conversion of phosphoryluse a to b, in vivo - Extracts of resting muscles contain predominantly phosphorylase *a.* During strong muscular contraction produced by strychnine or electric stimulation a large part of the enzyme is converted to the *b* form, presumably through the action of the PR enzyme *in vivo.* The sum of phosphorylase *a* and *b* does not differ significantly in resting and stimulated muscles. Determinations were carried out in crude, and crude dialyzed extracts and in the precipitate obtained with $(NH_4)_2SO_4$ at 0.41 saturation (Table 6). No crystals of phosphorylase a were obtained when its concentration fell below 25 per cent of the total phosphorylase.

Experiments on frogs gave results very similar to those obtained on rabbits. When electric stimulation was followed by a rest period of 10-15 minutes before the muscles were excised, the extract contained again preponderantly phosphorylase *a.* Conversion of the *b* to the a form has so far only been observed *in vivo* and nothing is known about its mechanism.

The progressive conversion of phosphorylase *a* to *b* in muscle as contraction continues might represent a regulatory mechanism, preventing exhaus-

Table 6. Effect of stimulation and recovery on phosphorylase-*a* content of muscle.

	Phosphorylase a + b per g muscle (*units*)	*Phosphorylase a in crude extract* (*per cent*)	*Phosphorylase a in dialyzed $(NH_4)_2SO_4$ precipitate* (*per cent*)
Rabbits			
12 resting	1,340	92	92
6 strychnine	1,420	10	8
4 electric stimulation	1,140	11	10
Frogs			
14 resting	1,290	86	91
7 electric stimulation	1,310	37	40
4 stimulated, 5′ recovery	1,330	43	64
7 stimulated, 10–15′ recovery	1,270	82	91

tion of the glycogen stores. The limiting reaction in the conversion of glycogen to lactic acid is the phosphorylation of fructosed-phosphate to fructose-I, 6-diphosphate. Consequently the equilibrium ester (glucose-6- and fructose-6-phosphate) accumulates during muscular contraction. The piling up of even more of this intermediate substance is avoided by a temporary inactivation of phosphorylase. The adenylic acid content of muscle is too low to permit full activation of phosphorylase *b*.

Liver phosphorylase and blood sugar formation - Until recently it was assumed that *a*-amylase plus maltase convert glycogen to glucose in the liver; the absence of dextrins and maltose from liver tissue and blood could not be easily reconciled with this assumption.

Glycogen added to dialyzed extracts of perfused liver tissue disappeared very slowly, indicating weak amylase activity. When inorganic phosphate and traces of adenylic acid were added to such extracts, glycogen disappeared rapidly and its disappearance was balanced by the sum of hexose phosphate and glucose which accumulated.

Fractionation of the liver extracts led to separation of the enzymes involved and it was shown that glucose was formed by the following reactions:

glycogen + phosphate ⇌ glucose-1-P (phosphorylase)
glucose-1-P ⇌ glucose-6-P (phosphoglucomutase)
glucose-6-P → glucose + phosphate (phosphatase)

The phosphatase seems to be specific for glucose-6-phosphate; its pH optimum is within the physiological range. The glucose formed diffuses into the blood stream and the phosphate can react again with glycogen.

It is the absence of phosphatase from skeletal muscle tissue which explains the fact that muscle does not contribute glucose to the blood. In the kidney, both the glycogen and phosphorylase content are very low and its contribution of glucose to the blood through the above system seems to be insignificant.

Part 3 – by Carl F. Cori

Plant phosphorylases - When Hanes first described the occurrence of phosphorylase in higher plants, he pointed out that there existed a close parallelism between the action of an enzyme system prepared from peas and potatoes and what was then known about the action of the corresponding enzyme system from animal tissues and from yeast. Hanes fractionated potato extract with ammonium sulfate and noted an initial lag period in the formation of starch from glucose-I-phosphate. In conformity with the results obtained with the animal enzyme he found that this lag period was abolished by the addition of a small amount of starch. Green and Stumpf found their purified potato phosphorylase preparations completely inactive in the direction of synthesis, unless a small amount of starch was added. Weibull and Tiselius showed that under certain conditions the reaction catalyzed by

Table 7. Concentration of substrates at which phosphoryiases show half maximal activity.

