I. STUDIES ON THE ACID PHOSPHATASES OF GREEN LEAVES.

II. STUDIES ON THE ROLE OF INDOLEACETIC ACID IN CELL ELONGATION.

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ABSTRACT

The bulk protein of green leaves has been shown to be dissociable and probably distinct from the acid phosphatase previously associated with it. This conclusion is based on the differential sedimentation rates in the analytical ultracentrifuge of the bulk protein and of phosphatase, on the relative amount of enzyme present in the bulk protein prepared by salt precipitation or by differential centrifugation, and on the comparative stability of the two proteins toward heat and acid.

Leaf phosphatase is further shown to be a mixture of isodynamic enzymes with different pH optima and Michaelis constants. A separation was accomplished by adsorption, dialysis, and by antigen-antibody reactions.

The phosphatases are shown to lose enzymatic activity during dialysis, but to be capable of reactivation by certain metal ions. Copper ions are the most effective activators. Comparison of the spectrographic analysis of the ash of dialyzed phosphatase with enzymatic activity, however, disclosed a direct relation between activity and the amount of iron and manganese, as well as copper. Various comparative aspects of the enzymes were also studied.

The influence of auxin on metabolic pathways is studied by a new method, in which the rate of incorporation of isotopically labeled intermediates into varied components of living tissue is studied as a function of the auxin supplied.

It is found that auxin is without effect on the total protein amino nitrogen, or on the rate of incorporation of the C^{14} label from glycine or leucine into tissue proteins of Avena and corn

coleoptiles. Hence, auxin has no effect on protein metabolism during cell elongation.

Similarly, auxin exerted only small effects on the rate of incorporation of C^{14} from acetate into the lipid constituents of Avena coleoptiles. Added auxin induced no important changes in the incorporation of the C^{14} of acetate or sucrose into the cell wall components of Avena. It was shown, however, that absence of auxin favors incorporation of the isotopic label of acetate or sucrose into the pectates and soluble polyuronide hemicelluloses, while the presence of auxin favors incorporation into the non-cellulosic polysaccharides. These effects are of small magnitude in relation to gross increase in cell length.

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PART I

STUDIES ON THE ACID PHOSPHATASES OF GREEN LEAVES

I. The problem.

The study of phosphorus metabolism is rapidly expanding because of general interest in phosphate intermediates, and in the transfer of energy within biological systems by way of energy-rich phosphate bonds. The conversion of carbon dioxide to the multitudinous compounds present in green leaves takes place in part by way of phosphorylated substances (1), and both aerobic and anaerobic breakdown of carbohydrates occurs with the same or similar compounds. The formation of proteins may occur in part by means of phosphorylated amino acids (2), and lipid synthesis from acetate, for example, may be mediated in certain instances through acetyl phosphate.

Phosphatases are involved in this phase of metabolism because they are the enzymes which catalyze the breakdown of these phosphorylated substrates. Hence they are near the hub of the metabolic wheel, and play a vital part in both the synthetic and degradative activities of cells.

Although phosphatase activity was first discovered in plants (3), most of the later research has been done on animal phosphatases, and indeed on the so-called alkaline phosphatases to the neglect of the acid. So scanty is our knowledge of the phosphatases of green leaves that Schäffner (4) was able to summarize the matter in two short paragraphs in the compendium on enzymes edited by Bamann and

Myrbäck (5).

The acid phosphatases of green leaves will be characterized in more detail in this part of the thesis. Since phosphatases are in general lacking in rigid substrate specificity, and have for that reason been classified on the basis of their pH optima, it was of interest to consider the possibility that the acid phosphatase activity of green leaves may result from more than a single enzyme. Experiments were designed to test this idea by means of various fractionation schemes. The relation of the phosphatase to a bulk protein found in green leaves by Wildman and Bonner (6) is also investigated. This protein, known as Fraction I, has been found to be electrophoretically homogeneous in the Tiselius apparatus, and certain preparations of it have been shown to exhibit strong phosphatase activity on a variety of substrates. The preparation of the enzyme free of Fraction I, and the degree of stability of the two proteins toward heat and acid is here reported.

It was observed that phosphatase activity is decreased in cytoplasmic solutions prepared in the presence of certain chelating agents. This fact suggested the possibility that metals might be involved as cofactors of the acid phosphatases as they had been known to be for the alkaline enzymes. An examination was therefore made of the metal relationships of the leaf phosphatases.

Some comparative characteristics of the enzyme, including its action as a phosphotransferase have been included in the final section.

II. Review of the literature.

1) Occurrence of phosphatases. In spite of the hundreds of papers which have appeared on the subject of the phosphatases, no comprehensive account of their classification or properties has yet emerged. Phosphatases are almost ubiquitous in cells and extracellular fluids, but the amounts and types of specificity are subject to large variations. Ignatieff (7) for example, reports phosphatases present in all tissues of the bean plant, although very little occurs in the flowers. Folley and Kay (8) note that dog skin and the aorta of several species have been reported to lack the enzyme. In general, those tissues which are metabolically most active are rich in phosphatase. The enzyme apparently occurs both in a soluble and in a particulate form, depending upon the source.

