"THE ENZYMIC HYDROLYSIS OF PHOSPHORIC ACID ESTERS

BY BARLEY EXTRACTS."

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EXPERIMENTAL WORK

INTRODUCTION

Phosphoric acid esters and the enzymes hydrolyzing them have assumed a very considerable importance, both an applied and purely scientific one, in the study of mammal and yeast physiology. In the higher plants little has been determined about their functions. Recognizing this, we commenced work on plant phosphatases choosing as our representative spermatophyte-barley.

The occurrence and necessity of the element phosphorus in the living cell was established by de Saussure (1804), Sachs (1860) and other investigators early in the 19th century. The discovery of its essentiality to life and its close association with carbohydrates, fats and proteins has led to the belief that its study would be a key to disclose much of the mechanism of living organisms.

Progress in the study of phosphorus metabolism has, however, been conditioned by a lack of knowledge of the chemistry of the element and its compounds as they occur in organisms. In recent years this state has been altered. Kay (1)[44] has reviewed our present knowledge of the phosphorus compounds of plants and animals.

Phosphoric acid esters first assumed importance in physiology following the important discoveries of Harden and his associates (2)[36] in 1905 on the general effect of the addition of sodium phosphate increasing the total fermentation produced by yeast juice on sugar. With these researches and the long series following the phosphorus metabolism of yeasts was intimately linked with the fermentation process through the esters of the mono- and di-saccharides. It is

sufficient to say here that other phosphoric esters, the phospho-
'lipides, phosphoproteins, meta-, ortho- and pyro-phosphates etc.
have assumed an import in many metabolic processes.

Prior to Harden's researches, Buchner (1897) had succeeded
in isolating from living yeast zymase, which, when freed from the
last trace of organized cell material was able to bring about
identical fermentation processes as had before deemed to be possible
only in the presence of active yeast cells. Enzyme chemistry
developed rapidly in the ensuing years and several observations
appeared on enzymes effecting the cleavage of organic phosphoric
acied esters (e.g. C. Neuberg and L. Karczag (3)). Their wide
distribution in the plant and animal kingdoms was soon indicated.
Phosphatases and their substrates have now, in addition to their
importance in fermentations, become significant in the study of
muscle contraction, ossification, in the work of the kidney and
in many other processes in mammal physiology. In plants we know
little or nothing of their relation to metabolic processes.
Conceivably they might be of importance in the transformation of
sugars apart from the photosynthetic process,-- in the germination
of malting barley, in sugar storage by sugar beets. If in mammals
they are intimately linked with the calcium compounds, might not
the same be true in plants, Many questions might be asked.

The writer while realizing the many problems presented by
phosphatases and their substrates in plants has sought only to

prepare the way for their future study. The work accomplished while

not far reaching is fundamental. Something has been gained

about the preparation of phosphatase extracts, and something

determined of their distribution through the development of the

barley plant.

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MATERIALS AND METHODS

(a) The Plant Material:

Seed of a pure line of Duckbill barley (a six-rowed variety of Hordeum vulgare L.) was obtained from the Department of Agronomy and sown, 4 seeds in each, in 8" clay pots in phosphorus free sand. One series received weekly Hoagland's complete nutrient solution and a second series received a similar solution in which potassium dihydrogen phosphate was replaced by potassium chloride. The complete and phosphorus deficient solutions were made up as follows:

Reserve Solution 1. KNO_3 67 g.)
) in 1 L.aq. dist.
                      $MQSO_4$ 100 g.)

"   "   2. $Ca(NO_3)_2.4H_2O$ 208 g.   in 1 L.aq. dist.

"   "   3. KH_2PO_4 50 g.   in 1 L.aq. dist.

"   "   4. KCh.   26 g.   in 1 L.aq. dist.

22 c.c., 26 c.c. and 12 c.c. of reserve solutions 1, 2 and 3 respectively added to sufficient aq. dist. to make 2 l. gave the phosphorus deficient nutrient solution.

Since the water requirements of the plants at different stages of growth varied so greatly and since the object in view was only to obtain normal and phosphorus deficient plants, fixed quantities of solution were not given the pots after the first few weeks. Rather, the amount required became a matter of judgement to see that the sand was uniformly moist in all the pots. Distilled water likewise was given as required.

First sowings were made November 2, 1935 and similar series were set up during the experimental period (until March 15, 1936) every three weeks.

The photoperiodic effects on the barley were very marked. Plants sown November 2, 1935, for example, came into head but a few days earlier than plants sown the second week in January. As a result where enzyme activities at different growth stages were to be compared, plants of a single sowing were used.

Plants of the second series showed marked phosphorus deficiency within five weeks. At eight weeks the deficiency was very pronounced with typical reddening of the foliage (from the leaf tips and edges back) with eventual necrosis of the lower leaves and a low dry weight.

The photo below indicates the difference between plants of the two series:

Photo

Plants receiving phosphate weekly grew rapidly and were highly succulent as a result no doubt of the combination of the high greenhouse temperatures and short photoperiods. Such a condition was favorable for aphis attacks for which pests the plants had to be continually sprayed.

Normal and deficient plants came into head about the same date. Accordingly, it was assumed that on a given date such plants of the same sowing were at the same developmental stage.

When germinating seed was required, dormant seed was placed between blotting paper in a dark room at $30^\circ \pm 3^\circ$ C. for five days.

(b) Materials and Methods Required for Enzyme Action *in vitro*:

Phosphatases effect the cleavage of phosphoric acid esters liberating free phosphoric acid. To determine their presence and activity the usual conditions for enzyme action *in vitro* must be set up.

To a substrate (some given phosphoric acid ester) in a solution buffered to a pH. suitable for reaction and set at a desirable temperature, is added a colloidal solution containing the enzyme previously extracted from the living plant tissue. At the end of a known period the phosphoric acid liberated is determined, assuming the amount of acid freed in unit time is indicative of the activity of the phosphatase.

In greater detail our procedure was as follows:
To 8 c.c. of phthalate, veronal, or glycine buffer, a trace of

toluene and 1 c.c. of 2% substrate in a test tube was added 1 c.c.
of enzyme extract (about 6 mgs. of dried extract) and the reaction
set at the desired temperature. At the end of a definite period,
usually 1 hour, 1 c.c. of trichloacetic acid was added, the mixture
centrifuged and the liberated phosphoric acid determined by the King ( )
colorimetric method. Series were run in duplicate.

After the conditions for enzyme action in vitro were set up
and a reaction liberating phosphoric acid shown, it was necessary
to show that the action is enzymic and not an ordinary catalyzed
chemical reaction.

Certain characteristics of enzyme preparation and their action
are used to determine the difference. In brief some of those are:
(1) the thermal instability of enzyme; enzymes are inactivated at
certain temperatures. (2) their sensitivity to C(H) and C(OH).
(3) their activation and inhibition by certain substances. (4) their
colloidal and protein nature as exhibited in extracts. (5) their
ability to be specifically absorbed, etc. (6) their sensitivity
to maceration, etc.

In addition to demonstrating the enzymic nature of a reaction,
it is sometimes desirable, though difficult of proof, to establish
the specificity of the enzyme involved--i.e. whether more than one
enzyme was acting on the substrate and if one, whether it is capable
of acting on other substrates. This is pertinent to phosphatase
investigations for it is not known just how many different phosphatases
act on phosphoric esters. Again, an enzymic estract might conceivably

act on a substrate in different ways--e.g. barley extract on a hexose
ester. In this case glucolysis i.e. enzymic degradation of the
hexose unit to lactic acid might liberate free phosphate; or, on
the other had a true phosphatase might act yielding free phosphate
but leaving the hexose unit intact. Which is true may be determined
by following the quantitative relations of the reaction. Further
complications may be readily introduced into the question of
specificity of enzymes.

(1) Preparation of Standard Buffer Solutions:

Sorenson 1909 (10)[84] and Michaelis 1909 (11)[58] recognized and
emphasized the fact of the profound influence of C(H) and C(OH) on
the activities of enzymes. As "regulators" for the production and
maintenance of a definite hydrogen ion concentration the standard
buffer solutions recommended by these men are widely employed in
enzyme chemistry.

The choice of buffers which can be employed in phosphatase
reactions are limited. Since free phosphate would interfere with
the presumably reversible reaction in which phosphate is one of the
end products phosphate buffers cannot be used. Additional free
phosphate would also decrease the reliability of its colorimetric
determinations. Further, many buffers have been found to inhibit
certain enzyme reactions and only those, found by trial to permit
suitable activity, can be considered.

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We have found several standard buffers to be suitable for our barley phosphatase reactions. (See also W.Jack (12).)

(a) $\frac{M}{5}$ Potassium acid phthalate and $\frac{M}{5}$ HCl buffer mixtures by Sorenson (10); range $_p$H 2.2--3.8; prepared as given in table by Clark (13).

(b) $\frac{M}{5}$ Potassium acid phthalate and $\frac{M}{5}$ NaOH buffer mixtures by Sorenson (10); range pH 4.0--6.2; prepared as given in table by C$_{lark}$ (13).

(c) 7.505 g. glycine + 5.85 g. sodium chloride in 1 l. + 0.1 N Sodium hydroxide; pH ra$_{nge}$ 11.2--8.3; prepared as given in table by Clark (13); buffer mixtures by Sorenson (10).

(d) .1 M glycine+ .1 M NaCh + 0.1 N HCh; range pH 1.1--3.6 prepared as goven in table by Clark (13); buffer mixtures by Sorenson (10).

(e) Michaelis' (14) veronal buffer.

(2) Protectants:

Enzyme chemists generally employ some protectant in reaction tubes and during autolyses to prevent the growth of, and hence the possible interference by, microorganisms. Anaesthetics and electrolytes, such as sodium chloride, in high concentrations, are frequently employed since reactions of an enzymic nature are not greatly inhibited by them. In our experiments we used C.P. toluene at a drop per tube. Chloroform, and concentrated sodium chloride were used with equally good results.

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(3)   Substrates:

Since esterases vary in their ability to hydrolyse their substrates, it was necessary for us to use several phosphoric acid esters.

.1.   Sodium glycerophosphate--British Drug Houses "C.C." quality, and phosphate free.

.2.   Sodium pyrophosphate--"C.P." and phosphate free obtained from "Physician's Pharmacy," Vancouver, B. C.

.3.   Calcium hexose diphosphate obtained from Brewing Co., Ontario, was an impure product with much insoluble matter and with a high percentage of Phosphoric acid.

.4.   Hexose monophosphoric acid. (Robison ester.) This ester was first isolated by Robison and co-workers (77) and is found along with the di-acid during yeast fermentation. Several methods have been used for its isolation. We used the following procedure by Raymond and Levene (76).

To live yeast and an excess of glucose, sodium phosphate was slowly added to maintain an optimum concentration and was followed by frequent colorimetric analyses for phosphate. While fermentation was still proceeding vigorously it was interrupted by adding trichloracetic acid and the mixture was centrifuged. The diphosphate and inorganic phosphate were precipitated by adding a solution of $BaCl_2$ equivalent to the phosphate used and then $Ba(OH)_2$ to pH 9. After being centrifuged the solution was clarified with charcoal and concentrated at reduced pressure to a small volume. The mono- phosphate was precipitated by adding an equal amount of 95% alcohol

and purified by repeated solution and precipitation.