Enzyme	*Substrate*	*Concentration (M per liter)*	*Author*
Muscle	glucose-1-phosphate	5.7×10^{-3}	Cori, Cori, and Green
Potato	,,	2.6×10^{-3}	Weibull and Tiselius
Muscle	inorganic phosphate	6.8×10^{-3}	Cori
Potato	,,	6.2×10^{-3}	Weibull and Tiselius
Muscle	glycogen	1.2×10^{-4}*	Cori, Cori, and Green
Potato	starch	2.4×10^{-4}*	Weibull and Tiselius

* Calculated per mole end-group, assuming that glycogen contains 10 per cent and starch 4.5 per cent end-groups.

potato phosphorylase was first order in either direction. The Michaelis-Menten constants of potato and muscle phosphorylase for substrates are very similar (Table 7).

Table 8. Differences in the action of muscle and potato phosphorylase.

Substance	*Effect on phosphorylase*	
	Muscle	*Potato*
Glucose (0.1 *M*)	inhibits	no effect
Cu^{++} (0.001 *M*)	inhibits	no effect
Reducing agents	activate	no effect
Adenylic acid	activates	no effect
Polysaccharides as primers	optimally primed by glycogen; not primed by short amylose chains	primed by starch or amylopectin more effectively than by glycogen; primed by short amylose chains

In spite of these and other similarities (nature of polysaccharide formed, effect of pH on equilibrium), there are certain important differences in the action of potato and muscle phosphorylase; these are summarized in Table 8.

Nature of polysaccharide formed - A summary of the properties of synthetic polysaccharides is given in Table 9. It is based on the work of Hanes, Hassid,

Table 9. Properties of natural and synthetic polysaccharides.

	X-ray diagram	*Rel. intensity of iodine color at 660 mμ*	*Hydrolysis by β-amylase*	*Average chain length*	*Rel. ability to activate muscle phosphorylase*
Starch (corn)	ring pattern	100	60	24–30	45
Amylopectin (corn)	diffuse	50	55	20–25	65
Amylose (corn)	ring pattern	310	100	250	10
Synthetic amylose					
potato phosphorylase	ring pattern	305	98	100	0
muscle phosphorylase	ring pattern	290	97	200	0
Glycogen (liver)	diffuse			12–18	100
Synthetic glycogen					
liver phosphorylase	diffuse				100

Bear, Cori and others. It seems clear that muscle and potato phosphorylase form a linear polysaccharide which closely resembles the amylose component of natural starch in all its properties.

In order to form a branched polysaccharide of the type of amylopectin or glycogen, a second enzyme is needed which forms a-1-6 glucosidic linkages at the points of branching. Such an enzyme has been found in both animal and plant tissues, but the mechanism of its action is not clearly understood. When crystalline muscle phosphorylase plus a second enzyme (called the branching factor and obtained from liver or heart) were allowed to act on glucose-1-phosphate, an autocatalytic type of curve was obtained (Fig.4). Traces of glycogen, introduced by the liver preparation, were present, but not enough to prime muscle phosphorylase when acting alone.

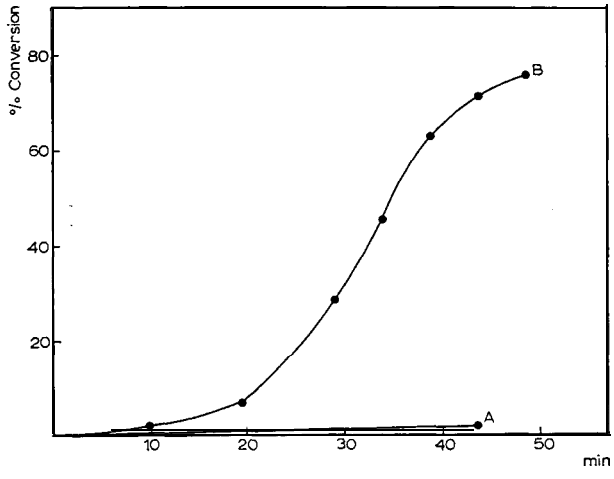


Fig. 4. Enzymatic synthesis of a branched polysaccharide from glucose-I-phosphate. In Curve B crystalline muscle phosphorylase was acting simultaneously with a supplementary enzyme prepared from liver. In Curve A the liver enzyme preparation was inactivated by heating before being added to muscle phosphorylase.