2) Classification. A classification of the phosphatases which is still widely accepted is that of Folley and Kay (8) shown in Tables 1a and 1b. The phosphomonoesterases, diesterases, pyrophosphatases, phosphoamidases, and metaphosphatases are here distinguished. The best known group, the phosphomonoesterases, is further divided into four types, depending upon the pH optimum, the requirement for magnesium, and the relative rate of hydrolysis of alpha and beta glycerophosphate. More recent schemes differ in detail, but have nothing more fundamental to offer.

TABLE 1a

A classification of the phosphatases after Folley and Kay (8).

	Class of enzyme	Substrates hydrolyzed	Examples of substrates
A	Phosphomono-esterases	Monoesters of phosphoric acid, including nucleotides	Glycerophosphate, phenylphosphate, adenine nucleotide
B	Phosphodi-esterases	Diesters of orthophosphoric acid	Diglycerylphosphoric ester
C	Pyrophosphatases (probably 3 subclasses)	Salts and symmetrical diesters of pyrophosphoric acid	Sodium pyrophosphate, diphenyl pyrophosphate
D	Metaphosphatases	Salts of metaphosphoric acid	Sodium metaphosphate
E	Phosphoamidases	N substituted amido phosphoric acids $NHR.P:O(OH)_2$	Phosphocreatine

TABLE 1b

Phosphatases of class A.

Sub-class	Occurrence	pH opt. (approx.)	Effect of Mg at opt. pH	Relative speed of hydrolysis of α and β glycerophosphate
I	Kidney, bone intestine, lung, mammary gland, blood plasma, etc.	9-10	activates	β > α
II	rice bran, pancreas, spleen, liver, kidney, etc.	4.5-5	doesn't activate	β > α
III	takadiastase	3-4	activates with α, depresses with β glycerophosphate	β > α
IV	mammalian erythrocytes, yeast	6	activates	α > β

3) Specificity. With the exception of adenosinetriphosphatases and metaphosphatases, most phosphatases show a remarkable lack of substrate specificity. Phytase, for example, which according to its name, ought to attack only phytin, hydrolyzes many other substrates as well. Owing to this lack of specificity, the phosphomonoesterases have been regarded as being isodynamic enzymes which, while attacking the same substrate, do so with a variety of pH optima. These optima have been reported to range from 2.7 to about 10. It was at one time convenient to distinguish acid and alkaline phosphatases on the basis of their optima with respect to neutrality, and although this concept no longer has its original value, it has been retained in the current literature.

4) Physical chemical properties. Phosphatases are enzymes which cleave the ester bond between the oxygen of an organic radical and the phosphorus of the phosphate radical (9). Comparatively little is known about the chemical nature and structure of the phosphatases. Although Van-Thoai <u>et al</u>. (10) claimed to have prepared a crystalline phosphomonoesterase, the statement was subsequently rescinded. Kunitz (11) has recently obtained a crystalline inorganic pyrophosphatase with a molecular weight of approximately 100,000 from yeast. H. and E. Albers (12) found the molecular weight of purified kidney phosphatase to be from 6,000 to 10,000 by means of diffusion.

Ultracentrifugation of rat serum disclosed that phosphatase sedimented with the large molecular weight components present, the globulins. Kabat also found this to be true (14), but if the kidney

tissue he used was first autolyzed, the enzyme remained in the soluble fraction. Kabat was of the opinion that the enzyme was merely "carried" by the larger proteins. On the other hand, Cattaneo (15) found that by fractionating horse serum with acetone-ether, almost all the phosphatase activity remained with the soluble components, the albumins. In view of some experiments to be reported on the purification of the enzyme by acid or heat treatment, in which some enzymatic activity remains with the aggregated proteins, depending upon the degree of washing, it is suggested here that the phosphatase is a very "sticky" enzyme which adheres to a variety of proteins. It is further suggested that this quality of the enzyme is responsible for the difficulty experienced in its purification, and the failure to obtain it in crystalline form.

Information on the structure of a phosphatase was obtained by Gould (16), who studied the enzymatic activity after the chemical modification of surface groups by means of ketene, phenylisocyanate, nitrous acid, and formaldehyde. The last three compounds react with free amino groups, while ketene reacts with the phenolic hydroxyl groups as well. The loss of activity by these treatments indicates that a free amino group and probably the phenolic hydroxyl are required by the enzyme.

Cedrangolo (17) reported the isoelectric point of horse serum phosphatase as 8.8 to 9.