Pryde (*93*) gives Robison's Method for the preparation of
hexose di- and mono-phosphoric acids by yeast fermentation. This
procedure has an advantage in its exactness and production of
higher yields. It is, however, much less rapid. The preparation of
the Neuberg (*93*) ester by hydrolysis of yeast diphosphoric acid is
also given by Pryde.

Pasternak (*69*) has recently prepared the Robison ester from
wheat flour and we prepared a small amount of it according to his
method. Wheat flour was hydrolyzed by 2% H_2SO_4 for five hours
at boiling temperatures, cooled and the hydrolysate neutralized to
phenolphthalein with baryta. Following filtration, the filtrate
was treated with two volumes of alcohol and purefied by further
precipitation from equal volumes of alcohol; from rotations of the
Barium salt and free acid and from the melting points of the
phenylhydrazine salt, Pasternak found his preparation to be
identical with Robison's ester.

Sodium phenyl phosphate, Sodium metaphosphate, hydroxy-x-quinoline
phosphate, etc. are obtainable in pure form, and are suitable substrates
for trial. Sodium hexosediphosphate may be obtained in relatively
pure form under the trade name "candiolin."

(4) Preparation and Purification of Enzyme Extracts:

The method used in obtaining an extract of phosphatase with
its associated materials depends essentially on its solubility in
water and insolubility in protein precipitants such as alcohol,

acetone, saturated ammonium sulphate solution, etc.

To transfer the enzyme from the plant source to an aquaeous
infusion some method of opening the cell structure must be found.
We tried autolysis (i.e. automatic dissolution of cells by unchecked
degrading enzymes such as the erepsins) and mechanical fragmentation.
The activity of extracts obtained with different autolyses and with
different periods of maceration in a ball mill, in a hand mincer and
in a mortar with sand and with glass wool, is given in TABLE I:

TABLE I

Extract Source: germinating seed--at 6 mgs. a tube.

Buffer: veronal--pH 5.6.   Time: 1 hour.

Substrate: 2% Sodium glycerophosphate. Temp.: 35° C.

TUBE NO.	EXTRACTION PROCEDURE.	pH	TOTAL P MGS. PER C.C.	INITIAL FREE P MGS. PER C.C.	FINAL FREE P	
					READING	P
1	Ball mill--4 hours.	5.6	.44	.08	22	.02
2	Ball mill--1/2 hour.	-	.44	.06	33	.00
3	Ball mill--1/4 hour.	-	.44	.06	33	.00
4	Mortar--sand--15 mins.	5.5	.44	a little	-	a little
5	Mortar--glass wool-- 15 mins.	-	.44	.06	33	.01
6	Mincer-autolysis-- 4 hours.	5.5	.44	.06	18	.05

The method adopted in subsequent work was to grind the plant

plant material twice in a hand mincer and followed by 10-15 minutes

maceration in a mortar; water was then added to 90% (only in the

case of seed had much water to be added) and using toluene as a

protectant autolysis allowed for 4-8 hours at constant temperature.

The autolysate was centrifuged for 5 minutes on a small

electrically driven centrifuge, filtered through a coarse filter paper

and from the filtrate by twice precipitating from 95% alcohol the

enzyme extract was obtained. The extract, dried over concentrated

H_2SO_4 had later to be ground finely, "dissolved" and the weight of insol-

uble matter determined. 4 mgs. of extract were generally added to

each tube, which amount gave a satisfactory reaction.

TABLE II

Extract Source: germinating seed.

Buffer: veronal.   Time: 1 hour.

Substrate: 2% sodium glycerophosphate.   Temp.: 30° C.

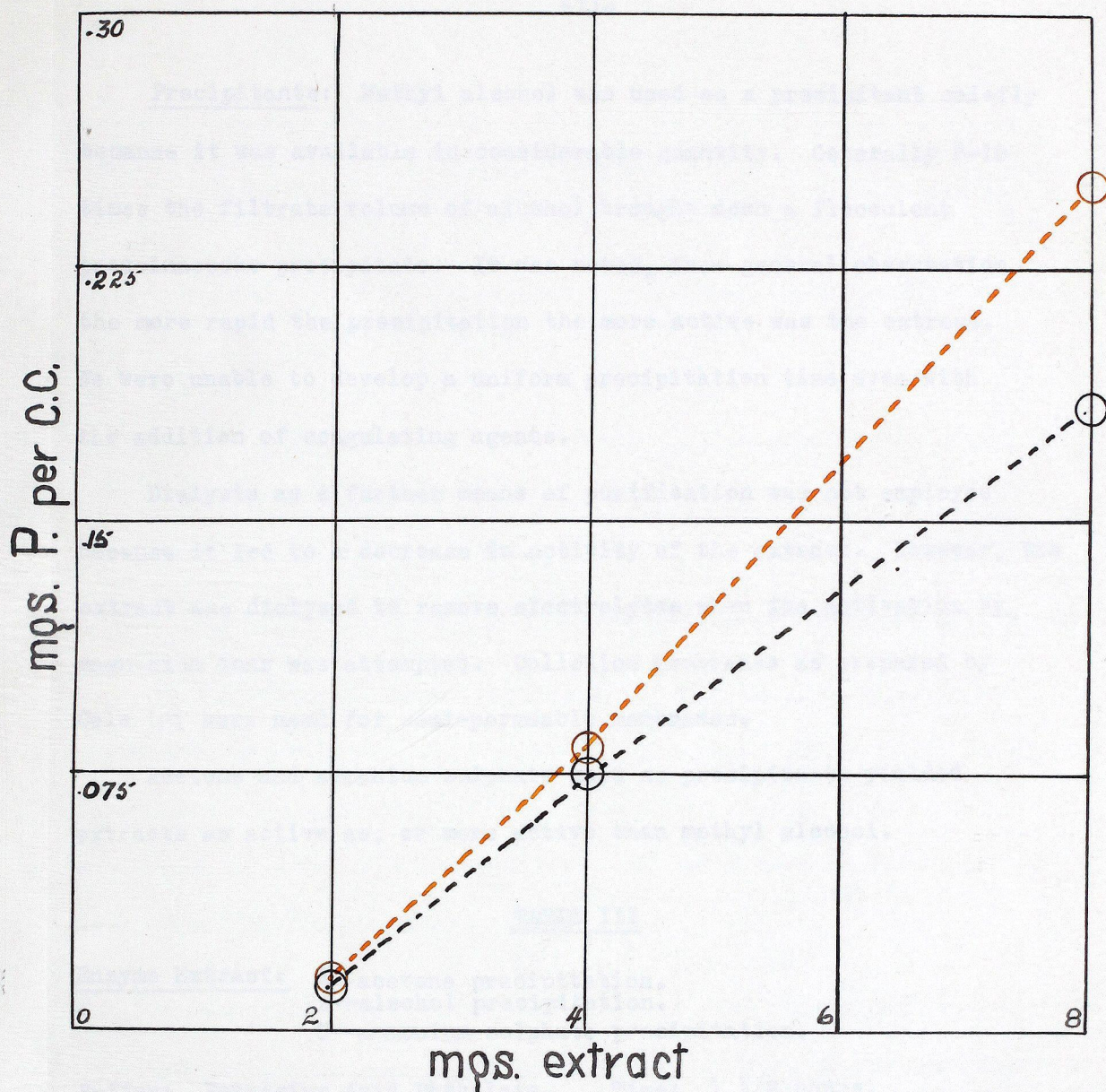| TUBE NO.	AMT. OF ENZYME MGS.	STATE OF ENZYME	pH.	TOTAL P MGS. PER C.C.	INITIAL FREE P	STANDARD P		LIBERATED P	
						READING	MGS. PER C.C.	READING	MGS. PER C.C.
1	8	Active	5.1	.44	little	20	.1	11	.18
2	8	"	5.7	.44	"	20	.1	8	.25
3	4	"	5.1	.44	"	20	.1	28	.07
4	4	"	5.7	.44	"	20	.1	25	.08
5	2	"	5.1	.44	trace	20	.1	---	little
6	2	"	5.7	.44	"	20	.1	---	"
7	4	Inactive	5.1	.44	little	20	.1	---	"
8	4	"	5.7	.44	"	20	.1	---	"

Fig.1: Enzyme Concentration Series

Extract Source: germinating seed

Substrate: 2% sodium glycerophosphate

Buffer: veronal ◯ ph 5.1 ◯ ph 5.7

Time: 1 hr.   Temperature: 30°C.

Precipitants: Methyl alcohol was used as a precipitant chiefly because it was available in considerable quantity. Generally 8-10 times the filtrate volume of alcohol brought down a flocculent proteinaceous precipitate. It was noted, from general observation the more rapid the precipitation the more active was the extract. We were unable to develop a uniform precipitation time even with the addition of coagulating agents.

Dialysis as a further means of purification was not employed because it led to a decrease in activity of the extract. However, the extract was dialyzed to remove electrolytes when its activation by magnesium ions was attempted. Collodion membranes as prepared by Cole (97) were used for semi-permeable membranes.

Acetone and ammonium sulphate used as precipitants yielded extracts as active as, or more active than methyl alcohol.

TABLE III

Enzyme Extract: A--acetone precipitation.
B--alcohol precipitation.
C--ammonium sulphate precipitation.

Buffer: Potassium Acid Phthalate.   Time: 1 1/2 hours.

Substrate: Sodium glycerophosphate. Temp.: 32° C.

TUBE NO.	pH	ENZYME EXTRACT	TOTAL P MGS. PER C.C.	INITIAL FREE P MGS. PER C.C.	STANDARD TUBES READING	P MGS. PER C.C.	EXPERIMENTAL TUBES READING	P MGS. PER C.C.
1	5.0	A	.44	--	20	.1	13	.15
2	5.6	"	.44	--	20	.1	7	.29
3	6.3	"	.44	--	20	.1	11	.18

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TABLE III (Cont.)

TUBE NO.	pH	ENZYME EXTRACT	TOTAL P MGS. PER C.C.	INITIAL FREE P MGS. PER C.C.	STANDARD TUBES		EXPERIMENTAL TUBES	
					READING	P MGS. PER C.C.	READING	P MGS. PER C.C.
1	5.0	B	.44	trace	20	.1	20	.10
2	5.6	"	.44	"	20	.1	25	.08
3	6.5	"	.44	"	20	.1	18	.11
1	5.0	C	.44	.05	20	.1	14	.14
2	5.6	"	.44	.05	20	.1	10	.20
3	6.3	"	.44	.05	20	.1	20	.10
4	5.6	-	.44	--	20	.1	--	--

In the study of mammal phosphatases, tissues in a solution of substrate have been found to split off phosphoric acid. While in tissue reaction the unknown factors are increased in number, they have an advantage in rapidity of preparation. We tried several reactions with tissues of the barley plant.

(5) Trichloracetic Acid: This acid is a general precipitant of proteins, congulating them in such a way as to render enzymes in the proteinaceous extract, inactive. Trichloracetic acid does not interfere with determination of free phosphorus by the King (45) method to any appreciable extent and it prevents the interference in this determination by protein through their precipitation. Hinsburg (39) using Fiske and Subarrow (31) colorimetric method reports that the use of trichloracetic acid may cause an error as high as 38%.