As shown in the last column of Table 9, branched and linear polysaccharides differ markedly in their activating effect on muscle phosphorylase. The autocatalytic type of curve would result from the increasing number of activating end-groups formed during the synthesis of a branched polysaccharide. It was suggested that a combined action of both enzymes was necessary for the formation of glycogen or amylopectin. Haworth and collaborators have, however, reported the conversion of amylose to amylopectin

by the action of an enzyme prepared from potato, a sort of cross-linking enzyme which would break the long amylose chains into smaller fragments and rearrange them in a laminated pattern.

Connected with the problem of formation of α-1-6 linkages is that of their disruption. Neither α- nor β-amylase can split this linkage. Recently an enzyme present in muscle has been investigated in this laboratory which in combination with phosphorylase is able to cause an almost complete degradation of glycogen. Phosphorylase alone cannot degrade branched polysaccharides beyond the branch points. In fact, the limit dextrin formed from amylopectin or glycogen by either potato or muscle phosphorylase is larger than that formed by β-amylase. The question whether the same enzyme is involved in the formation and disruption of the 1-6 linkage has not been settled.

Theory of the action of phosphorylase - In the phosphorolysis of giycogen or starch the reactants are (1) inorganic phosphate, and (2) the terminal glucose unit of the polysaccharide chains. The chain molecule is thereby shortened, one glucose unit at a time, but the concentration of terminal glucose units remains the same until the limit of degradation is reached.

The theory of the action of phosphorylase is based on the assumption that the terminal glucose unit is also a reactant in the reverse direction; in this case the polysaccharide chains would be lengthened by successive additions of new glucose units from glucose-1-phosphate, again without any change in the concentration of the terminal glucose units. The reaction catalyzed by phosphorylase in the direction of increasing chain length might therefore be expressed as follows.

Terminal glucose (of n chain units) + glucose-r-phosphate + terminal glucose (of n +1chain units) + phosphate, etc.

The following observations are in accord with this formulation. (1) Muscle as well as potato phosphorylase remain inactive- when glucose-1-phosphate alone is added; they require the further addition of polysaccharide as an essential reactant before new polysaccharide can be formed. (2) When increasing amounts of glycogen or starch are added, the rate of polysaccharide formation from glucose-1-phosphate increases in a manner which is characteristic for a reacting molecule (see Fig. 2.) (3) Branched polysaccharides are more strongly activating than linear polysaccharides, the effect being roughly proportional to the number of end-groups present. (4) The equilibrium of the reaction is independent of the concentration of polysaccharide. This would follow from the fact that the concentration of one of the two

reactants in either direction, the terminal glucose units, remains constant. Hence this term cancels out when the usual mass-law expression is written and the equilibrium constant, K = (phosphate)/(glucose-I-phosphate). (5) For the same reason, the reaction in either direction, although involving two reactants, has been found to be kinetically of the first order.

Several experiments have been devised to test this theory. The reaction, glucose-r-phosphate ⇌ polysaccharide + phosphate, in the presence of ^{32}P, should lead to an incorporation of ^{32}P in glucose-I-phosphate. Such an exchange occurred in the presence but not in the absence of polysaccharide primer, showing that the latter was an essential reactant for polysaccharide synthesis from glucose-1-phosphate.

A further consequence of the theory presented above is that the length of the newly formed polysaccharide chains should depend on the ratio of molar concentrations, glucose-I-phosphate which reacted/terminal glucose units of added primer. When this ratio is large, long chains, when it is small, short chains should be formed. This has been shown to be the case by a number of investigators, who used iodine color as a measure of chain length. A comparison has also been made of the chain length calculated from the above ratio and that actually found by end-group assay, with fair agreement.

A special case arises when the limit dextrin of glycogen (formed by exhaustive treatment with phosphorylase) is used as primer and an amount of glucose-1-phosphate is added which would allow no more than one chain unit to be added for each primer end-group. At the start the reaction can proceed only in the direction of synthesis, since there are no end-groups present in the limit dextrin which can be removed by phosphorolysis. As the reaction proceeds, cleavable end-groups are added, but the rate of phosphorolysis close to the limit of degradation is very slow. If polysaccharide synthesis consists in the addition of glucose units to primer end-groups, one would expect under these special conditions a shift in the equilibrium to the side of polysaccharide formation and a dependence of the equilibrium on the concentration of added primer. Hestrin who carried out this experiment (unpublished) found both these predictions fulfilled.

There is thus experimental support for the idea that polysaccharides act as primers in the direction of synthesis because they enter stoichiometrically into the reaction catalyzed by phosphorylase.

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