5) The role of metals in alkaline phosphatases. Considerably more information is available on the role of metals, but unfortunately this subject is confused by the numerous contradictions

that are found in the literature. It is very difficult to assess the value of metals in enzyme systems merely on the basis of their ability to inhibit or enhance activity. For example, carbonic anhydrase, which has zinc as an integral part of the enzyme molecule, is also inhibited by the addition of zinc (18). Many workers have concluded that magnesium is required for alkaline phosphatase activity since it is capable of increasing the activity of slightly active preparations, yet Abul-Fadl and King (19) report that thoroughly dialyzed kidney phosphatase cannot be reactivated by magnesium alone, but requires an electrodialyzable organic cofactor which is thermostable. D. Albers (20) also found that kidney phosphatase dialyzed for 162 hours against water was not restored by the addition of magnesium, but if the dialysate of another preparation were added, 20% reactivation occurred.

Massart and Vandendriessche (21) found that alkaline yeast phosphatase was activated by magnesium, manganese, cobalt, nickel, or ferrous iron. Bamann and Heumüller (22) state that manganese is far more effective as an activator than magnesium for alkaline liver phosphatase.

Roche _et al_. (23) dialyzed dog intestinal phosphatase against water extensively, and found that the amount of zinc remaining associated with the protein was unchanged, but that the magnesium content had decreased by one third to one half. Zinc has also been claimed to be an activator (24), but Hove _et al_. (25) found it to activate only crude preparations, and that it was inhibitory if the enzyme were extensively dialyzed. This evidence suggests that the zinc may com-

bine with a natural inhibitor, rather than function as a required cofactor. Sadisivan (26) reports that an alkaline phosphatase in Penicillium, if inactivated by cyanide, is restored to activity by zinc preferentially above other metals. Cohn et al. (27) state that alkaline plasma phosphatase is inhibited by zinc, but is activated by cadmium, a metal which is generally inhibitory to phosphatases.

6) Organic cofactors. v.Euler and Hahn (28) found that intestinal phosphatase purified by alcohol precipitation and electrophoresis was inactive until a partially purified preparation heated to 100°C. was added. The addition of the dialysate ash was without effect. On the basis of ultracentrifugal analysis, Ek and v.Euler (29) assumed this coenzyme to be a polypeptide of approximate molecular weight 900.

H. Albers et al. (30) claim to have dissociated an acid and alkaline phosphatase by means of dialysis into their respective apo- and co-enzymes. The addition of the acid coenzyme to the alkaline apoenzyme reconstituted the original alkaline enzyme with the same pH optimum and substrate specificity; similarly the acid phosphatase was reconstructed from acid apoenzyme and alkaline coenzyme. This coenzyme was not magnesium. Kutscher and Schrier (31) found that the dialyzable material which reactivates muscle phosphatase was not magnesium since this metal was lacking in the concentrated dialysate. Schales and Mann (32) dialyzed alkaline kidney phosphatase against water and then against alkaline buffer. They found that the second dialysate was more effective in restoring activity than the first.

Although Roche (33) firmly believes that amino acids, particularly alanine, are required with magnesium for reactivation

of extensively dialyzed alkaline phosphatases, D. Albers (34), among others (35), found amino acids to be inhibitory on the same enzymes. Cloetens (29) also fails to find reactivation with amino acids, and reports that the activity of a dialyzed enzyme can be restored merely by returning to an alkaline pH.

Contradictory claims have also been made regarding the role of ascorbic acid in phosphatase activity. Thannhauser _et_ _al_. (36) found an increase in the activity of serum phosphatase with ascorbic acid, but Albers failed to find the same effect. Giri (37) reported that both crude and dialyzed acid or alkaline phosphatases were not affected by ascorbic acid or by copper ions singly, but that the addition of both resulted in inhibition which could be prevented by glutathione, cysteine, or cyanide.

7) The role of metals in acid phosphatases. In his review, Roche (38) states that acid phosphatases are not metal enzymes, and that they cannot be reactivated by any means once they have become inactivated. Courtois and Kharsand (39), however, report that an acid phosphatase in green leaves with a pH optimum of 4.0 - 4.3 was activated by magnesium, zinc, nickel, cobalt, or manganese. A second enzyme with an optimum of pH 4.7 - 5.2 was only slightly activated by magnesium, nickel, or cobalt, and was inhibited by zinc and manganese. Unfortunately, no data or details of inactivation are given.

Kutscher and Schreier (31) reported an acid phosphatase from muscle which required magnesium for reactivation when using glycerophosphate as a substrate. The dialyzed enzyme was equally active with or without magnesium, however, when phenyl phosphate was

the substrate. The dialysate was concentrated and found to have no magnesium. Axelrod (40) observed large fluctuations in the activation of citrus phosphatase with magnesium, but in one instance reported a 48% increase.