(6) Method of Determining Free Phosphoric Acid: Since Bell and Doisy (7)

proposed their method for the colorimetric determination of phosphoric

acid in 1920 many modifications of it have appeared. In our work we

used a recent modification by of King (45). Materials required were

as follows:

    .1. 72-60% perchloric acid.

    .2. 5% ammonium molybdate (phosphate free).

    .3. 0.2% 1:2:4 aminonaphtholsulfonic acid (.5 gms. of

the 1:2:4 of the acid, 30 gms. sodium bisulphite and 6gms. sodium

sulphate dissolved in aq. dist. to make a volume of 250 c.c.; left

stand overnight, filtered and prepared every 2 weeks.)

    .4. Standard Phosphate 2.1935 g. KH_2PO_4 in 500 c.c.

aq. dist. to give 1 mg. phosphorus per c.c.

  To 10 c.c. of reaction mixture obtained after centrifuging the

trichloracetic acid precipitate and similarly to tube containing 10 c.c.

of standard phosphate or some dilution of it, was added.

    1 c.c. perchloric acid followed by gentle agitation.

    1 c.c. ammonium molyhdate " " "   "

    0.5 c.c. 1:2:4 acid   " " "   "

  5 minutes was allowed for the blue color of the phosphomolybdic

complex to develop and the reaction tube read against the standard

tube in the colorimeter.

  According to de-Beer's Law the absorption of light by solutions

is directly proportional to the concentration of the coloring substance.

The amount of phosphate in the reaction tube may thus be computed from

the equation $C_2:C_1 = L_1:L_2$ where C_2 is the phosphate concentration of

the standard and L_2 the tube length of the standard (in m.m.) as read

on the colorimeter, and were C_1 is the phosphate concentration of the reaction tube (the unknown) and L_1 its tube length.

Our colorimetric determinations were made with a Kleth-Bio colorimeter used and cared for in the manner described by Coles [94]. The accepted reading on a tube was the average of three.

Total Phosphorus was also determined by the King [45] method.

(7) pH Determinations: With few exceptions determinations of pH were made electrometrically using a Leeds and Northrup Co. quinhydrone pH indicator. Possible "drift" and "salt error" were minimized by frequently checking the pH determined electrometrically with La Motte color standards. Temperature corrections were made from the table provided. pH mearsurements above 8 were determined by the La Motte set. Leeds and Northrup Co. [95] in their valuable notes have described the use and care of the quinhydrone pH indicator. Coles [94] also deals with its use. Though only small amounts of extract are available, 1/2 generally goes into pH determination tubes. B.D.H. capillator, colorimetric system of pH determination requires only small amounts of mixture, as also do several recently developed micro-electrodes for use on the gurnhydrone. (Pierce and Montgomery [74].)

EXPERIMENTAL RESULTS

(a) To demonstrate the existence of an enzymic reaction, with phosphoric acid esters as substrates, in which one of the end products is free phosphoric acid:

Free phosphoric acid is not liberated except in the presence of the active enzyme extract. From the data in Table IV it is seen that combinations of buffer and substrate without active extract, as of buffer and active extract without the substrate yield no phosphoric acid; that the buffer plays little part in the reaction other than regulation of pH is indicated in the liberation of phosphoric acid by a combination of enzyme and substrate with buffer absent.

TABLE IV

Extract Source: germinating seed.   Time: 1 hour.

Substrate: 2% Sodium glycerophosphate.   Temp.: 26° C.

Buffer: Potassium Acid Phthalate; pH 5.5.

TUBE NO.	BUFFER 10 C.C.	SUBSTRATE 1 C.C.	EXTRACT 1 C.C.	EXTRACT BOILED 1 C.C.	TOTAL P MGS. PER C.C.	LIBERATED P MGS. PER C.C.
1	+	+	+	−	.44	.27
2	+	+	−	+	.44	trace
3	+	−	+	−	.00	.00
4	−	+	+	−	.44	.04
5	+	+	−	−	.44	.00

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The thermal instability of an ester cleaving substance is shown in a consideration of Table V and Figure 2. The data, while not very satisfactory from the point of view of completeness is sufficient to demonstrate the progressive inactivation of the enzyme at temperatures above and below a broad optimum around 26-37° C.

TABLE V

Conditions as in Table IV.

Extract Source: A-germinating seed.

B-leafy tops.

TUBE NO.	TEMPERATURE ° C.	EXTRACT	TOTAL P MGS. PER C.C.	INITIAL P MGS. PER C.C.	LIBERATED P MGS. PER C.C.
1	7°	A	.44	--	.05
2	26°	A	.44	--	.22
3	37°	A	.44	--	.25
4	60-65°	A	.44	--	.04
5	37°	B	.44	.05	.10
6	65°	B	.44	.05	.06
7	100°	B & A	.44	.05	.05
1 (a)	* 37°	A	.35	.05	.15

* Tube 1 (a) is Tube 1 after 1 hour at 7° C.

The Significance of pH: The hydrogen and hydroxyl ion concentration of a medium is a fundamental influence in most enzyme reactions. Activity pH curves comparing relative activity with the pH may be constructed to demonstrate a characteristic optimal region
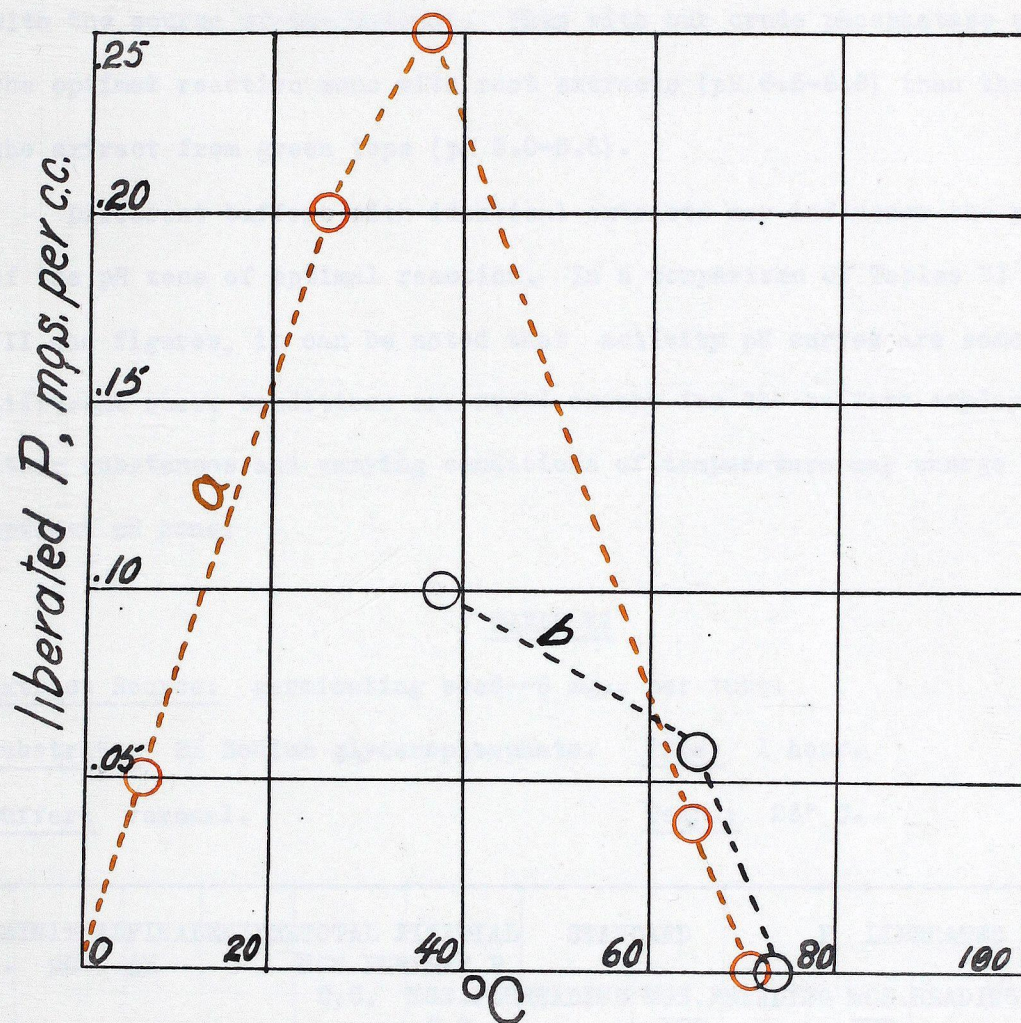
Fig 2: Activity~Temperature Series
Extract Source: (a) germinating seed (b) tops
Substrate: 2% sodium glycerophosphate
Buffer: Phthalate   Time: 1 hr

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of action. (Figures 3 and 4).

With crude extracts and often with highly purified ones the position of the reaction optimum in relation to the pH scale may vary considerably with the source of the extract. Thus with our crude phosphatase extracts the optimal reaction zone with root extracts (pH 6.5-6.8) than that with the extract from green tops (pH 5.0-5.6).

Different buffers with identical extracts may influence the position of the pH zone of optimal reaction. In a comparison of Tables VI and VII and figures, it can be noted that activity pH curves are somewhat different where conditions are equal except for the buffers employed. Other substances and varying conditions of temperature may charge the optimal pH zone.

TABLE VI

Extract Source: germinating seed--6 mgs. per tube.

Substrate: 2% Sodium glycerophosphate.   Time: 1 hour.

Buffer: veronal.   Temp.: 26° C.

TUBE NO.	INITIAL pH	FINAL pH	ENZYME	TOTAL P MGS. PER C.C.	INITIAL FREE P MGS.PER C.C.	STANDARD		P LIBERATED			
						READING	MGS.PER C.C.	READING	MGS. PER C.C.	READING	MGS. PER C.C.
1	3.2	3.2	+	.44	--	20	.1	30	.066	31	.06
2	4.4	4.5	+	.44	--	20	.1	27	.074	27	.074
3	5.2	5.2	+	.44	--	20	.1	22	.09	23	.09
4	5.8	5.8	+	.44	.02	20	.1	18	.11	17	.10
5	6.8	6.7	+	.44	--	20	.1	18	.11	19	.11
6	7.5	7.4	+	.44	.02	20	.1	25	.08	29	.050
7	8.0	7.8	+	.44	.02	20	.1	37	.054	27	.054
8	5.8	5.7	-	.44	--	20	.1	--	trace	--	trace

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TABLE VII

Extract Source: A - germinating seed (acetone precipitation)--6 mgs.
B - dormant seed--6mgs. per tube.

Substrate: 2% Sodium glycerophosphate.

Time: 1 hour. Temperature: 26° C.

TUBE NO.	INITIAL pH	FINAL pH	ENZYME	TOTAL MGS. PER C.C.	P INITIAL MGS. PER C.C.	STANDARDS		P LIBERATED			
						READING	MGS. PER C.C.	READING	MGS. PER C.C.	READING	MGS. PER C.C.
1	3.1	3.1	A	.44	--	20	.1	35	.05	38	.05
2	4.0	4.1	A	.44	--	20	.1	21	.1	18	.11
3	5.5	5.4	A	.44	--	20	.1	9	.24	11	.18
4	6.5	6.5	A	.44	--	20	.1	11	.18	11	.18
5	7.0	7.0	A	.44	--	20	.1	25	.08	33	.06
6	8.0	7.7	A	.44	--	20	.05	25	.05	28	.07
7	10.0	9.5	A	.44	--	20	.05	26	.05	26	.08
8	5.5	5.5	-	.44	--	20	.05	--	trace	--	trace
1	5.5	5.5	B	.44	little	20	.1	11	.18	--	--
2	6.5	6.5	B	.44	--	20	.1	12	.16	--	--
3	5.5	5.5	-	.44	--	20	.1	--	--	--	--

The optimal reactionzones for all our phosphatase extracts lie on the acid side of neutrality. Correlated with this is the fact that the reaction of the crude cell sap of barley is acid. (pH 5.0-6.8). This does not preclude the possibility of barley phosphatases with optimal reaction zones on the basic side.

