STUDIES ON THE CHEMISTRY OF THE LIVING BARK OF THE BLACK LOCUST TREE IN RELATION TO FROST HARDINESS. VI. AMYLASE AND PHOSPHORYLASE SYSTEMS OF THE BARK TISSUES [1]

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While studying the relation of the chemical composition of the living bark of the black locust tree (*Robinia pseudoacacia*) to its frost resistance, SIMINOVITCH *et al.* (**4, 26, 27**) have observed marked seasonal fluctuations in the starch and sucrose contents of this tissue. The analytical data were suggestive of the existence of a temperature-sensitive starch-sucrose interconversion process, low temperature in late fall promoting a disappearance of starch and warm temperature of early spring promoting the production of starch with a proportionate and simultaneous disappearance of sucrose. Because of the pronounced seasonal changes in the nature of the carbohydrate reserves in the black locust tree bark it was of interest to study enzyme systems which might be involved. Further interest was attached to such a study because little information concerning the enzyme systems present in the living bark of trees is available. The presence of amylase and phosphorylase systems in the bark tissue of the black locust tree is established and characterization studies on these systems are presented here.

The enzymatic degradation of polysaccharides of the starch-glycogen type may be hydrolytic, as catalyzed by amylases, or phosphorolytic, as catalyzed by phosphorylase. The mode of action of the amylases has been the subject of many studies and of several reviews (**3, 10, 22, 25**). Two types of amylase, α and β, are recognized. The α-amylases degrade starch preferentially by hydrolyzing glycosidic linkages far removed from the terminal units. With prolonged incubations, however, α-amylase causes considerable conversion of starch to maltose together with the liberation of a small amount of glucose. β-Amylase degrades starch by hydrolytic cleavage of successive maltose units progressing from the non-reducing ends of the polysaccharide chains. This action is stopped at the branching points of the amylopectin component of starch, but the degradation of the linear component approaches completion. The depolymerizing action of the amyl-

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ases on starch is apparently not reversible, *i.e.*, the equilibrium lies far toward hydrolysis, so synthesis is not observed with these enzymes.

The phosphorolytic cleavage of starch or glycogen involves consumption of one molecule of inorganic phosphate for each glucosidic bond which is broken. CORI and co-workers (**5**) showed that the first product of phosphorolysis of glycogen in animal tissues is glucose-1-phosphate. This reaction is reversible (**6, 7**) and the phosphorylase enzyme catalyzes also the synthesis of polysaccharide from glucose-1-phosphate. The occurrence of phosphorylase in plant tissues was first reported by HANES (**11, 12**) who found it in peas and potatoes. Subsequently the enzyme has been found in many other plant and animal tissues. The reactions catalyzed by the plant and animal phosphorylases are similar. The synthesis of polysaccharide occurs by the endwise apposition of successive glucose units from glucose-1-phosphate to the non-reducing ends of pre-existent starch or glycogen molecules (**15**) which serve as starters. HANES (**12**) and CORI *et al.* (**8, 9**) showed that at a given pH the phosphorylase reaction approaches the same equilibrium ratio of inorganic phosphate to glucose-1-phosphate from either direction. This ratio is independent of the concentration of starch or glycogen but it is pH dependent, varying from about 10.8 at pH 5.0 to 3.1 at pH 7.0 (**12**). HANES and MASKELL (**13**) showed that the equilibrium ratio of similarly charged inorganic and organic phosphate ions is constant over the entire pH range of enzyme activity.

The phenomena of essential sulfhydryl groups in enzymes and of the inhibition of enzymes by sulfhydryl reagents have been much investigated and the subject has been discussed in reviews by OLCOTT and FRAENKEL-CONRAT (**20**), by SINGER (**28**), by MASSART (**17**) and more recently in a comprehensive review by BARRON (**2**). Much of the pioneer work in this field was carried out by RAPKINE (**23, 24**), who used chiefly the reactions between sulfhydryl groups and the alkylating agents, iodoacetate and iodoacetamide, for the detection and study of essential sulfhydryl groups in enzymes. It has since been found that these reagents do not react with all sulfhydryl groups, and their specificity has been questioned (**2**). A number of other reagents are now preferred for the study of sulfhydryl groups in enzymes. They include oxidizing agents (such as iodine, oxidized glutathione, iodosobenzoate, and porphyrindin), and mercaptide forming agents (such as heavy metals, *p*-chloromercuribenzoate, and organic arsenicals). These two groups of reagents are of particular interest because their reactions with sulfhydryl groups are reversible. Thus enzymes inactivated by these reagents may frequently be reactivated by reducing agents or thiols. The reactivations help to confirm the mode of action of the inhibitors.

An apparently selective inhibition of amylase in the presence of phosphorylase by iodine was observed by HANES (**11**) in aqueous extracts of peas. NAKAMURA and MARUO have (**19**) described a similar technique for detecting phosphorylase in the presence of amylases. They used mercury compounds as selective inhibitors of the amylases in enzyme preparations

from several plants. Both the iodine-potassium iodide reagent and the mercury compounds are known to react with sulfhydryl groups. Thus the qualitative observations suggest that the activity of the amylase systems is dependent on the integrity of sulfhydryl groups within the enzyme molecules, while that of the phosphorylases is independent of sulfhydryl groups. The observations would be explained also, if the phosphorylases contain sulfhydryl groups which, though essential for enzyme activity, are less reactive chemically than those of the amylases. The possibility of obtaining complete inhibition of the locust bark amylase without inhibiting the phosphorylase was explored. Iodine, iodoacetate, *p*-chloromercuribenzoate, phenylmercuric chloride, and phenylmercuric nitrate have been used as sulfhydryl reagents. Cysteine has been used in efforts to reverse the enzyme inhibition produced by these reagents. The results of the experiments with sulfhydryl reagents on the amylase and phosphorylase systems of the living bark of the black locust tree are reported here as a part of the characterization of these enzyme systems.