Some of the reported effects of metal activation may be due to a shift in the pH toward the pH optimum. Frankenthal (41) reported such a shift in a system containing magnesium or manganese and a tumor triphosphatase. H. Neumann (42) showed that the pH optimum of alkaline intestinal phosphatase was not changed by dialysis, nor after purification with acid or ethanol. The addition of the inhibitors cyanide, fluoride, carbonate, oxalate, strontium, barium, silver, aluminum, or copper did not shift the optimum, nor did the activators magnesium, manganese, cobalt, or nickel.

8) Reported fractionation of leaf phosphatases. It is apparent that the conflicting account of activators and inhibitors is not conducive to a clear understanding of the structure or of the physiological significance of the phosphatases. Interesting papers by a group of Japanese workers are also open to question, since Bamann was unable to confirm some of their results (43). Kobayashi (44) claimed to have resolved a phosphatase from rice bran with an original pH optimum of 5.6, into an inhibitor, "X", and an enzyme with an optimum of 3-4. This "X" substance was not adsorbed on kaolin at pH 4.1, but was on C γ alumina gel at pH 7. "X" was thought to be attached to the protein since the original enzyme migrated to the cathode on cataphoresis, but to the anode after purification by adsorption. Inouye (45), by means of the same

adsorption technique, found a phosphatase with an optimum of 2.7-3.0. By adding 1/4, 1/2, or 1 part of "X" back to the purified enzyme, he was able to shift the pH optimum to 4.5, 5.0, and 5.5 respectively.

Our knowledge of the phosphatase enzymes must therefore be placed historically in an "age of confusion".

An attempt has been made in this thesis to study the properties of the acid phosphatases of green leaves, so that an integrated and consistent pattern may soon emerge.

III. The relation of phosphatase to the bulk protein of green leaf cytoplasm.

1) Preparation of the bulk protein. The cytoplasmic proteins of green leaves were obtained in general by the procedure devised by Wildman and Bonner (5). The midribs were removed with a scalpel to facilitate grinding, and the leaf blades chopped into pieces approximately 1-2 cm^2. These pieces were dispersed in an Eppenbach stainless steel colloid mill with M/2 potassium maleate buffer of pH 7 in the ratio of 2g. of leaves to 1 ml. of buffer. The operations were performed in a cold room at $4^{o}C$. and the mill was chilled with circulating ice water. The slurry was passed through sharkskin filter paper in a basket centrifuge. This treatment removes unbroken cells, cell walls, and large aggregates of chloroplasts. The solution contains, depending upon the species used, whole or broken chloroplasts, grana, nuclear and wall fragments, various insoluble substances, and all the soluble components not adsorbed or bound in some way to the remaining precipitate. This crude suspension is used to grind the remainder of the leaves, fresh buffer being added

only when the slurry becomes too thick to grind. This whole protoplasm, as it is called, is again passed through the basket centrifuge. It is frozen by layering in flasks at -40°C., and stored in a deep freeze at -20°C.

The cytoplasmic proteins were prepared by thawing whole protoplasm, and centrifuging for one hour at 20,000g. This treatment is in general sufficient to remove almost all the residual green material which has been aggregated to a large extent by the process of freezing and thawing. It is sometimes necessary to remove this green particulate matter, for example with sugar beet leaf cytoplasm, by centrifugation at 25,000 rpm for one hour in the Model L Spinco preparative ultracentrifuge. A clear amber solution can be removed with a syringe from the middle portion of the centrifuge tube. This solution, free from particulate matter, is called whole cytoplasm.

Wildman and Bonner prepared Fraction I from whole cytoplasm by the addition of ammonium sulfate to 35% saturation. The protein so precipitated was removed by centrifugation at 20,000g. for one hour. The remaining solution contains the proteins known collectively as Fraction II. All operations on these proteins were performed with solutions at 0°C.

As has previously been mentioned, the studies of Wildman and Bonner indicated that Fraction I contained phosphatase activity. Nevertheless, it was apparent that if Fraction I and the enzyme were different, some evidence of their dissimilarity should be obtainable by means of techniques not yet employed. Since salt fractionation and electrophoresis failed to distinguish the two, the use of a method

which depended on separation by mass differences was thought to be warranted, and this possibility was tested with the analytical ultracentrifuge.

2) Evidence obtained by the analytical ultracentrifuge for the separation of the enzyme and the bulk protein. The separation cell used in the analytical ultracentrifuge is a truncated cone with a porous barrier at right angles to the imposed gravitational field which is directed toward the base of the cone. This system permits the separation of molecules which are sufficiently different in mass and shape. The faster moving components may flow through the porous plate, leaving the slow, smaller molecular weight substances on the opposite side. Following centrifugation, it is possible to remove the contents of the top compartment without disturbing the contents on the opposite side of the barrier. An experimental means is thus provided for the separation of protein mixtures.