All our pH activity curves (figures 3 and 4) possess relatively broad zones of optimal reation. This is in striking contrast to the activity pH curves for mammal phosphatases where .2 pH may cause large differences.

Suitable salt mixtures whose acidity does not alter appreciably over a certain range by the addition of acids, bases or amphoteric substances are used in reaction tubes to prevent an alteration of acidity due to products already present or products formed in the course of enzyme reaction. The addition of a substrate such as sodium glycerophosphate of alkaline reaction will tend to raise the pH. Similarly other substrates and enzyme solutions may alter the initial pH (W. Jack (16)).

Over most of their range the buffers we selected were very suitable, changing but little during the course of reaction. Tables VI and VII.

With the question of the influence of materials concomitant with the enzyme reaction are to be considered specific activators and inhibitors. Lohmann (50) and others have shown that a specific activator of yeast and mammal phosphatases is magnesium in the ionic form. By dialyzing our extracts to remove electrolytes then adding a magnesium chloride solution we were able to activate barley phosphatase. Table VIII and Figure 5.

TABLE VIII

Extract Source: A - germinating seed (dialyzed)--6 mgs.
　　　　　　　　　B - normal root (dialyzed)--6mgs.
　　　　　　　　　C - germinated seed (not dialyzed)--6 mgs.

Buffer: Phthalate.　　　　　　　　　　　　Time: 1 hour.

Substrate: 2% Sodium Glycerophosphate.　Temp.: 26° C.

TABLE VIII - Continued:

TUBE NO.	pH	M$_G$CL$_2$ 6 mgs. PER C.C.	EXTRACT	TOTAL P MGS. PER C.C.	FREE P MGS. PER C.C.	STANDARD		LIBERATED P	
						READING	MGS $\overset{p}{\wedge}$ PER C.C.	READING	MGS. PER C.C.
1	5.6	+	A	.44	---	20	.1	9	.23
2	5.6	—	A	.44	---	20	.1	18	.11
3	5.6	+	B	.44	---	20	.1	14	.14
4	5.6	—	B	.44	--	20	.1	33	.06
5	5.1	+	A	.44	---	20	.1	15	.13
6	5.1	—	A	.44	---	20	.1	33	.06
7	5.6	—	C	.44	trace	20	.1	12	.17
1*	5.6	+	A	.37	--	20	.1	20	.10
2*	5.6	—	A	.37	--	20	.1	35	.06

* Substrate: Sodium pyrophosphate.

From several series, dialysis of dormant seed extracts seemed to increase their activity and it was thought that a dialyzable, thermostable inhibitor of barley phosphatases had been found. A later series did not confirm these results and the use of impure toluene as a protectant cast doubt on the former findings. The question of activators other than magnesium and inhibitors was left in abeyance.

(a) Distribution of Phosphatases in Barley:

A substance in barley precipitated as a protein sensitive to maceration and capable of catalyzing a reaction in which free phosphate
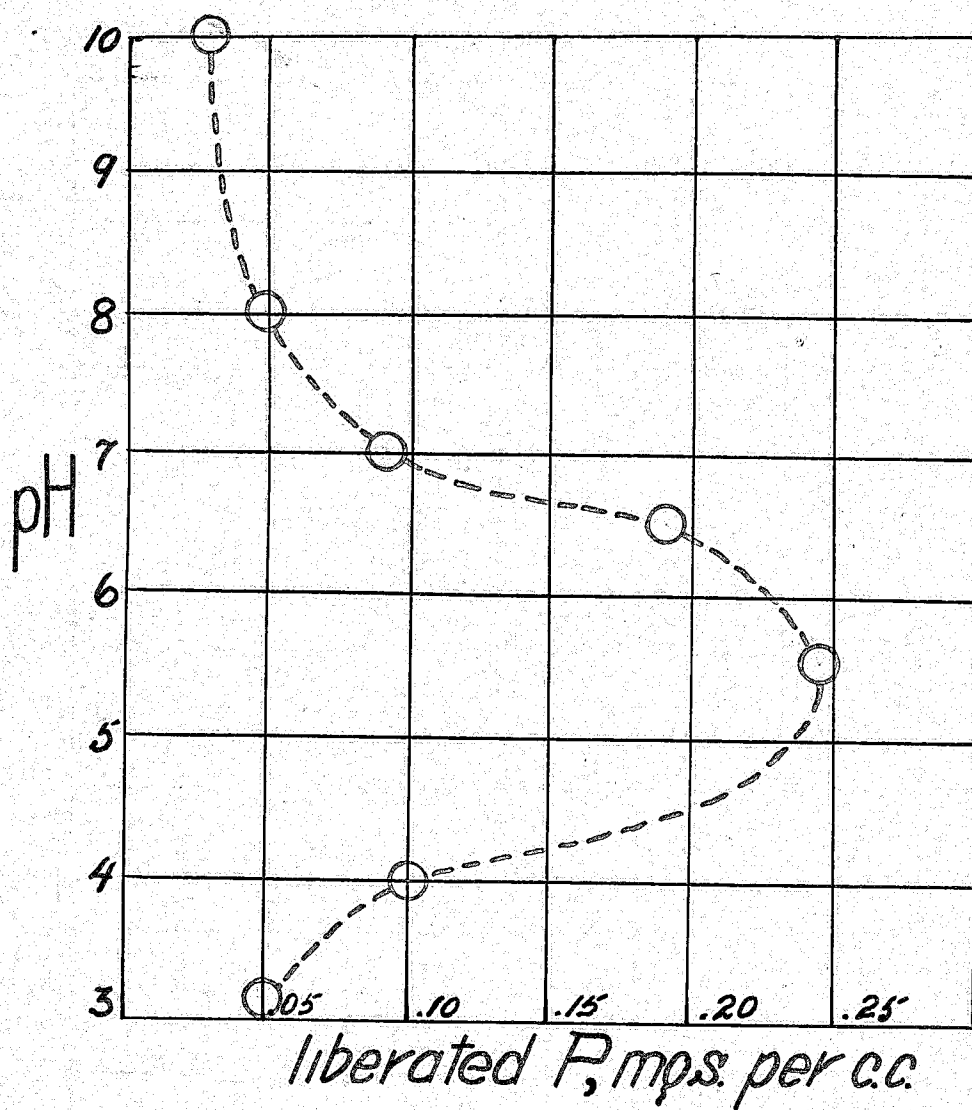
Fig.3: Activity-pH Series
Extract Source: germinating seed
Substrate: Na glycerophosphate   Time: 1 hr.
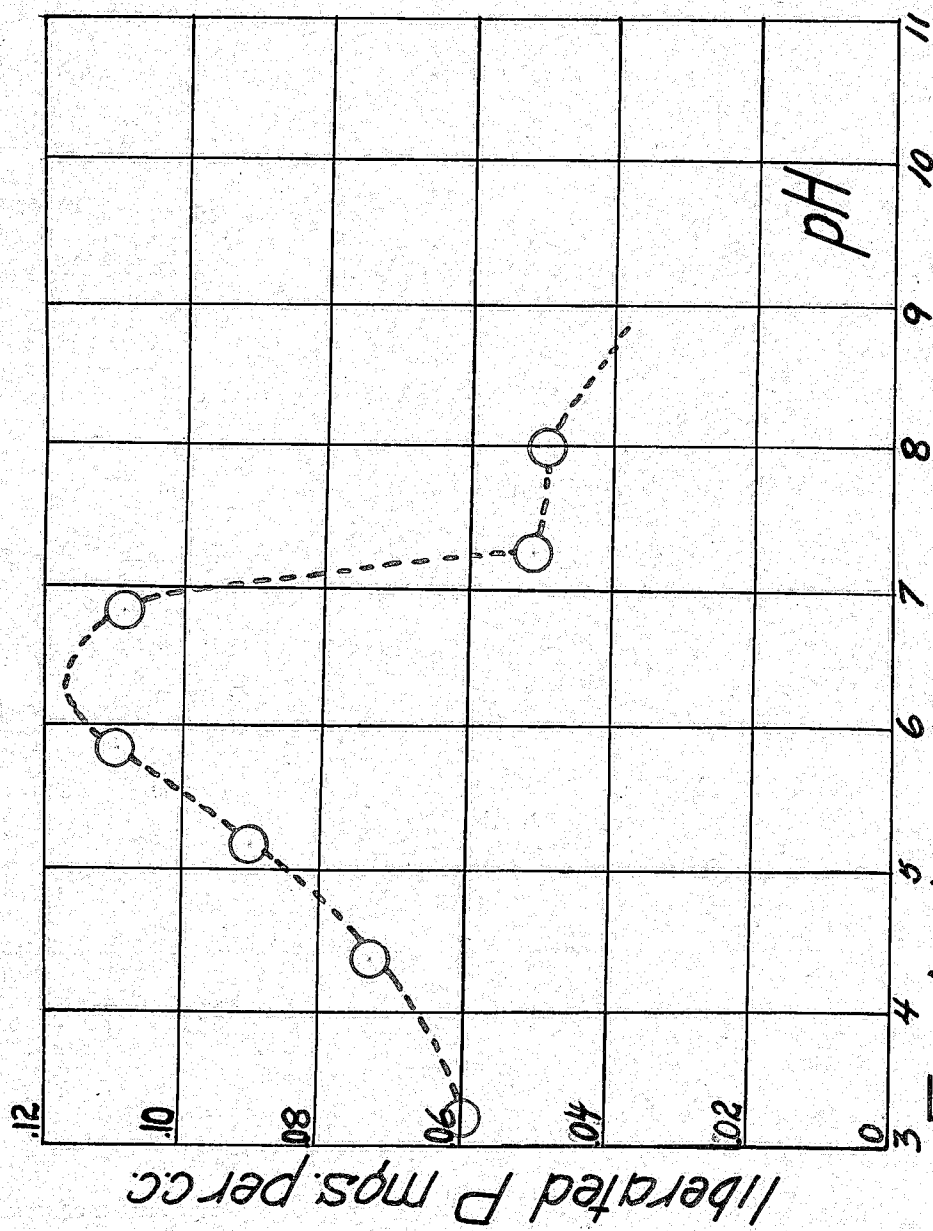Buffer: Phthalate   Temperature: 26°C.

Fig. 4: Activity-pH Series

Buffer: veronal; conditions otherwise as in Fig. 3

liberated P mgs. per c.c.

pH

was split from phosphoric acid esters, a substance with activity optima

at definite pH and temperature zones and activated by magnesium chloride,

we believed to be a true phosphatase. (Its determination as such,

however, was not quite complete--discussion, page   ).