Materials and methods

TISSUE EXTRACTS.—The dead outer bark of black locust tree logs was removed and the inner, living bark was ground off with a motor-driven wire brush wheel. Samples of the freshly shredded tissue were soaked in three to four volumes of cold distilled water (5° C) for 2–3 hours with periodic trituration and the tissue was squeezed to remove as much as possible of the water extract. The residual tissue was washed with two volumes of cold distilled water, the washings combined with the original extract. The extract was filtered to remove tissue debris.

Amylase activity in the extract was demonstrated qualitatively by incubating aliquots with starch solution and periodically testing a few drops of the reaction mixture with iodine-potassium iodide reagent for evidence of starch degradation. The tests indicated that a highly active amylase system occurs in this tissue.

Qualitative tests for phosphorylase activity were made by incubating aliquots of the tissue extracts with glucose-1-phosphate and a trace of starch primer. At intervals of approximately five minutes a few drops of the test solution were treated with iodine-potassium iodide reagent for evidence of starch synthesis. The amylase activity of the crude tissue extracts was sufficiently great to destroy the starch primer and any synthetic starch and the tests remained negative. Following the method of HANES (**11**), however, it was found that low concentrations of iodine-potassium iodide reagent added to the enzyme-substrate mixture inactivated the amylase system to a marked degree, presumably by reaction with sulfhydryl groups, while leaving strong phosphorylase activity. In this manner it was possible to obtain intense blue color tests, similar to those given by the linear component of starch, for the synthetic polysaccharide with the iodine-potassium iodide reagent.

AMYLASE PREPARATION.—The proteins from the tissue extract were fractionally precipitated by addition of cold saturated neutral $(NH_4)_2SO_4$. The precipitates were taken up in Mc'Ilvaine citrate-phosphate buffer, pH 6.6, and dialyzed at 5° C during three days against water followed by three changes of the buffer, pH 6.6, diluted 1 to 10. The fraction precipitated between 36 and 44% saturation with $(NH_4)_2SO_4$ showed the greatest amylase activity and was used for the amylase experiments.

Estimates of amylase activity were obtained by measuring the increase in reducing sugars after aliquots of the enzyme preparation were incubated with a standard solution of starch. The reducing power was measured by oxidation with alkaline ferricyanide and titration of the resulting ferrocyanide with 0.01 *N* ceric sulfate according to the method of HASSID (**14**). Enzyme action in the sample aliquots was stopped by transferring them to test tubes containing the alkaline ferricyanide reagent. Enzyme activity is expressed as the volume of 0.01 *N* ceric sulfate equivalent to the reducing sugars produced per mg. of nitrogen in the enzyme preparation during the time the enzyme was allowed to act.

PHOSPHORYLASE PREPARATION.—Since the polysaccharide synthesizing action of phosphorylase is inhibited by inorganic phosphate, enzyme preparations containing phosphate buffer were not used for the phosphorylase experiments. The total proteins were precipitated from the tissue extract by addition of 600 gm. solid $(NH_4)_2SO_4$ per liter of extract, separated by filtration, and taken up in 0.05 M citrate buffer, pH 6.7. The solution was dialyzed for 40 hours against cold, flowing distilled water and for an additional 45 hours against two changes of the citrate buffer. Sediment was removed by centrifugation and the supernatant was used for the phosphorylase experiments. This preparation contained amylase as well as phosphorylase but, where starch primer was added and the initial rates of inorganic phosphate formation from the glucose-1-phosphate substrate were determined, the amylolytic action was considered non-determinant in the studies of phosphorylase activity.

Phosphorylase activity was usually estimated by measuring the rate at which the release of inorganic phosphate from glucose-1-phosphate was catalyzed by the enzyme preparations. Unless 0.1% or more of soluble starch was added to the enzyme-substrate mixtures the reaction was characterized by an induction period. Because the reaction approaches a state of equilibrium as inorganic phosphate accumulates, the initial rate of increase in concentration of inorganic phosphate was generally used as the measure of phosphorylase activity. In one experiment reported here phosphorylase activity was demonstrated by measuring the disappearance of inorganic phosphate and the production of glucose-1-phosphate when an aliquot of the enzyme was incubated with starch and phosphate buffer.

ANALYSIS FOR PHOSPHORUS COMPOUNDS.—Inorganic phosphate and glucose-1-phosphate were estimated by a modification of the method of LOWRY and LOPEZ (**16**). An aliquot of 1–3 ml. from the enzyme-substrate mixture

was mixed with three volumes of 8 to 10% (w/v) trichloracetic acid in a small centrifuge tube. The solution was adjusted to pH 4.0 with 1 *N* sodium acetate, the amount of sodium acetate required having been determined by titrating a separate sample to a pale green color with brom cresol green. These neutralized samples were heated in a boiling water bath for one minute, cooled, and centrifuged. Aliquots of supernatant were transferred to 25-ml. volumetric flasks and to each flask 5 ml. of 0.1 M acetate buffer, pH 4.0, 2.5 ml. of 1% ascorbic acid in acetate buffer, 2.0 ml. of 2.5% ammonium molybdate, and distilled water to dilute to 25 ml. were added. The color was allowed to develop for 5–15 minutes and the optical density was measured at 670 mμ in a Coleman Model 11 photoelectric spectrophotometer. Blank samples were obtained by processing aliquots of the enzyme-substrate mixture immediately after the glucose-1-phosphate was mixed with the enzyme. The inorganic phosphate equivalent to the observed optical density was estimated by reference to a standard curve.