Ammonium sulfate precipitated Fraction I from Turkish tobacco was subjected to centrifugation in a separation cell by Dr. S. J. Singer. Although Fraction I is homogeneous electrophoretically, it shows evidence of more than one component in the analytical ultracentrifuge. The largest single entity present, which is also the fastest moving component, will be called the bulk protein. Centrifugation was continued until the bulk protein was present in the top compartment in from one to five percent of its concentration in the original mixture. The slower moving components in the top compartment were present in from ten to fifty percent of their original concentrations. Concentration estimates were made by Dr. Singer

from the scanning patterns.

At this point it is possible to test the hypothesis that the bulk protein is itself the phosphatase, since not more than from one to five percent of the total phosphatase activity should now be present in the top compartment. Any value in excess of this could only be due to the splitting off of a phosphatase moiety from the bulk protein, or to the non-identity of the phosphatase with the bulk protein.

Enzymatic analyses were made on the mixture put into the cell, and on an aliquot from the top compartment after centrifugation. The original mixture was held at room temperature for one hour, since during the run, the contents of the separation cell were at room temperature for a similar period. For assay, one tenth ml. aliquots were incubated with 4 mg. of sodium nitrophenylphosphate (NPP) in 0.1M sodium acetate buffer of pH 5.0. The nitrophenol liberated was determined by the increase in optical density following the addition of 10 ml. of 0.2N NaOH. Density determinations were made in a Klett photoelectric colorimeter using a No. 42 filter. Figure 1 presents the data from this assay.

It can be seen from these data which result in a linear plot of hydrolytic activity, that the degree of hydrolysis is a function of the amount of enzyme present. The top compartment, which contains the smaller molecular weight components, retains about 20% of the total phosphatase activity present in the original mixture, and this is in excess of what might be expected if the bulk protein were indeed identical with the phosphatase.