The following tables IX-XIV illustrate the qualitative distribution

of phosphatases through the development of normal and phosphorus deficient

barley from seed to heading. While a few short series not given, were

run at other stages of development the date is sufficient to indicate the

distribution of the enzyme, in a gross way at least, throughout growth

and in all parts of the plant. Table XII summarizes this data. Many

omissions are obvious, particularly in regard to the pyrophosphatases.

TABLE IX

Extract Source: A - germinating seed.
                   B-- dormant seed.

Buffer: Potassium acid phthalate.   Time: 1 hour.

Substrate: Sodium pyrophosphate .06 M.   Temp.: 26° C.

TUBE NO.	EXTRACT SOURCE	pH	TOTAL P MGS. PER C.C.	FREE P MGS. PER C.C.	STANDARDS		LIBERATED P	
					READING	MGS. P PER C.C.	READING	MGS. PER C.C.
1	A	5.0	.37	--	20	.1	33	.06
2	A	5.4	.37	--	20	.1	25	.08
3	A	6.1	.37	trace	20	.1	18	.11
4	A	6.7	.37	--	20	.1	18	.11
5	A	7.2	.37	trace	20	.1	28	.07
6	-	5.4	.37	--	20	.1	--	--
1	B	6.1	.37	+	20	.1	35	.055
2	B	6.7	.37	+	20	.1	35	.06
3	B	5.4	.37	+	20	.1	39	.05
4	-	5.4	.37	-	20	.1		

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TABLE X

<u>Extract Source</u>: A - germinating seed--6 mgs. per tube.
  B - dormant seed--6 mgs. per tube.

<u>Substrate</u>: 2% NA glycerophosphate. T_{ime}: 1 hour.

<u>Buffer</u>: Potassium Acid Phthalate. T_{emp}.: 26° C.

SUBSTRATE	ENZ-YME	pH	TOTAL P MGS. PER C.C	INITIAL FREE P	STANDARD		LIBERATED P	
					READING	MGS. P PER C.C.	READING	MGS. PER C.C.
Sodium glycero-phosphate	+	5.5	.44	---	20	.1	7	.29
" "	+	5.9	.44	---	20	.1	10	.20
Calcium hexose-diphosphate	+	5.6	.32	.25?	20	.1	6?	.08?
" "	+	5.9	.32	.25?	20	.1	6?	.08?
Sodium hexose-diphosphate	+	5.3	.34	.05	20	.1	9	.17
" "	+	5.7	.34	.05	20	.1	9	.17
Sodium hexose-mononophosphate (Pasternak)	+	5.6	.22	.04	20	.1	20	.10
" "	+	5.9	.22	.04	20	.1	20	.10
Sodium hexose-diphosphate		5.6	.34	.05	20	.1	9	.18
" "		6.0	.34	.05	20	.1	12	.11
Calcium hexose-diphosphate		5.5	.32	.25?	20	.1	6?	.08
" "		5.9	.32	.25?	20	.1	6.5?	.06
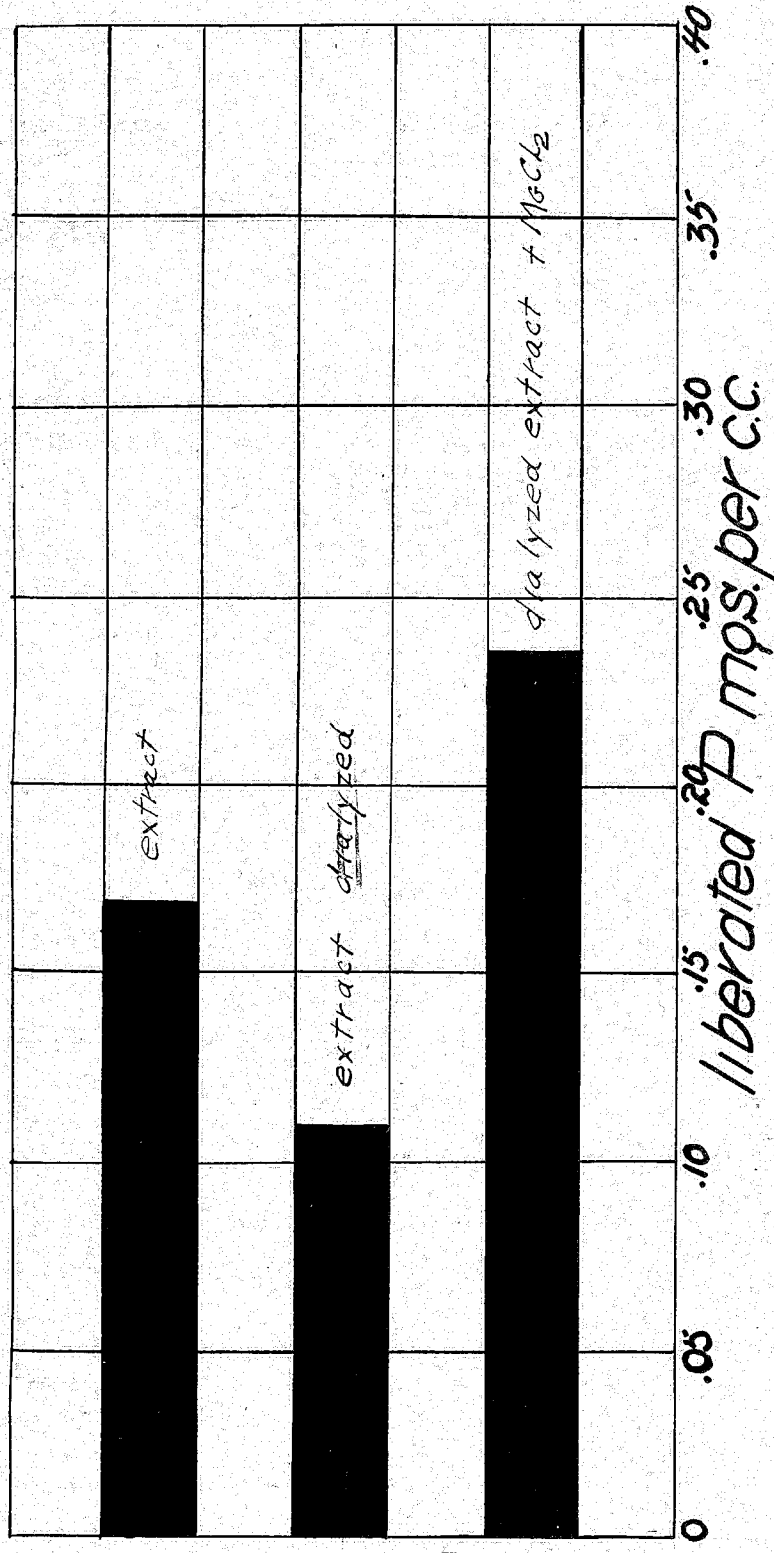
Fig. 5 Magnesium Activation Series

liberated P mgs. per c.c.

Extract Source: germinating seed Temperature: 26°C
Substrate: 2% sodium glycerophosphate Time: 1 hr.

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TABLE XI

<u>Extract Source:</u> A - Roots of Normal Plants--6 mgs. per tube.
B - Tops of Normal Plants--6 mgs. per tube.

<u>Substrate:</u> Sodium glycerophosphate. <u>Time:</u> 1 hour.

<u>Buffer:</u> Potassium acid phthalate. <u>Temp.:</u> 26° C.

TUBE NO.	EXTRACT	pH	TOTAL P MGS. PER C.C.	INITIAL FREE P MGS. PER C.C.	STANDARD		FINAL P	
					READING	MGS. P PER C.C.	READING	MGS. P PER C.C. LIBERATED
1	A	5.0	.44	--	20	.1	25	.08
2	A	5.3	.44	--	20	.1	22	.09
3	A	5.8	.44	--	20	.1	12.5	.16
4	A	6.5	.44	--	20	.1	13	.15
5	A	7.0	.44	--	20	.1	14	.14
6	-	5.8	.44	--	20	.1	--	---
1	B	5.0	.44	--	20	.1	15	.13
2	B	5.3	.44	--	20	.1	13	.15
3	B	5.8	.44	--	20	.1	20	.10
4	B	6.5	.44	--	20	.1	20	.10
5	B	7.0	.44	--	20	.1	35	.06
6	B	5.8	.44	--	20	.1	--	---

TABLE XII

Buffer: Potassium Acid Phthalate.

Temp.: 26° C.   Time: 1 hour.

EXTRACT SOURCE	SUBSTRASE	OPT. pH	TOTAL P PER C.C. MG.	INITIAL FREE P	P LIB-ERATED MG.	% OF ESTER HYDRO-LYZED
Dormant Seed.	2%Sodium glycero-phosphate	5.5-6.0	.44	--	.18	40%
	2% Sodium hexose-diphosphate	5.5-6.0	.34	--	.18	53%
Dormant Seed.	2%Calcium hexose-diphosphate	5.5-6.0	.32	--	.08	25%
	.06M Sodium pyro-phosphate	6.0-6.5	.37	--	.10	27%
Germi-nating Seed.	2% Sodium glycero-phosphate	5.5-6.0	.44	--	.29	66%
	2% Sodium hexose-diphosphate	5.5-6.0	.34	--	.22	65%
	2% Calcium hexose-diphosphate	5.5-6.0	.32	--	.08	25%
	2% Robison ester	5.5	.22	--	.10	45%
	.06M Sodium pyro-phosphate	6.0-6.5	.37	--	.11	30%
Roots of Phos-phorus Defic-ient Plants	2% Sodium glycero-phosphate	6.0	.44	--	.08	18%
	2% Calcium hexose-diphosphate	6.0-6.5	.34	--	.05	14%
	.06M Sodium pyro-phosphate	6.5	.37	--	.07	18%

(Table XII Continued)

TABLE XII (Continued)

EXTRACT SOURCE	SUBSTRASE	OPT. pH	TOTAL P PER C.C. MG.	INITIAL FREE P	P LIBERATED MG.	% OF ESTER HYDRO-LYZED
Roots of Normal Plants	2% Sodium glycero-phosphate	6.0	.44	--	.16	36%
	2% Calcium hexose-diphosphate	6.5	.32	--	.08	25%
	.06M Sodium pyro-phosphate	6.5	.37	--	.11	30%
Tops of Phosphorus Deficient plants	2% Sodium glycero-phosphate	5.5	.44	--	.07	15%
Tops of Normal Plants	2% Sodium glycero-phosphate	5.7	.44	trace	.15	34%

TABLE XIII

Tissue: A - Normal Roots--15 c.c.   Time: 1 1/2 hours.

        B - Normal Roots inactivated--15 c.c.

                                   Temp.: 30° C.

TUBE NO.	pH	TIS-SUE	SUBSTRATE 10 C.C.	STANDARD		INITIAL FREE P		LIBERATED P	
				READING	MGS.P PER C.C.	READING	MGS. PER C.C.	READING	MGS. PER C.C.
1	6.7	A	10% Glucose	20	.1	13	.15	20	.00
2	6.7	A	4% Sodium glycero-phosphate	20	.1	11	.18	12	.08
3	6.5	B	10% Glucose	20	.1	30	.07	22	.00
4	6.5	B	4% Sodium glycero-phosphate	20	.1	30	.07	21	.00

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Maccerated tissues where inorganic phosphorus was not too high nad to which substrate was added, gave reactions similar to those given by extracts. The use of tissues introduces more unknown factors and their only advantage is in speed of preparation. In mammals where, usually, highly specialized parts are more definitely set apart, tissue reactions have proved of value (Bodansky (9)). For what they are worth then the data in the Tables XIV-XVII are presented:

TABLE XIV

Tissues: A - roots of Normal plants.
B - roots of Normal plants previously heated to 100° C. for
C - roots of phosphorus deficient plants.   (3 minutes.
D - roots of phosphorus deficient plants previously heated to
100° C. for 3 minutes.