When estimating the concentration of inorganic phosphate plus glucose-1-phosphate (7-minute acid hydrolyzable phosphate) the trichloracetic acid was used to hydrolyze the phosphate ester. The acid-treated aliquots were placed in a boiling water bath for 7 minutes. The hydrolyzed samples were then cooled, neutralized to pH 4.0 with 1 *N* sodium acetate and diluted to a definite volume (*e.g.*, 25 ml.) in volumetric flasks. These diluted samples were then centrifuged and aliquots of the supernatant were analyzed for inorganic phosphate according to the method previously described.

Experimental results

Effects of temperature, pH, and other environmental conditions upon the activities of locust bark amylase and phosphorylase

Optimal temperature for amylase activity.—Figure 1 shows the effect of temperature on the locust tree amylase. Incubation times of 27 minutes (curve A) and 64 minutes (curve B) were employed. During the shorter incubation period maximal hydrolysis occurred at or above 55° C. With the longer incubation period there was definite evidence of enzyme inactivation at 55° C, with maximal hydrolysis occurring at 50° C.

In figure 2 the data of the 27-minute incubation period experiment are examined graphically for compliance with the Arrhenius equation which may be written,

$$\log k = C - \frac{A}{2.3\,RT}$$

where k represents the rate of starch hydrolysis at absolute temperature, T, C is a constant, and A is the activation energy. A plot of log k against the reciprocal of T should give a straight line with slope $\frac{-A}{2.3\,R}$ and thence the value of A may be estimated from the observed value of the slope. The

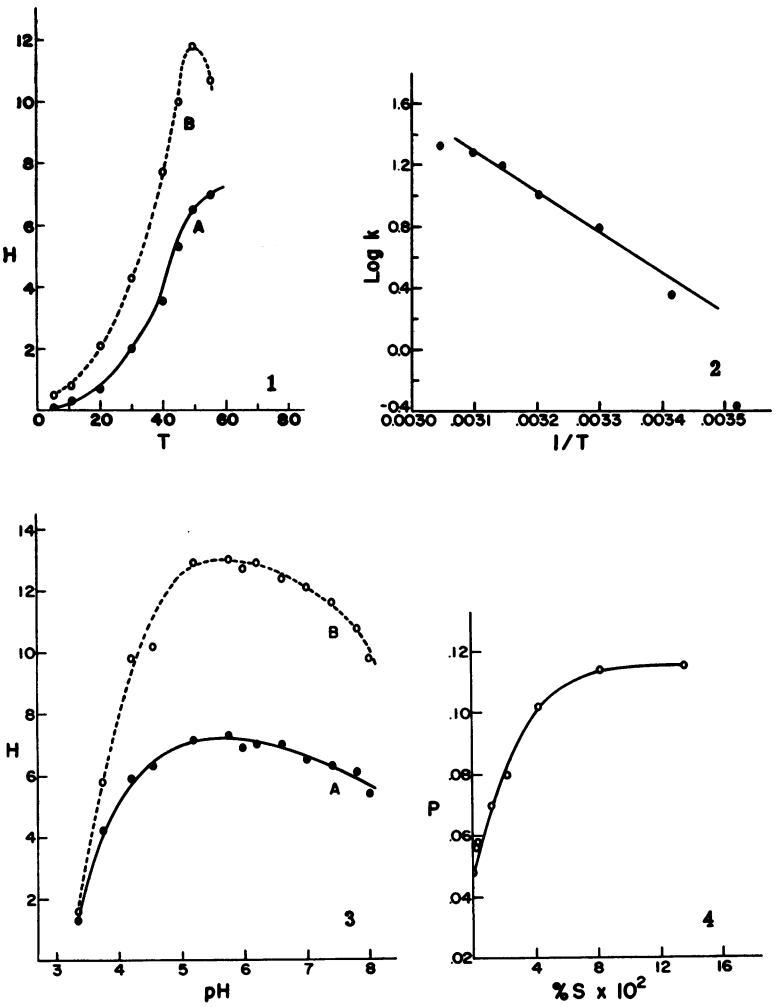

FIG. 1. Effects of temperature, T (°C), on hydrolysis, H, of starch by locust tree amylase, at pH 6.6. H is expressed as ml. of 0.01 *N* ceric sulfate equivalent to increase in reducing sugars per mg. of nitrogen in the aliquot of enzyme preparation employed. Curves A and B refer to reaction times of 27 and 64 minutes, respectively. Each sample contained 1 ml. of enzyme preparation, 5.0 ml. of 1% soluble starch and 3 ml. of Mc'Ilvaine citrate-phosphate buffer, pH 6.6.

FIG. 2. Graphic estimation of the activation energy of the locust tree amylase hydrolysis of starch from the slope of the plot of the logarithms of reaction rates, K (glucose equivalent formed in identical enzyme substrate aliquots after 27 minutes incubation), versus the reciprocal of the temperature, T (in degrees absolute).