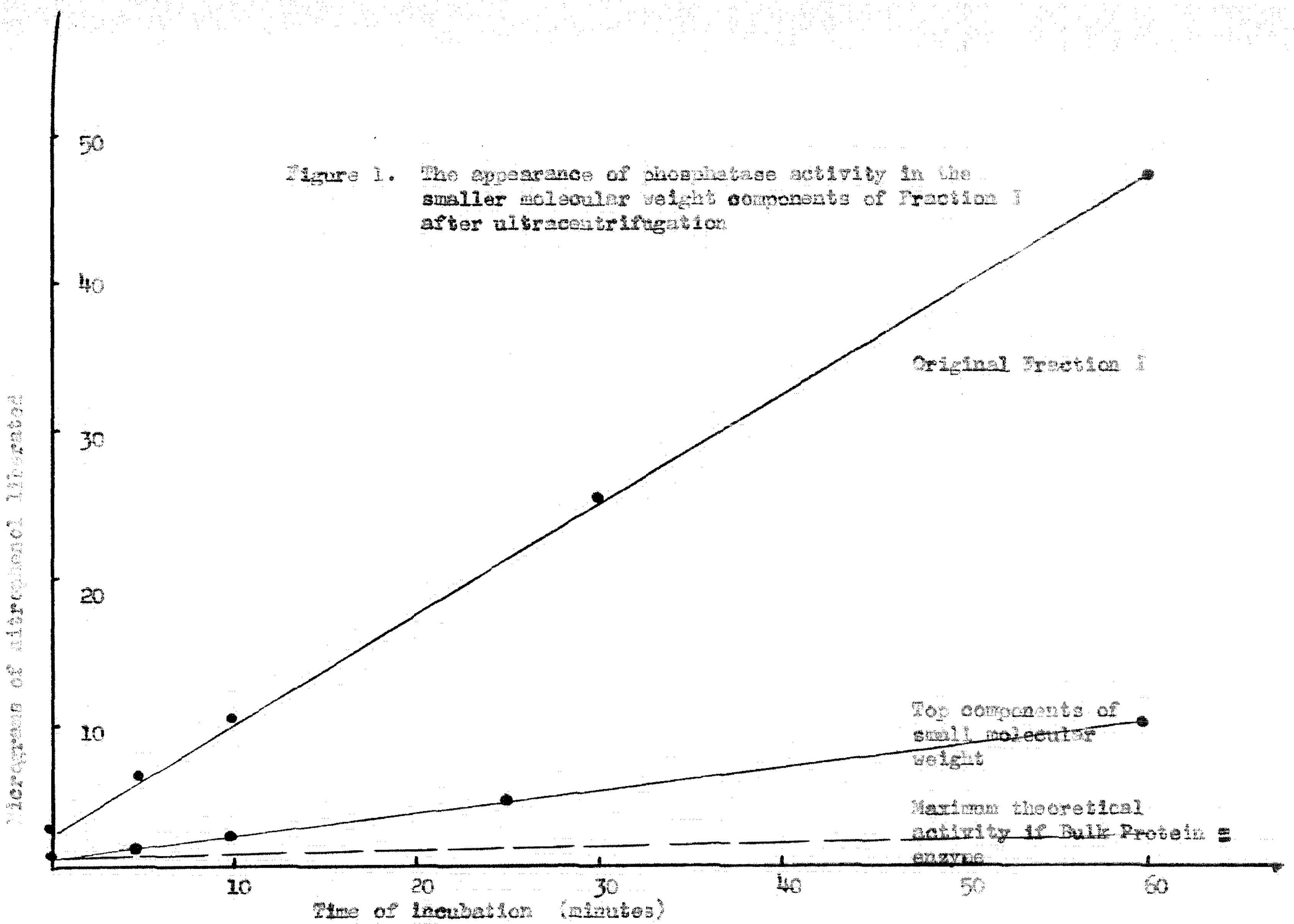

Figure 1. The appearance of phosphatase activity in the smaller molecular weight components of Fraction I after ultracentrifugation

3) Comparative stability of the enzyme and bulk protein toward heat. We may interpret the above experiment as presumptive evidence that the enzyme and the bulk protein are not identical. Further evidence for this dissimilarity was obtained by treatment of whole cytoplasm with heat. For example, 20 ml. of Turkish tobacco whole cytoplasm was heated with stirring in a thin walled test tube at 58°C. for three minutes. The tube was cooled rapidly and centrifuged to remove the voluminous tan precipitate. The precipitate was washed three times by resuspending in 0.1M acetate buffer of pH 5, centrifuging, and made to a volume equal to that of the supernatant from the heat treatment. Protein was determined on both samples by adding trichloracetic acid (TCA) and heating to 90°C. for five minutes. The precipitate was washed twice with additional TCA, transferred to tared beakers, and dried to constant weight at 100°C. Enzyme activity was assayed as previously described.

Table 2 shows that although a considerable amount of enzyme was destroyed by the heat, approximately 15% of the original protein retained 52% of the original phosphatase activity. By this means an approximate fourfold purification of the enzyme was accomplished.

The amount of enzyme precipitated by the heat treatment is variable, and depends to a large extent on the species of plant used. In no case is it possible to remove all the activity from the precipitate without also losing most of the activity in the supernatant. Of the protein remaining after heating, no trace of the bulk protein could be detected in the analytical ultracentrifuge (46).

TABLE 2

The resistance of phosphatase to heat as compared with the bulk protein.*

Treatment	Minutes hydrolyzed	Optical density	µg. NP[1] liberated	Protein mg./ml.	Specific[2] activity
Original	0	.108	7.5	6.7	
	10	.673	64.5		8.5
	20	.939	---		
Supernatant	0	.077	5.3	1.0	
	10	.435	35.0		29.7
	20	.668	63.2		

---

1. NP: nitrophenol
2. Specific activity: $\frac{\text{µg. NP liberated}}{\text{mg. protein x time}}$

* 0.1 ml. of enzyme used

4) Comparative stability toward acid. The stability of the bulk protein and of the enzyme in the presence of acid is also markedly different. Spinach leaf cytoplasm was dialyzed at 4°C. against Sørensen's 0.1M citrate buffer of pH 2.0 to 4.0. After 22 hours, the contents of the dialysis sacs were centrifuged for one hour at 20,000g. in order to remove the proteins aggregated by the acid treatment. The pH of the resulting solutions was measured, as well as the volumes recovered. Duplicate samples of 4 ml. each were used to determine the TCA precipitable protein concentration. An aliquot of the original cytoplasm was kept in an ice bath during the time of dialysis, and TCA protein was also determined on this sample.

As can be seen from Table 3 at pH 4.1, 22% of the original protein retained 74% of the original phosphatase activity, so that again a fourfold purification resulted. Similar results have been obtained with sugar beet leaf cytoplasm, and in fact, the simple addition of acid, rather than prolonged dialysis, is sufficient to obtain the same result.

It is known from experiments performed in this laboratory (46) that the bulk protein is unstable under acid conditions. No bulk protein was detectable in the analytical ultracentrifuge in the sample whose final pH was 4.1. These results would also indicate then that a distinction can be made between the bulk protein and the enzyme on the basis of their stability in acid.

5) The relative amount of enzyme present in Fractions I and II prepared by salt precipitation. The last two experiments

TABLE 3

The purification of phosphatase at the expense of the bulk protein by means of acid.

pH of dialyzed protein	ml. recovered	protein mg./ml.	total protein mg.	% recovered
2.3	20.6	2.15	44.3	33
2.6	20.2	1.75	35.4	26
3.2	21.0	1.28	26.9	20
3.6	21.2	1.25	26.5	20
4.1	21.1	1.40	29.5	22
original	20.0	6.70	134.0	100

	µg. NP liberated after minutes:			specific activity	total[1.] activity	% total activity
	0	10	20			
2.3	1	3	5	1.86	82.5	18
2.6	1	5	8.5	4.28	149	30
3.2	1	6	11	7.80	210	42
3.6	1	7.2	16	12.0	318	64
4.1	1	9.2	19	12.8	378	74
original	4	16.0	29	3.74	502	100

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1. Total activity: specific activity x total protein.

reported, while distinguishing the enzyme and bulk protein by their stability, do so by denaturing a considerable amount of the original proteins in solution. Fractionation by milder procedures would of course be superior in deciding what the relation of the enzyme and the bulk protein actually is in the native state. Two such methods are available which are less likely to denature the proteins after the initial grinding. The first is by salt fractionation as was originally done by Wildman and Bonner in the preparation of Fractions I and II. The second is by differential centrifugation.