Substrate: 2% Sodium glycerophosphate.

Time: 1 1/2 hours.   Temperature: 30° C.

TUBE NO.	pH	TISSUES 10 C.C.	SUBSTRATE 2 C.C.	INITIAL P		STANDARDS		LIBERATED P	
				READING	MGS. PER C.C.	READING	MGS. PER C.C.	READING	MGS. PER C.C.
1	6.8	A	+	20	.1	20	.1	11	.08
2	6.6	B	+	40	.05	20	.1	40	.00
3	6.0	C	+	.00	.---	20	.1	22	.09
4	6.0	D	+	.00	---	20	.1	trace	---
5	6.8	A	---	.20	.1	20	.1	24	some

TABLE XV

Tissue: A - active root tissue of Normal plants.
          B - inactivated root tissue of normal plants.
          C - active root tissue of P deficient plants.
          D - inactivated root tissue of normal plants.

Substrate: 2% Sodium glycerophosphate.

Time: 1 hour.   Temperature: 30° C.

TUBE NO.	pH	TISSUE 10 C.C.	SUBSTRATE 2 C.C.	STANDARDS		INITIAL P		LIBERATED P	
				READING	MGS.P PER C.C.	READING	MGS. PER C.C.	READING	MGS. PER C.C.
1	6.8	A	+	20	.1	20	.1	11	.08
2	6.6	B	+	20	.1	40	.05	40	.00
3	6.0	C	+	20	.1	00	.--	22	.09
4	6.0	D	+	20	.1	00	--	trace	00
5	6.8	A	+	20	.1	20	.1	22	00

TABLE XVI

Tissues: Substrate: 2% Sodium glycerophosphate.
A - P deficient; tops active.
B - P deficient tops; inactivated by boiling.   Time: 1 1/2 hours.
C - Normal plants; tops active.
D - Normal plants; tops inactivated.   Temp.: 26° C.

TUBE NO.	pH	15 C.C. TISSUE	SUBSTRATE 4 C.C.	TOTAL P PER C.C. MG.	INITIAL FREE P	STANDARDS		LIBERATED P	
						READING	MGS.P PER C.C.	READING	MGS. PER C.C.
1	5.6	A	+		trace	20	.1	25	.08
2	5.4	B	+		trace	20	.1	40	.05
3	5.4	C	+	10	.20	20	.1	7	.07
4	5.5	D	+	10	.20	20	.1	18	.19
5	5.5	A	aq.dist.		trace	20	.1	--	.02

TABLE XVII

Tissue: A - normal barley kernel--15 c.c.   Time: 1 1/2 hours.
        B - P deficient barley kernel--15 c.c.
Buffer: Phthalate.   Temp.: 30° C.

TUBE NO.	TISSUE	SUBSTRATE 2C.C.	STANDARDS READING	MGS.P PER C.C.	INITIAL FREE P MGS. PER C.C.	LIBERATED P MGS. PER C.C.
1	A	Sodium glycero-phosphate 2%	20	.1	.05	.48
2	A	Sodium pyro-phosphate .06M	20	.1	.05	.39
3	B	None	20	.1	.05	trace
4	B	Sodium glycero-phosphate	20	.1	traces	little

Several points are worth noting. Free phosphate, for an example, did not increase markedly until the ester substrate had been added. Again heating tissues to temperatures of 60° and over caused a decrease in phosphate. Just at what temperatures this process occurred were not determined. In view of the doubt cast on the occurence inorganisms of a synthesizing "phosphatase," should this process be a constant thing in tissues its further investigation might be warranted.

Several attempts were made by addition of glucose and phosphate to extracts to obtain an enzymic synthesis of phosphoric acid esters but they were not successful. The only encouraging results we obtained in this direction were with green leaf tissues containing considerable free

esters phosphate or to which dextrose and phosphate had been added
artificially at a temperature of 60° C. or over. Such conditions led to
a disappearance of free phosphate. The nature of the reaction was not
determined. Tables XIV-XVII.

DISCUSSION

Different preparations from the same part of the barley plant, using the same procedure, gave extracts of satisfactory but not uniform activity. More active extracts might be prepared using precipitating agents such as acetone or ammonium sulphate or using sodium chloride solution, rather than water in extraction. Lohmann $(^{50}_{c})$ has prepared highly pure extracts of phosphatases using absorption methods. Harden and Macfarlane (36) have found the mechanism supplying inorganic phosphate in yeast to be highly sensitive to grinding and usually nearly destroyed while the zymase and co-enzyme system passed with at least 1/2 of its original activity.

Sensitivity to grinding may have been an explanation for the lack of uniformity in different extracts. It was noted too that activity varied with the length of time taken by precipitates to fluctuate in the MeOH.

We found Sorenson's glycine and potassium acid phthalate and Michaelis' veronal (Sodium diethylbarbiturate) buffers to be suitable for our use.

Of our substrates sodium glycerophosphate and sodium pyrophosphate were the only "C.P." products. The others were of untested purity.

Davies (24) and other investigators have found the King (46) colorimetric determination of inorganic phosphorus to be a suitable method in phosphatase investigations. Unless some critical comparison of current colorimetric methods is undertaken, and, unless it is shown to be at fault its use, because of its convenience, will be wide.

In several cases our enzyme extract hydrolyzed its own weight of substrate in one hour. With phosphatases, never with a high active content in extract, this is satisfactory.

From our tests we concluded that the barley plant possessed true phosphatase systems liberating phosphoric acid from ester substrates. The catalyzer we showed to be thermally unstable above 65° C. with optimal reactions about 26°-37° C. pH activity curves were determined showing broad optimal zones. While optimal activity was always in acid media the possibility of alkaline phosphatases is not precluded for no enzyme extractions were made in alkaline media. Precipitating by usual protein precipitants indicated the protein and colloidal nature of the extract. In common with other phosphatase extracts ours was sensitive to maceration and activated by magnesium ions.

One point in proof of the identity of our enzymes is lacking. The hexose unit in the sugar phosphates might be attacked glucolytically to yield lactic acid and free phosphate. By following the products of the reaction quantitatively, the proof would be complete. In another manner a similar end could be achieved. Glucolysis occurs only in the presence of a dialyzable thermostable co-enzyme. If following dialysis phosphoric acid esters were cleaved glucolysis as a possible factor would be removed.

Demonstration of specific phosphatases in our extracts is lacking. Lohmann (57) by differential absorption was able to separate pyro-phosphatase and sugar phosphatases in muscle. Levene by reactions in point of time was able to establish the identity of three separate phosphatases acting on phospholipides. Hotta (38) believing the

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specificity of phosphatases was determined not alone by the nature of the bond uniting the phosphoric acid but also by the nature of the alcoholic residue divided phosphatases into (a) phosphodiesterases (b) phosphomonoesterases (c) pyrophosphatases (d) amidophosphatases. The presence of a metaphosphatase in corn has been suggested by Menjdahl and Weissflag (⁊). Waldschmidt-Leitz (⁊⁊) upholds the determination of Levine and Lohmann only.

The distribution of phosphatases has been shown to be very wide and their functions are just becoming known. In mammals and invertebrates (Roche) different phosphatases are found in many organs and tissues, muscle, bone, kidney, blood, brain, intestinal mucose, and tumours. In yeasts and Aspergillus oryzeae, the source of takadiastase, phosphatases hydrolyzing many substrates are found. Phosphatases are found in rice and jackbean. From our tests it would seem that in barley, also, their distribution is wide, and are to be found in roots, tops and seeds and at all stages in development.

CONCLUSION

The results of our work must be regarded as essentially qualitative. Because of the very considerable variations in conditions of extraction and reaction quantitative results cannot be deduced from the data. Many refinements in technique are obviously necessary for further work, We do, however, submit the following conclusions:

  1. The barley plant possesses true phosphatase systems, some of which, at least, are activated by magnesium ions.
  2. Phosphatases are widely distributed throughout the developmental cycle from seed to seed and occur in phosphorus deficient as well as in normal plant, though possibly less active in the former.

DISCUSSION

.1. INTRODUCTION

In view of the rapidly increasing numbers of researches on phosphatases and their related metabolic exchanges, recent pertinent literature is difficult to appraise. The following discussion is, as a consequence, very limited both in general outlook and in the number of papers reviewed.

"Enzymes may be defined as definite material catalyzers of organic nature with specific powers of reaction formed indeed by living cells, but independent of the presence of the latter in their operation." (Waldschmidt-Leitz) With some important differences they are much in keeping with Ostwald's (ω) definition of a catalyst. (a) their action consists of accelerating reactions probably already in progress and which are, theoretically reversible (Van't Hoff.) (b) the enzyme is not a product, is required in small amounts only, and does not act in molecular quantities. (c) in a general way the rate of reaction depends on the concentration of the enzyme. (d) most enzyme reactions are hydrolytic. Unlike most catalysts of the usual type (a) their action is specific. (b) some particular optimum pH and temperature is required for their action. (c) enzymes are usually slowly destroyed in the process of reaction, and optimum conditions are those in which the enzyme reacts most quickly. (d) enzymes are readily subject to inactivation or acceleration by concomitant substances. (e) while the primary function of enzymes relates essentially to energy transfer the accompanying heat effect is slight.

Although at the present time differences of opinion as to the mode of

-2-

enzyme action exist only in details as Waldschmidt-Leitz states
the foundations for another important phase of the above definitions
are much less assured, namely in regard to the assumption of a definite
material nature for these catalyzers.

Crystalline enzyme preparations have been prepared (Summers' urease
(56), Northrop's pepsin (68).) Levene and Halberger (47), however, have
concluded that the chief components of the crystals are extraneous
proteins. Waldschmidt-Leitz and Purr (87) have separated "crystalline"
trypsin into four by special adsorption agents.

The prevailing concept of the material nature of enzymes is that of
the Wilstatter school. This school considers that enzymes are composed
of a colloidal bearer and a specific active group which enables them to
be bound to the substrate and the composition of which at the same time
conditions the colloidal nature of the entire complex.

Evidence in support of the Wilstatter theory has been gained
chiefly from well known and verified absorption experiments where enzymes
in a high state of purity have been obtained through use of absorbents
such as kaolin and alumina. In support of this theory that enzymes are
colloidal in character with an active specific group are the recent
observations of Bredig and Gerstner (13) wherein a diphenylamine group
added to cotton made a catyst splitting carbonic acid from B-heto-
carbonic acid, and those of Langenbeck (40) on activating groups within
the active enzyme proper. Fischgold's and Ammon's (94) theory of
esterase activity through displacement of H_2O on the enzyme surface
depends on the Wilstatter theory.