FIG. 3. Effects of pH on the hydrolysis, H (units same as in fig. 1), of starch by locust tree amylase. Temperature of incubation was 39° C. Curves A and B refer to reaction times, 30 and 60 minutes, respectively. Each sample contained 1 ml. of enzyme preparation, 3 ml. of Mc'Ilvaine citrate-phosphate buffer, and 5 ml. of 4% soluble starch.

FIG. 4. Effects of soluble starch (primer) concentrations, % S, on the amount of inorganic phosphorus, P, released from glucose-1-phosphate during 30 minutes incubation

experimental data appear to comply with the equation at intermediate temperatures (30–50° C). The activation energy, A, calculated from the line drawn through four points, is 12,000 calories per mole.

OPTIMAL PH FOR AMYLASE ACTIVITY.—The manner in which the activity of the amylase at 39° C and incubation times of 30–60 minutes was affected by pH, is shown in figure 3. There was low enzyme activity and probable inactivation of the enzyme at pH 3.4. There is a wide plateau of maximal activity in the region between pH 5.3 and 6.0 which suggests the probability of the presence of two or more amylases having different pH optima.

EFFECT OF STARCH CONCENTRATION ON PHOSPHORYLASE ACTIVITY.—In the absence of sufficient starch to fully " prime " the reaction the rate at which release of inorganic phosphate from glucose-1-phosphate was catalyzed by the locust tree phosphorylase was characterized by an induction period. Amylolytic splitting of the suboptimal amount of polysaccharide with a consequent increase in the number of terminal non-reducing units may account for an autocatalytic effect which followed. The induction period was eliminated by adding sufficient soluble starch. In figure 4 the effect of the concentration of the starch primer on the amount of inorganic phosphate released during 30 minutes at 30° C is shown. A maximum of activity was approached when the concentration of starch in the sample mixtures exceeded 0.1%.

EFFECT OF TEMPERATURE ON PHOSPHORYLASE ACTIVITY.—In figure 5 the effects of temperatures, ranging from 5–45° C, on the amounts of inorganic phosphate liberated from glucose-1-phosphate during 45 minutes by locust tree phosphorylase are compared. While in this experiment the activity was greatest at the highest temperature employed, the curve appeared to be approaching a maximum in this temperature region. In a subsequent experiment a maximal activity was obtained at 50° C while at 55° C the enzyme was rapidly inactivated.

EQUILIBRIUM OF THE PHOSPHORYLASE REACTION.—The reversibility of the reaction catalyzed by the locust tree phosphorylase is demonstrated in figure 6. Experiments were made at 30° C and pH 6.7, and equilibrium was approached from either direction. Although there was no evidence of interference by a phosphatase sensitive to fluoride, NaF was added as a precautionary measure in these experiments. When glucose-1-phosphate was the substrate (fig. 6 A) the observed final ratio of inorganic phosphate to glucose-1-phosphate was 4.1. For the experiment with starch and inorganic phosphate as initial substrate (fig. 6 B) the final ratio of inorganic phosphate to glucose-1-phosphate was 5.9. The first value, 4.1, agrees more closely with the equilibrium ratio at this pH reported for other phosphorylase systems (**9, 12**). It is possible that in the latter case amylolytic de-

time at pH 6.6 and 30° C by locust tree phosphorylase. P is expressed as mg. phosphorus released per mg. of nitrogen in the aliquot of enzyme preparation employed. Each sample contained 0.5 ml. of phosphorylase preparation, 0.3 ml. of 1.5% dipotassium glucose-1-phosphate in 0.05 M citrate buffer, pH 6.6, and an additional 0.3 ml. of the citrate buffer solution containing from 0.0 to 0.5% soluble starch.