Turkish tobacco cytoplasm was brought to 10% saturation at 4°C. with a saturated solution of ammonium sulfate. This treatment aggregates the residual green material when centrifuged for one hour at 20,000g. The supernatant solution was then brought to 35% saturation with additional salt, and the precipitated Fraction I removed by centrifugation. The precipitate was dissolved in 0.1 ionic strength cacodylic acid buffer of pH 6.93, and both Fractions I and II were dialyzed against two changes of cacodylic acid buffer in order to remove the residual ammonium sulfate. TCA protein weights were obtained and aliquots incubated with NPP as usual.

Table 4 shows the data from this experiment. It can be seen that Fraction II, while constituting approximately 13% of the total protein, contains 56% of the total enzyme activity. Fraction I, on the other hand, which in this case represents about one half of the original protein, has only 12% of the phosphatase present originally. Since both the specific and total activity of Fraction II is greater than Fraction I, it is apparent that the enzyme in tobacco

TABLE 4

The enrichment of phosphatase activity in Fraction II as compared with Fraction I after salt fractionation.

Treatment	Protein mg./ml.	Total volume ml.	Total protein mg.	% of total* protein
Whole cytoplasm	15.5	330	5100	100
Fraction I	39.2	64	2500	49
Fraction II	4.3	160	690	13.5

	µg. NP liberated after minutes:			Specific activity	Total activity	% total activity
	0	5	15			
Whole cytoplasm	3	25	61	2.84	14,500	100
Fraction I	5	19	42.5	.72	1,800	12.4
Fraction II	2.5	28	64.5	11.8	8,140	56.2

* It is apparent that some denaturation occurred during salt fractionation, since the recovery of Fractions I and II was incomplete.

cytoplasm is not preferentially associated with Fraction I.

6) The relative amount of enzyme present in the bulk protein and in the remaining cytoplasm obtained by ultracentrifugation. Differential centrifugation, a procedure which is even milder than salt precipitation, gave results which support most strongly the concept of two separate entities. Frozen Turkish tobacco whole protoplasm was thawed and centrifuged in the Spinco for one hour at 25,000 rpm. The supernatant was removed with a syringe and centrifuged for an hour at 40,000 rpm, decanted, and again spun at 40,000 rpm for 2 1/2 hours. The protein pellet obtained was carefully rinsed with 0.1 ionic strength maleate buffer of pH 7.4, and allowed to dissolve overnight in a small quantity of buffer at 0°C. The dissolved protein was decanted to clean tubes, centrifuged for 20 minutes at 12,000 rpm to remove any particles, and the remaining solution centrifuged for two hours at 40,000 rpm. The translucent brown pellet obtained is considered to be once cycled bulk protein. It is again dissolved in buffer, centrifuged for 20 minutes at 12,000 rpm, and the supernatant spun at 40,000 rpm for two hours. The supernatant solution resulting from the first centrifugation at 40,000 rpm corresponds in a rough way to the Fraction II obtained by salt fractionation in that most of the bulk protein has been removed. This supernatant, the original cytoplasm, and the twice cycled bulk protein were analyzed for phosphatase activity using NPP as substrate in pH 5.0 acetate buffer.

Table 5a shows that while this procedure is not particularly effective as a means of purification of the phosphatase, it is very efficient in demonstrating that the enzyme does not travel with the

TABLE 5

The enrichment of phosphatase activity in Fraction II as compared with Fraction I after differential centrifugation.

a

Treatment	Protein mg./ml.	Minutes of hydrolysis	µg. NP liberated	Specific activity
Whole cytoplasm	11.0	0	1.5	
		10	20.5	1.73
		20	30.5	
Bulk protein	13.2	0	1.0	
		10	3.0	0.15
		20	4.0	
Fraction II	7.6	0	1.5	
		10	20.0	2.44
		20	30.5	

b

Treatment	Protein mg./ml.	Minutes of hydrolysis	µg. NP liberated	Specific activity
Whole cytoplasm	8.0	0	5.5	
		5	34.0	7.12
		10	64.5	
Bulk protein	17.0	0	4.0	
		5	13.5	1.12
		10	26.0	
Fraction II	4.6	0	5.5	
		5	32.0	11.5
		10	63.5	

large molecular weight bulk protein, since the Fraction II components have approximately 12 times as much phosphatase activity as the bulk protein fraction.