.2. ENZYMIC KINETICS

Enzymic reactions while essentially catalytic do not necessarily follow the laws of chemical dynamics as formulated for catalysts of the usual sort. The complicated and imperfectly understood composition of enzymic reaction systems together with our ignorance of the chemical nature of the effective catalyzers and the influence of adventitious impurities upon their affinity often results in preventing a classification of even the simpler transformations along basic principles. The validity of older kinetic-reaction research applying the mass low in one form or another is in many cases very limited because of the many unknown factors disregarded. Waldschmidt-Leitz ( ) has reviewed examples best supported by experimental evidence.

Metabolic exchange systems in which phosphatases are involved are complex and new factors and relationships are being discovered from time to time and for this reason the kinetics of phosphatase system have not been worked upon to any extent.

The kinetics of glycerophosphate cleavage by kidney phosphatase has been examined in detail by Jacobsen (40). The determining factors he found to be the liberation of free phosphate and the concentration of the substrate. The usual curves indicating formation of substrate enzyme complex through induction periods were given.

Martland and Robison (53) studied the course of sodium glycerophosphate hydrolysis by bone phosphatase in the absence and presence of inorganic phosphate. They found that even without the addition of phosphates the velocity constant calculated according to the equation for unimolecular reactions decreased more rapidly than could be

accounted for by inactivation of the enzyme; they advanced the hypothesis

that a resynthesis between phosphoric acid and glyceral was occurring.

They demonstrated the influence of phosphate in the reaction, but were

unable to demonstrate the possibility of a resynthesis working with

glyceral and phosphate in consentration employed in previous hydrolysis.

Erdtman (29) using kidney extract from hogs found the velocity

constant in absence of activating agents to decrease rapidly. He

attributed the fact at least in part to enzyme destruction and to in-

activiting products of hydrolysis.

Belfanti, Contardi and Ercoli (6) found a similar rapid decrease

in the velocity constant and believed it to be in agreement with the

Schutz Rule, which implies the reaction velocity was not only

proportional to the substrate concentration but inversely to the

quantity already transformed. These workers postulate that in addition

to an enzyme-substrate complex there is an enzyme-phosphate complex

capable of hydrolyzing action.

The dependence of the activity of most enzymes upon pH was first

brought out by Sorenson (1909) and Michaelis (1909). A characteristic

optumal reaction region is generally apparent and the activity pH ratios

can be expressed in pH activity curves. Many factors as has been

previously pointed out disturb or distort the measurement. In this regard

concomitant substances such as buffers, activators, inhibitors are

important as well as, in addition, the stability of the enzyme itself.

at a given pH.

The theoretical significance attached to activity--pH curves for

enzymes themselves are to be regarded as amphoteric electrolytes,

which according to the pH action varying capacities on anions, kations

a undissociated niclecules. At first, Michaelis implied that the undis-

sociated material was to be considered as the catalytically active

portion.

These results, however, have been strongly questioned by many

workers including Michaelis himself. Kuhn (88) finds that the H ions

themselves influence the decomposition velocity of the enzyme substrate

combination. Northrop (05) working with proteolytic enzymes found the

dissociation of the substrate to be influenced by the H ions; his data

showed activity-pH curves to be coincident with dissociation curves of

the substrate.

Michaelis (59) also used the behaviour of enzymes in an electrical

field and their absorption affinities to explain their electro-chemical

nature. In acid medium in an electrical field he showed the amphoteric

enzyme particles moved to the cathode, in alkaline medium to the anode,

and in the intermediate region corresponding to their isoelective point

no migration at all. His work on electro-chemical absorption i.e.

on the enrichment of dissolved materials on the surface of solid

bodies, has been fundemental in absorption methods of enzyme purification

so extensively used by the Wilstattler school.

In principle every reaction is reversible (Nernst) and according

to the mass law the concentration of reacting substances determines

not only the velocity but also the direction of the reversible process.

Theoretically these enzymes may catalyze reactions not only in the

direction of hydrolysis but also in the direction of synthesis.

Numerous examples of synthetic action by enzymes are known (Bayliss (4)

on enzymic syntheses.)

The recognition of the synthesizing action of enzymes has led to the question as to whether or not enzyme equilibrium is to be regarded as identical with that effected by other catalysts. "According to the laws of thermodynamics it was expected that this would be so, if the enzyme were incapable of binding an essential portion of the reacting components. The results obtained with enzymic equilibria have shown that this requirement is not fulfilled in all enzymic reactions even though the point of the enzymic equilibrium cannot be definitely determined. According to Euler ($\mathcal{PP}$) the equilibrium position of enzymic reaction is not determined as an ideal catalysis by the concentration of the substances concerned but by the relation of their affinities to the enzyme itself. Bayliss ($\mathcal{4}$) emphasizes the non-necessity of assuming special synthesizing enzymes and that enzymes must accelerate both hydrolytic and synthesizing aspects unless they carry the reaction to completion under the conditions present.

Several references in the literature are found on enzymic syntheses of phosphoric acid esters. Martland and Robison (53) have explained abnormal drops in velocity constant during hydrolyses to resymthesis of glycerol and H_3PO_4. Nagai (65) has found the ability of kidney and liver of pigeons to esterify H_3PO_4 to be 14-78% greater in a condition of B-avitaminosis and attributes this to acceleration of a phosphatase Hemini (N) and Tsukitari (37) were unable to demonstrate the presence of a phosphatase in Rhizopus (Mucor) tritici, a fungus grown under a variety of conditions.

Rumstrom, Linnesstrand and Borii ( ) obtained esterification of

H_3PO_4 in haemolyzed blood in presence of co-zymase. Pett and Wynne (72)

were unable to demonstrate synthesis of the esters by bacteria.

According to Waldschmidt-Leitz (70) the differentiation of a separate

phosphatese is not to be accepted. Rather it seems attention should

be paid to the conditions necessary for synthesis on the part of

phosphatases--a complex of conditions probably, involving the presence

or absence of definite activators, inhibitors and accompanying reactions.

Lohmann (51) found phosphorylation of glycogen in muscle to occur only

in the presence of a complete co-ferment system (adenylpyrophosphate M_G).

.3. ACTIVATION AND INHIBITION

In addition to the influence of hydrogen and hydroxyl ions,

substrates, etc., activation of enzymes by specific activators,

inhibition by inhibitors of a specific nature can be demonstrated.

Waldschmidt-Leitz (88) classes these in four ways.

(1) Activation by Inorganic Ions: e.g. the stability of enzyme-

substrate complex appears, for example, using saccharan, to decrease

in the following sequence in the presence of these ions - NO_3^-, Cl^-,

Bi^-, SO_4^-. The mode of activation is not clear.

(2) Specific Activation is characterized by the fact that it is

incapable of expressing itself except with respect to definite

particular affinities of an enzyme or else with a definite stage of the

reaction which it accelerates. One of the best demonstrated examples is

the activation of pancreatic trypsin by enterokinase from the

intestinal mucous. Waldschmidt-Leitz (88) believes there is formed in

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this case a compound of enzyme and activation. Co-zymase, dialyzable,

and thermotable is an activator of this class (Harden and Young (34) .)

Myrbach (63) and Myrbach and Euler (64) believe co-zymase to be a nucleotide

closely related to adenylic acid and capable of being hydrolyzed by

phosphatases. Anti-enzymes, about which there is little known, might

be recognized as attributible to specific activators.

(3) Non-specific activation and Inhibition: It has been shown by

Wilstattier, Waldschmidt and Memmen (98) that activation phenomena in

solutions of lipase are based merely on specially favorable conditions

for the contact of water soluble enzyme with its insoluble substrate.

The activating effect of gall salts, etc. is due to the production of

colloid particles which exert and absorbent action with respect to

enzyme and substrate thereby facilitating the reaction.

(4)   Toxic Influence of Heavy Metal Salts is common to all enzymes to

a greater or lesser degree. Euler and co-workers (88) have demonstrated

the "poisoning" of saccharase to be accompanied by a corresponding

decrease in heavy metal ions and further that activity could be

renewed by addition of H_2S to precipitate the metals.   The effect is

apparently upon dissociation of enzyme-substrate complex.

(5)   Inhibition and Activation of Enzymes by Definite Salts on Organic

Addition Substances: Many reports on this class of enzyme activation

and inhibition occur in the literature. Only a few of these, pertinent

to phosphatases will be mentioned.

(a) Magnesium Activation of Phosphatases: Erdtman (22), using

kidney extracts, first reported magnesium activation of phosphatase;

later work found it to be an activator of phosphatases generally.

Lohmann (50) found that hexosediphosphate loses no phosphate when added to dialyzed muscle extracts; when magnesium ions are present one molecule is lost, thereby becoming Embden ester, and equilebrium mixture of aldo-hexose with a small amount of keto-hexose. Embden ester is then dephosphorylated in the presence of adenylpyrophosphate. Further the enzymic change of morganic pyrophosphate to orthophosphate is, according to Lohmann, carried out only in the presence of magnesium.

(b) Activation of Phosphatases by Arsenate: Pett and Wynne (72) investigating the oft reported influence of arsenate and arsenite on the enzymatic breakdown of phosphoric esters could find no activity increase for phosphatase in general, but did find activation by arsenate in special cases. Wherever arsenate activation occurred arsenate less rapidly gave a similar result. Harden and Young (34) showed arsenate and arsenite ions accelerated the liberation of inorganic phosphorus from hexosediphosphate by yeasts. Meyerof (56) working with heart and muscle extracts, Macfarlane (52) and Harden (35) with yeast believed the effect to be exercised on the glycolytic and not the phosphatase system. Braunstein and Lewitow (7) observed progressive diminution of inorganic arsenate in a mixture of yeast, sugar, arsenate, toluene and aq. dist. and suggested the formation of labile hexosearsenates.

(c) Activation and Inhibition by Potassium Cyanide: Warburg (88) concluded hydrogen cyanide in the presence of phosphate showed down alcoholic fermentation by yeasts and suggested it affected chiefly the mechanism for liberation of phosphate from hexosediphosphate. Miss Patterson (71) has in some detail investigated the effects of KCN and HCN on alcoholic fermentations generally but has not as yet determined the

systems upon which they might act.

(d) Influence of Oxalates: Several workers mention the effects of oxalates on phosphatase systems, noting chiefly the inhibiting action. Balfanti, Ercoli and Contardi (6) found that when extracts of liver, kidney or bone phosphatase are allowed to act on glycerophosphate in alkaline medium with oxalate, the hydrolysis at first proceeds slowly then little by little accelerates and finally proceeds as if the oxalate were no longer present until the hydrolysis of the substrate is complete. In the presence of phosphate, added artificially, sodium oxalate does not manifest andy inhibitory action. It was suggested that the explanation of these phenomena was that the inorganic phosphate set free from glycerophosphate gradually displaces the oxalate ions from the inactive enzyme--oxalate complex giving rise to an active enzyme--phosphate complex capable like the free enzyme of uniting with substrate and hydrolyzing it. Bodansky (7) and others mention the inhibitory effects of oxalates on phosphatase.

(e) Fluoride: Fluoride has been known as an activator of lipase and several other enzymes for some time and recently phosphatase has been added to this list. Loevenhardt and Pierce (-), Smith and Lantz (8), Auhagen and Grzycki (/) found kidney phosphatase to be unaffected by sodium fluoride; found yeast phosphatase to be highly sensitive, and takaphosphatase from Aspergillus oryzeae less so.