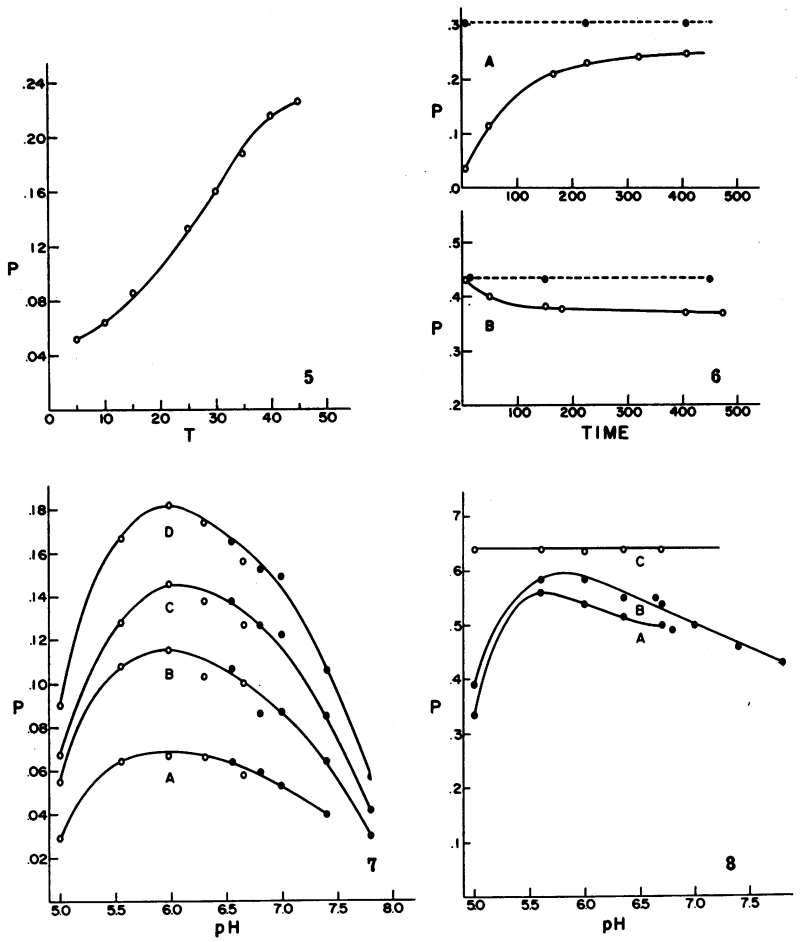

FIG. 5. Effect of temperature on locust tree phosphorylase activity at pH 6.6. Amounts of inorganic phosphate, P (units same as in fig. 4), released from glucose-1-phosphate during a 45-minute incubation time at various temperatures. Each sample contained 0.5 ml. of the enzyme preparation, 0.3 ml. of 1.5% dipotassium glucose-1-phosphate in 0.05 M citrate buffer, pH 6.6, and 0.3 ml. of 0.5% soluble starch in citrate buffer. The whole samples were used for the analyses.

FIG. 6. Approach to equilibrium from either direction of the reaction catalyzed by locust tree phosphorylase at 30° C. Dashed lines represent concentration of inorganic plus 7-minute hydrolyzed phosphate. Full line represents concentration of inorganic phosphate. P is in same units as in figure 4. In figure 6 A the samples contained 8 ml. of enzyme preparation, 3 ml. of 1.4% dipotassium glucose-1-phosphate in 0.5 M citrate buffer, pH 6.7, 3.0 ml. of 1% soluble starch in citrate buffer and 1.0 ml. of 1.0 M sodium fluoride. In figure 6 B the samples contained 10 ml. of enzyme preparation, 3.0 ml. of 1.0 M sodium fluoride in 0.05 M citrate buffer, pH 6.7, 2.0 ml. of 0.1 M phosphate buffer, pH 6.6, and 10.0 ml. of 4% soluble starch.

FIG. 7. Effects of pH on net rate at which the release of inorganic phosphate, P (units same as in fig. 4), from glucose-1-phosphate, is catalyzed by locust tree phosphorylase. Temperate was 30° C. Curves A, B, C, and D refer to 15, 30, 45, and 60

struction of the starch substrate stopped further phosphorolysis, short of equilibrium.

EFFECT OF pH ON PHOSPHORYLASE ACTIVITY.—In figure 7 the effect of pH on the early progress of the reaction catalyzed by the locust tree phosphorylase at 30° C is shown. The substrate for each sample was prepared by adjusting 1.5 ml. of an approximately 1.5% solution of dipotassium glucose-1-phosphate to the required pH with hydrochloric acid and diluting to 4 ml. with distilled water. Each substrate sample was mixed with 5.0 ml. of 0.5 M citrate buffer or veronal buffer (**18**) of the required pH, 1.0 ml. of 1% soluble starch and finally 2.0 ml. of enzyme preparation. When incubated for periods of 30, 45, or 60 minutes (curves B, C, and D, respectively) the greatest net liberation of inorganic phosphate occurred at pH 6.0. For a 15-minute incubation (curve A) the accumulation of inorganic phosphate was as great at pH 6.3 as at pH 6.0. Equal concentrations of inorganic phosphate at pH 6.0 and 6.3 represent a closer approach to equilibrium at pH 6.3 (**12**). Therefore, the apparent optimum at pH 6.0 may not indicate a maximum of enzyme activity. It appears that this phosphorylase is most active at some pH between 6.0 and 6.5.

Analyses of aliquots from the previously described samples after prolonged incubations (10–21 hours) showed that pH dependent equilibrium ratios were approached. From figure 8 it is seen that between pH 5.6 and 7.8 the final concentration of inorganic phosphate decreased with rising pH. This is consistent with the expected higher equilibrium ratios at lower pH levels (**9, 12**). The low level of inorganic phosphate released at pH 5.0 may have been due to a combination of low enzyme activity and enzyme inactivation. It was not possible to estimate the actual equilibrium ratios from these results because the sum of inorganic plus 7-minute hydrolyzable phosphate was determined only during the early stages of the reaction and prior to possible volume alteration due to evaporation of water during the prolonged incubations.

IDENTIFICATION OF PRODUCTS OF ENZYME REACTIONS BY PAPER CHROMATOGRAPHY

The products of the reactions catalyzed by the locust tree amylase and phosphorylase systems were separated and identified by descending paper chromatography (**21**). The solvent combinations used were a 40% (by volume) *n*-butanol, 10% ethanol and 50% water mixture or a 40% *n*-butanol, 10% propionic acid and 50% water mixture. The products were detected

minutes incubation times respectively. Open circles refer to experiments conducted in citrate buffers, filled circles to those in veronal buffers.

FIG. 8. Effects of pH on final concentrations of inorganic phosphate and glucose-1-phosphate in presence of locust tree phosphorylase at 30° C. Curves A and B refer to final concentration of inorganic phosphate, P (units same as in fig. 4), after 600 and 1300 minutes incubation time respectively. Curve C refers to sum of inorganic and 7-minute hydrolyzable phosphorus present.

on the paper chromatograms by spraying with either a 1:1 mixture of freshly prepared 2 *N* silver nitrate and concentrated NH_4OH or a mixture containing 2 gm. of trichloracetic acid, 0.2 gm. of *p*-amino dimethyl aniline monohydrochloride and 50 ml. of water. The products were identified by comparison with simultaneously developed chromatograms of mixtures of known sugars. Unhydrolyzed starch could be identified at the starting point on all chromatograms of the enzymatic digests.

With prolonged incubations (*e.g.*, 19 hours) the chief products of the action of the amylase on starch were found to be maltose and glucose, with maltose predominating. There were also diffuse spots of low R_F values indicating dextrins. With shorter incubation periods (*e.g.*, 6 hours) the only products detected were maltose and dextrins. The results were identical whether citrate or phosphate buffers were employed in the reaction mixtures.

The products of the action of crude phosphorylase preparations on glucose-1-phosphate were starch, dextrins, and maltose. The maltose and dextrins probably resulted from the action of the amylase, which is always present, on the synthetic starch.

The action of sulfhydryl reagents upon the amylase and phosphorylase enzymes

The inhibition of amylase.—Attempts to demonstrate inhibition of the locust tree amylase by iodoacetate or ferricyanide were unsuccessful. However, the locust tree amylase was inhibited by iodine-potassium iodide

TABLE I

INHIBITION OF AMYLASE.

Inhibitor	Ml. of .01 *N* $Ce(SO_4)_2$ per mg. N_2		
	30 min.	60 min.	90 min.
Control (no inhibitor added)	4.55	9.45	14.1
2.5×10^{-5} M I_2–7.5×10^{-5} M KI	.88	1.55	2.3
0.5×10^{-4} M I_2–1.5×10^{-4} M KI	....	.25	.25
0.75×10^{-4} M I_2–2.3×10^{-4} M KI	.20	.15	.15
1.0×10^{-4} M I_2–3.0×10^{-4} M KI	....	....	....
2.5×10^{-4} M C_6H_5-Hg-Cl	.35	.6	.9
2.0×10^{-4} M C_6H_5-Hg-NO_3	1.05	1.70	2.55
2.5×10^{-4} M p-Cl-Hg-C_6H_4-COOH	.35	.3	.4

Inhibition of locust tree bark amylase at 35°C and pH 6.3 by iodine-potassium iodide reagent, phenylmercuric chloride (C_6H_5-Hg-Cl), phenylmercuric nitrate (C_6H_5-Hg-NO_3) and *p*-chloromercuribenzoate (*p*-Cl-Hg-C_6H_4-COOH). In each experiment the sample contained amylase preparation equivalent to 10 mg. protein nitrogen, 5 ml. of Mc'Ilvaine citrate phosphate buffer, inhibitor in amount to yield the final concentration shown and distilled water to give a total volume of 20 ml.

reagent, phenylmercuric chloride, phenylmercuric nitrate, or *p*-chloromercuribenzoate. Some representative data are shown in table I. In all experiments the buffer, substrate, inhibitors, and distilled water were mixed before the enzyme was added. The results indicate that 0.5×10^{-4} M iodine in 1.5×10^{-4} M potassium iodide or approximately 2.5×10^{-4} M concentrations

of the mercury compounds produced almost complete inhibition of the enzyme. The similarity in the effective equivalent concentrations suggests that the different inhibitors react with the same groups in the enzyme molecules.

REVERSAL OF AMYLASE INHIBITION.—In figure 9 are shown the results of an experiment in which inhibition of the locust tree amylase by phenylmercuric nitrate was reversed with cysteine. Analyses of aliquots from sample C subsequent to the cysteine addition were corrected for the reducing action of the cysteine. Inhibition of the amylase by phenylmercuric nitrate was completely reversed by the cysteine. Similar reactivations by cysteine were observed when *p*-chloromercuribenzoate and phenylmercuric chloride were the inhibitors used.

THE INHIBITION OF PHOSPHORYLASE.—An attempt to demonstrate inhibition of the black locust tree phosphorylase by iodoacetate was, as in the case of the amylase, unsuccessful. However, as is shown in figure 10, the phosphorylase was inhibited by iodine-potassium iodide reagent. The excess starch added in these experiments prevented the amylase present from influencing the phosphorylase action.

In qualitative experiments with crude enzyme preparations, sulfhydryl reagents had appeared to give selective inhibition of the amylase. Therefore, to determine whether complete inhibition of the amylase could be achieved without any inhibition of the phosphorylase, the effects of different concentrations of the iodine-potassium iodide reagent on the amylase and phosphorylase activities of a single enzyme preparation were compared. The results, expressed as per cent. inhibition of enzyme activity, are shown in figure 11. The percentage inhibition of the amylase activity was estimated from the increase in reducing carbohydrate in the different samples after incubating for 40 minutes at 35° C. The percentage inhibition of the phosphorylase was estimated by comparing the quantities of inorganic phosphate liberated from glucose-1-phosphate during the 32-minute period between the first and second samplings in the preceding experiment (fig. 10). From figure 11 it is evident that it would be possible to obtain complete inhibition of the amylase with iodine-potassium iodide reagent while considerable phosphorylase activity remained. However, it appears to be impossible to obtain complete inhibition of one enzyme without partial inhibition of the other.

REVERSAL OF PHOSPHORYLASE INHIBITION.—Figure 12 shows the results of an experiment in which the phosphorylase was inhibited with phenylmercuric nitrate and reactivated with cysteine. In similar experiments it was found that the phosphorylase could be inhibited with phenylmercuric chloride and reactivated with cysteine. It was also shown that the enzyme was inhibited by *p*-chloromercuribenzoate, but no attempt was made to demonstrate reversal of this inhibition.