(f) Sulfhydryl groups: Phosphatases generally are inhibited by sulfhydryl groups at their pH optimum. Schaffner and Bauer (//) . Cysteine inhibits yeast phosphatase most at pH 6.1 and at pH 8.8 with kidney phosphatase. Dislysis renders the enzyme more susceptible.

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Reactivation with iodoacetic occurred with kidney phosphatase. Mowatt
and Stewart ( ) find on the other hand that iodoacetic acid prevents
gluolysis, and destroys glutathione--the activator of glyoxalase.

.4. Methods of Determining Orthophosphate Acid.

Jack (76) has at some length discussed the various methods used
in the determination of micro quantities of orthophosphoric acid and
their mention will suffice here. Bell and Doisy (7), Briggs ( ),
Fiske and Subarrow (31), Martland and Robison (53) and King (45) have
proposed colorimetric methods. Kirk (46) proposes a very convenient
gasometric method. A micro-gravimetric method has been suggested by
Plimmer (75). Titrametric methods have been put forward by Neumann (75) 1902
and Monasteric (60). Davies and Davies (23), Hinsberg (39) and Pett (73)
have examined the nature of interference by extraneous substances to
reaction in the colorimetric methods and have suggested modifications.
Valuable service would be rendered in a critical comparison of these
various micro-methods. Hinsberg (39) has done something towards this end.

Emmert (28) and Litynski (47) has put forward colorimetric methods
suitable for use with vegetable tissue.

Total phosphorus and organic P, by first combusting materials,
may be determined by the above colorimetric methods. Elek and Hill
(24), Fulcher (-), Garelli (-) have proposed estimation of organic P
by first combusting materials in a micro-bomb.

Lohmann (50), Boyland (11) and Hinsberg (39) give micro-methods for
determination of pyrophosphoric acid.

Menjdahl (54) for metaphosphoric acid and for phosphorores and hypo-
phosphorores acids.

Microdeterminations, gravimetric and colorimetric, are given
through the literature on phosphatases. Harden (34), Pryde (63), Raymond
and Levene (76); for determination of hexosemonophosphoric acid see
Cori and Cori (21).

Determination of small quantities of phosphatides may be made by
methods of Bloor and Snider (83), Backlin (-), Jewett (63), Norberg and
Leavill (77), Jordan and Chibnall (50), Levene and associates (49).

Hexoses may be determined by the Hagedvorn and Jensen (34) method
or by Hanes (32) modification of it. Several colorimetric methods for lactic
acid determinations are available.

.5. THE ROLE OF PHOSPHATASES IN METABOLISM

The role of phosphatases in metabolism is a multiple one as might
be gathered from its wide occurrence in organisms. The chief current
investigations on the part they play in metabolic exchanges may be
resolved as they concern (a) yeast fermentation (b) the chemistry of
muscle (c) the chemistry of ossification (d) the work of the kidney.

(a) Their significance in yeast fermentation:

Harden and co-workers in a series of researches have been able to
show that the addition of soluble phosphate to a yeast juice, hexose
fermentation produces an equivalent amount of carbon dioxide and alcohol
quantitatively expressed in the equation, thus:

I. $2 C_6H_{12}O_6 + 2 R_2HPO_4 \longrightarrow 2 CO_2 + 2 C_2H_5OH + 2 H_2O + C_6H_{10}O_4 (PO_4R_2)_2.$
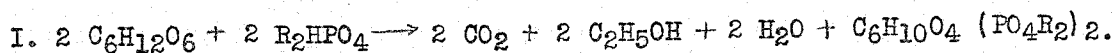
It has been shown that the phosphate is indispensable to the process

and that at least three stages occur in the process.

(1) A period coincident with the increased fermentation during which

free phosphate rapidly diminishes. (2) A period of uniform activity

where only small amounts of free phosphate occur. (3) A period of

lessened activity and rapid increase of free phosphate. An enzymic

hydrolysis similar to the last stage is effected after removal of the

co-enzyme of zymase indicating the presence of a phosphatase, the action

of which might be represented as:

II. $C_6H_{10}O_4 (PO_4R_2)_2 + 2 H_2O \longrightarrow C_6H_{12}O_4 + 2 R_2HPO_4$
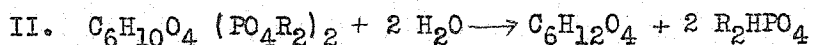
Harden ( ) gives the following simple explanation of the sequence of

events during fermentation: "The rapid diminution in the amounts of free

phosphorus during stage (1) corresponds with the occurrence of reaction I.

During the whole period of fermintation the enzymic hydrolysis of the

hexose proceeds according to equation II. Up to the end of stage (2)

the phosphate thus produced enters into the equation according to

equation II, with the sugar which is present in excess and is thus

reconverted into hexose phosphate, so that, as long as alcoholic

fermentation is proceeding freely no accumulation of free phosphate can

occur." As soon as alcoholic fermentation ceases, however, phosphate

accumulates, there being no hexose present with which it might react.

Harden has shown recently three types of fermentation in yeasts.

(a) the relatively slow fermentation, without addition of PO_4. (b)

the more rapid process by adding PO4. (c) rapid fermentation by addition

of Arsenate by either stimulating phosphatase activity or by the

formation of more labile hexosearsenates.

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The rate of hexose monophosphoric and pyrophosphoric acids in yeasts has largely to be worked out.

(b) Muscle Contration:

That phosphates play a part in muscle contraction is evidenced by the facts: (1) that free phosphate and lactic acid are liberated in equimolecular amounts. (2) that by adding phosphate all glycogen is converted to lactic acid. (3) that during muscular work ther is an increase in the secretion of phosphate into the urine. (4) that performance of muscular work is augmented by addition of phosphate.

In a long series of researched Embden, Meyerhof and their associates have established an intimate connection between the conversion of carbohydrates into lactic acid in the contracting muscle and the presence of phosphates in the latter. Experiments were made indicating that a hexosemonophosphate (the lactocidogen) is found as an intermediate compound in the breakdown of glycogen to lactic acid. The changes may be represented in two phases (a) the anaerobic and (b) aerobic.

anaerobic
(contactile)

$$5\ C_6H_{10}O_5 + 5\ H_2O + 4\ K_2HPO_4 \longrightarrow 4\ C_6H_{11}O_5(PO_4K_2)\ +\ C_6H_{12}O_6 + H_2O$$

glycogen.   hexosemonophosphate. glucose.

$$4\ C_6H_{11}O_5(PO_4K_2) \longrightarrow 8\ C_3H_6O_3 + 4\ K_2HPO_4 + C_6H_{12}O_6$$

lactic acid.   glucose.

aerobic
(recovery)

$$8\ C_3H_6O_3 + 4\ glucose + K_2HPO_4 + 6O_2 \longrightarrow 4\ C_6H_{11}O_5(PO_4K_2)\ +\ 6CO_2 + 10\ H_2O$$

The glucose freed during the contractile period is oxidized during the recovery period; then 1/3 of the glycogen is burned.

That there are enzymes in muscle capable of bringing about the changes outlined above has been demonstrated by Lohmann ( ), Meyerhof ( ), etc. They have shown it possible to separate completely the lactic acid producing enzyme free from muscle carbohydrate. A dialyzable thermostable co-enzyme has been discovered. The preparations split hexose under special conditions and readily act on starch and glycogen. In the complex is an enzyme splitting hexose mono-and di-phosphoric acids.

Embden and Zimmermann (27), Lohmann (50) have found in addition to lactocidogen in muscle other phosphorus containing compounds, adenylic acid, and pyrophosphoric acid occur. Eggleton and Eggleton (23) reported the presence of phosphagens. Lohmann (50) has shown a definite pyrophosphatase may act on adenylpyrophosphoric acid and pyrophosphoric acid, to convert them to the orthophosphoric form. Meyerhof and Lohmann (57) investigating phosphagens (unstable compounds of inoyanic phosphate and creative or orginine) have found muscle immersed in sodium salts of monacetic acid and fatiqued, contract without utilization of glycogen or formation of lactic acid; ammonia was produced, phosphagen decreased, phosphoric acid increased, creature phosphate $\longrightarrow$ creature $+ H_3PO_4$ creature $\longrightarrow NH_3 + x$.

$H_3PO_4 +$ hexose $\longrightarrow$ hexosephosphate $\longrightarrow H_3PO_4 +$ lactic acid.

lactic acid $\longrightarrow CO_2 + H_2O$.

Eggleton and Eggleton (ibid) demonstrated aerobic resynthesis of phosphogens. Nachmanschen (69) anaerobic resynthesis after relaxation.

(c) Chemistry of Ossification:

Marrowless, dry bone consists of some 60-70% of Ca.Mg. phosphates

and carbonates deposited in a protein matrip. Of these ~~mayamic~~ constituents calcium phosphate is most important and investigations have chiefly revolved around the problem of its deposition. Several theories have been advanced and considerable experimental work done on the problem. The chief of theories involve the presence of born phosphatases. (Tell and Robison (73) .)

       (d) Significance of Phosphatases in the Kidney:

Eichholtz, Robison and Bruell (73) have attached special significance to phosphatases of the kidney in regard to the excretion of phosphate in the urine and some of the most recent work on phosphatases is on kidney phosphatase.

Davies (24) has investigated phosphatases of the spleen.

Magai (65) and other Japanese workers are studying the importance of Phosphoric acid esters and their hydrolyzing and synthesizing enzymes in diet--in B-avitaminasis particularly.

               .6. PROBLEMS

From the study of work being done on mammal and yeast phosphatases and from our own brief investigation we are confronted with numerous questions concerning the rôle of these enzymes and their substrates in the higher plants.

The rôle of these enzymes in yeast fermentation and in muscular work has been closely linked with carbohydrate metabolism particularly with the utilization of carbohydrates in the production of free energy.

Might there not be a close relationship in the higher plants? With the

process of carbon assimilation it has been suggested that phosphorus

is not necessary. (   ). In the transformation and

synthesis of sugars apart from the photosynthetic process e.g. as

occurs in malting barley, in sugar beet storage, etc., phosphatases

might play a part. Indirect evidence in support of this suggestion is

the universal occurrence of traces of phosphate in starch of plant origin.

Willis, Reland and Gray (92) working with soyabean have determined

an intimate connection between Calcium, magnesium and phosphate, both

in absorption and processes within the plant.

Demonstration of natural substrates in the higher plants, on which

phosphatases might act is limited. Burkard and Neuberg (16) have shown

hexosediphosphate to be present in sugar beet leaves, and Cockefair (20)

in other plants; Menjdahl and Weissflag (55) have indicated the presence

of meta- and pyro-hosphates. Phosphagens have not been reported for

plants.

Phosphorus metabolism in general, and phosphorus absorption more

particularly in the higher plant is notably influenced by changes in

radiant energy. (Barton-Wright (97).) Might not phosphatases and their

substrates, directly or indirectly, be involved in this relationship?

Some enzymes are light sensitive. Are plant phosphatases similarly

constituted?

The study of phosphatases might have a directly practical value in

the malting of barley. Quality in malting barley is determined to a

great extent by the ability of the seed to hydrolyze its starch reserves

to yield sugars. Could an intimate relationship be established between

quality and phosphatase activity a ready test for malting quality might

be devised.

This brief speculation may at least indicate the variety and number of problems relating to plant phosphatases.

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