Discussion

The observed variations in activity of an enzyme under different conditions of temperature and pH can represent the combined effects of inacti-

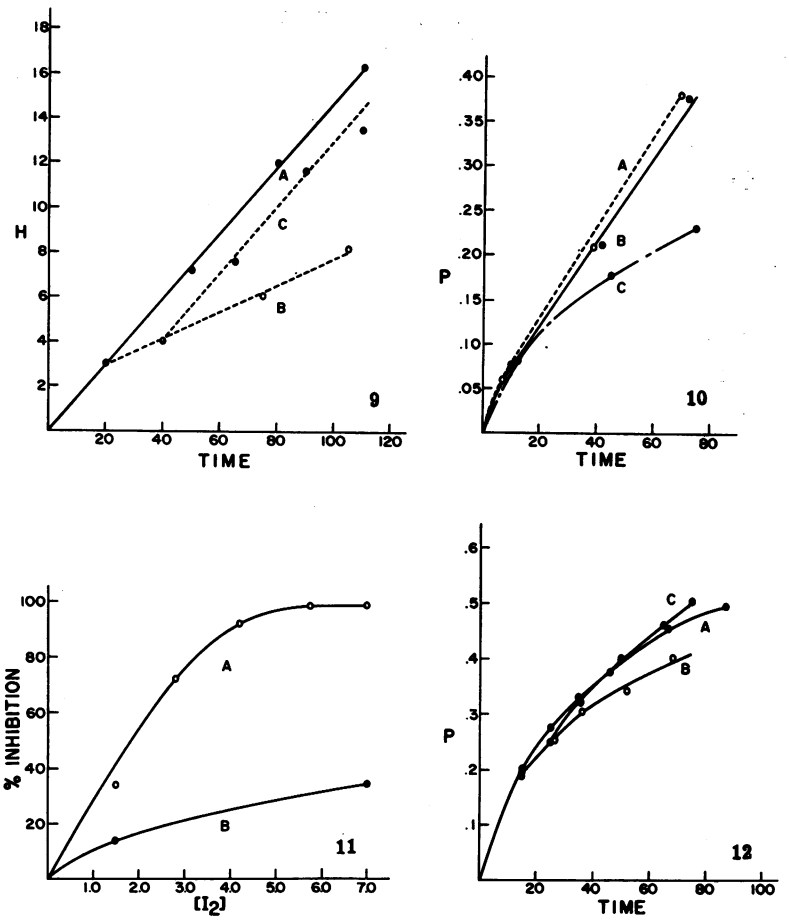

FIG. 9. Reversible inhibition of locust tree amylase with phenylmercuric nitrate and cysteine at 35° C and pH 6.3. Increase in hydrolysis, H (units same as in fig. 1), versus time (minutes) of incubation. Curve A—no inhibitor added. Curve B—inhibitor added at final concentration of 2×10^{-4} M after 20 minutes. Curve C—inhibitor added as in curve B followed 20 minutes later by addition of cysteine at final concentration of 1.65×10^{-2} M. Each sample contained amylase preparation equivalent to 10 mg. of protein nitrogen and 0.15 gm. wheat starch. Citrate buffer concentration in the final solutions (25 ml.) was 0.02 molar.

FIG. 10. Inhibition of locust tree phosphorylase, at 30° C and pH 6.7, with iodine-potassium iodide reagent. P is expressed in same units as in figure 4. Time is in minutes. Curve A is a control with starch added at 6 minutes after zero time. With curve B, starch was added at 8 minutes and inhibitor at a final concentration of 1.9×10^{-5} M $I_2 + 5.4 \times 10^{-5}$ M KI was added at 9 minutes after zero time. For curve C, starch was added at 11 minutes and inhibitor at final concentration 0.9×10^{-4} M I_2 $+ 2.7 \times 10^{-4}$ M KI was added at 12 minutes after zero time. Each sample contained enzyme preparation equivalent to 1.45 mg. of protein nitrogen, 1.0 mg. of soluble starch, and 7.5 mg. of glucose-1-phosphate (neutralized) in 5.5 ml. of 0.01 M citrate buffer. Inorganic phosphorus is corrected for reagents blank at zero time. Zero time was taken from the moment that the enzyme and glucose-1-phosphate were mixed.

vation and actual changes in activity. In the experiments reported here no attempt was made to differentiate between these two phenomena.

Measurements of the activity of amylases based on the increase in reducing sugars do not, of course, identify or classify the mode of action of the enzyme or enzymes (*e.g.*, α or β). The appearance of high concentrations of maltose while considerable high molecular weight material remained in the enzyme substrate mixtures suggests that an enzyme of the β-amylase type was present. Since the formation of glucose from starch is believed to be characteristic of α-amylase (**22**), the chromatographic detection of glucose after long incubation suggests that an α-amylase was also present. Thus, it is probable that amylases of both the α and β types occur in the locust tree tissue.

The action of the locust tree phosphorylase appears to be similar to that of other phosphorylases from plant and animal sources (**8, 9, 11, 12**). The synthesis of polysaccharide is dependent upon the presence of a small amount of polysaccharide to act as a primer. The reaction is reversible and approaches an equilibrium which is independent of the concentration of polysaccharide but varies with pH.

The inhibitions of these enzymes by sulfhydryl reagents indicate that both the amylase and the phosphorylase systems of the black locust tree are dependent on the integrity of sulfhydryl groups. Failure of efforts to inhibit the enzymes with iodoacetate or ferricyanide do not discredit this conclusion because some protein sulfhydryl groups do not react readily with these reagents (**2**). Since concentrations of the iodine-potassium iodide reagent which gave complete inhibition of the amylase gave only partial inhibition of the phosphorylase, it appears that the sulfhydryl groups of the phosphorylase are not as reactive as those of the amylase. However, there is not sufficient difference in reactivity to permit quantitative inhibition of the amylase without some concomitant inhibition of the phosphorylase. There is a possibility that the difference in reactivity with sulfhydryl reagents may

FIG. 11. Comparison of inhibition of locust tree amylase and phosphorylase in a single crude enzyme preparation with varying concentrations of iodine–potassium iodide reagent, concentration of I_2 in the incubating solutions is given as milliequivalents $\times 10^{-4}$ of iodine added per milligram of nitrogen in the aliquot of enzyme preparation used. Curves A and B refer to amylase and phosphorylase activities, respectively. In the amylase inhibition experiments each sample contained tissue extract equivalent to 7.2 mg. protein nitrogen, 150 mg. wheat starch in total volume of 25 ml. of 0.01 citrate buffer, pH 6.7. Phosphorylase inhibition results are taken from data of figure 10.

FIG. 12. Reversible inhibition of locust tree phosphorylase, at 30° C and pH 6.7, with phenyl mercuric nitrate and cysteine. Units are same as for figure 10. Curve A is a control. In curves B and C phenyl mercuric nitrate (final concentration $= 1 \times 10^{-4}$ M) was added at 15 minutes after zero time. In curve C cysteine (final concentration $= 1.1 \times 10^{-2}$ M) was added at 27 minutes after zero time. Each sample contained phosphorylase preparation equivalent to 4.3 mg. protein nitrogen, 20 mg. of soluble starch, and 30 mg. of glucose-1-phosphate in 12 ml. final volume of 0.01 citrate buffer. Zero time is taken as moment at which enzyme-starch mixture is added to glucose-1-phosphate solution.

play a role in the regulation of metabolic processes. Natural sulfhydryl reagents acting as inhibitors of specific enzymes may be involved, for example, in the seasonal changes in the chemical constitution of the protoplasm (**27**). The phosphorylase inhibitor reported by ARREGUIN-LOZANO and BONNER (**1**) may be such a sulfhydryl reagent, being elaborated in potatoes during storage at warm temperatures and absent when the potatoes are stored at cold temperatures.

These characterization studies on the amylase and phosphorylase systems of the living bark of the black locust tree have yielded no definite clues as to whether or how these enzymes may be involved in the temperature sensitive reversible deposition (or mobilization) of starch which occurs in these tissues and which apparently play a minor role in the abilities of the locust bark to withstand injury by freezing.

Summary

Amylase and phosphorylase enzyme systems have been detected in the living bark tissue of the black locust tree.

The optimal temperature for the amylase system during a short incubation period (27 minutes), at pH 6.6, was greater than 55° C. With a longer incubation time (64 minutes) there was pronounced inactivation of the enzyme at 55° C and maximum activity was observed at 50° C. The activation energy for the amylase catalyzed reaction was approximately 12,000 calories per mole. The optimal pH range in citrate-phosphate buffer for the amylase system at 39° C was 5.3–6.0. The products of hydrolysis of starch by the locust tree amylase were maltose, glucose, and dextrins.

Synthesis of polysaccharide from glucose-1-phosphate by the locust tree phosphorylase was dependent on the presence of a small amount of polysaccharide primer. The reaction catalyzed by the locust tree phosphorylase was reversible and approached an equilibrium ratio of inorganic phosphate to glucose-1-phosphate which was independent of the concentration of starch. This ratio was lowest at the highest pH values. The optimal temperature for action of the locust tree phosphorylase during 45 minutes, at pH 6.6, was approximately 50° C. The optimal pH in citrate buffer, as revealed by the initial rate of release of inorganic phosphate from glucose-1-phosphate at 30° C, was approximately 6.3.

The amylase system of the living bark of the black locust tree was inhibited by iodine-potassium iodide reagent, *p*-chloromercuribenzoate, phenylmercuric chloride, and phenylmercuric nitrate, but was not inhibited by iodoacetate or ferricyanide. The inhibitions produced by *p*-chloromercuribenzoate, phenylmercuric chloride, and phenylmercuric nitrate were readily reversed by an excess of cysteine.

The phosphorylase of the living bark of the black locust tree was inhibited by iodine-potassium iodide reagent, *p*-chloromercuribenzoate, phenylmercuric chloride, and phenylmercuric nitrate, but it was not inhibited by iodoacetate. The inhibitions produced by phenylmercuric chloride and phenylmercuric nitrate were readily reversed by cysteine.

In experiments with crude enzyme preparations containing both amylase and phosphorylase it was shown that concentrations of the iodine-potassium iodide reagent which gave complete inhibition of the amylase gave only partial inhibition of the phosphorylase.

These studies indicate that both the amylase and the phosphorylase systems of the black locust tree are dependent on the integrity of sulfhydryl groups. However, the essential sulfhydryl groups of the phosphorylase are not as chemically reactive as those of the amylase. It is suggested that differential sensitivity of enzymes to natural sulfhydryl reagents may be involved in the regulation of metabolic processes of plants and particularly in controlling the seasonal changes in composition of the protoplasm of the black locust tree living bark.

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