It has been possible on another occasion to obtain by the same method a sample of Turkish tobacco bulk protein which also had almost no phosphatase activity. It is usual for a fair amount of enzyme to be present in Spinco prepared bulk protein. The reason for the variation in enzyme content of the leaves is not known, for it occurs even with plants grown in the Earhart Laboratory. Data from a similar experiment will therefore be given showing that while the absolute values of specific activity are different, the conclusions to be drawn are exactly the same, namely, that most of the enzyme is found in the fraction which is not enriched in the bulk protein. These results are presented in Table 5b.

7) Conclusions. We have seen that the bulk protein of tobacco leaves can be prepared by differential centrifugation with greatly reduced phosphatase activity. Salt precipitated Fraction I has also been shown to contain less phosphatase than the Fraction II components. The separation of the bulk protein from other substances by means of the separation cell of the analytical ultracentrifuge has indicated that more phosphatase appears in the smaller molecular weight components than could be accounted for if the heavier bulk protein were indeed the enzyme. From these data, we may conclude that while a tenuous bond may exist between the bulk protein and the enzyme, the bond is one which is readily broken by procedures generally considered to be the least likely to cause protein denaturation. That

the bulk protein and the enzyme have widely different stability characteristics in the presence of heat or acid may be considered contributory evidence for the independence of the two proteins.

IV. The separation of isodynamic acid phosphatases from the cytoplasm of green leaves.

Phosphomonoesterases typically exhibit a broad spectrum of substrate specificity. Many enzymes are known which are highly specific not only for a particular substrate, but indeed for a particular steric configuration. Since some enzymes which were once thought to have relatively wide requirements have been shown subsequently to be a mixture, it is reasonable to assume that the acid phosphatase of green leaves may be in fact composed of numerous individual phosphatases. Salt fractionation disclosed no evidence for the occurrence of more than one phosphatase, so an attempt was made to detect isodynamic phosphatases by means of adsorption on a polystyrene resin, by acid dialysis, and by means of serology.

1) Separation by means of an ion exchange resin. The resin, Dowex 50 of 300-400 mesh, was prepared according to the directions of Moore and Stein (47). A glass chromatographic adsorption column 0.9 x 15 cm. was packed with about 10 cm. of Dowex by passing in a slurry under gentle pressure. The column was left at 0°C. for 30 minutes, and 0.5 ml. of sugar beet cytoplasm was applied to the surface. After the cytoplasm had run into the column, 12 ml. of 0.1M citrate buffer of pH 4.9 was added gradually, and a slight suction applied. The eluate was collected in lots of approximately 1 ml., and then 10 ml. of 10% sodium tartrate of pH 7.7 was added. Fractions of this eluate

were collected as above. 0.5 ml. aliquots of the various fractions were incubated with 4 mg. of NPP in 2 ml. of 0.1M acetate buffer of pH 5.2. These data are given in Table 6. Most of the phosphatase activity was present in the second tube from the citrate elution, and no enzyme was recovered beyond the third tube even though an additional 8.2 ml. of buffer was passed through the column. The tartrate solution, however, was able to elute additional enzyme, most of which appeared in the third tube of this series.

That the two eluates might be in fact two isodynamic enzymes was tested by determining their pH optima. An experiment similar to that described above was performed with leaf cytoplasm from Maryland Mammoth tobacco. Tubes 2 and 3 from the citrate elution were pooled, and tubes 3, 4, and 5 from the tartrate elution. Aliquots were incubated with NPP, in acetate buffer from pH 3.6 to pH 6, and in borate buffer in the more alkaline region. The pH of the mixture was determined at the same time that an aliquot was removed to determine the amount of nitrophenol liberated. Figure 2 is a graph of the results of this experiment, and indicates an optimum of about 5.7 - 5.8 for the citrate eluate, and about 5.0 - 5.1 for the tartrate eluate. Since a sharp separation was obtained by adsorption on the Dowex, and the eluates removable with two buffers of different pH reveal more than one pH optimum, it is concluded that at least two isodynamic phosphatases have been separated by this technique.

2) Separation by acid dialysis. A second way of demonstrating the occurrence of isodynamic phosphatases in leaf cytoplasm made use of the observations already reported on the purifica-

TABLE 6

The separation of isodynamic phosphatases of leaf cytoplasm on Dowex 50.

Tube no.	Ml. collected	µg NP liberated in 20 minutes
	Citrate elution	
1	1.0	0
2	1.2	300
3	1.3	10
4	1.3	0
5	1.3	0
6	1.1	0
7	2.5	0
8	2.0	0
	Tartrate elution	
1	0.9	0*
2	1.2	0
3	2.3	35
4	1.5	12
5	2.0	6
6	1.0	4
7	1.0	2

* 40 minute hydrolysis.

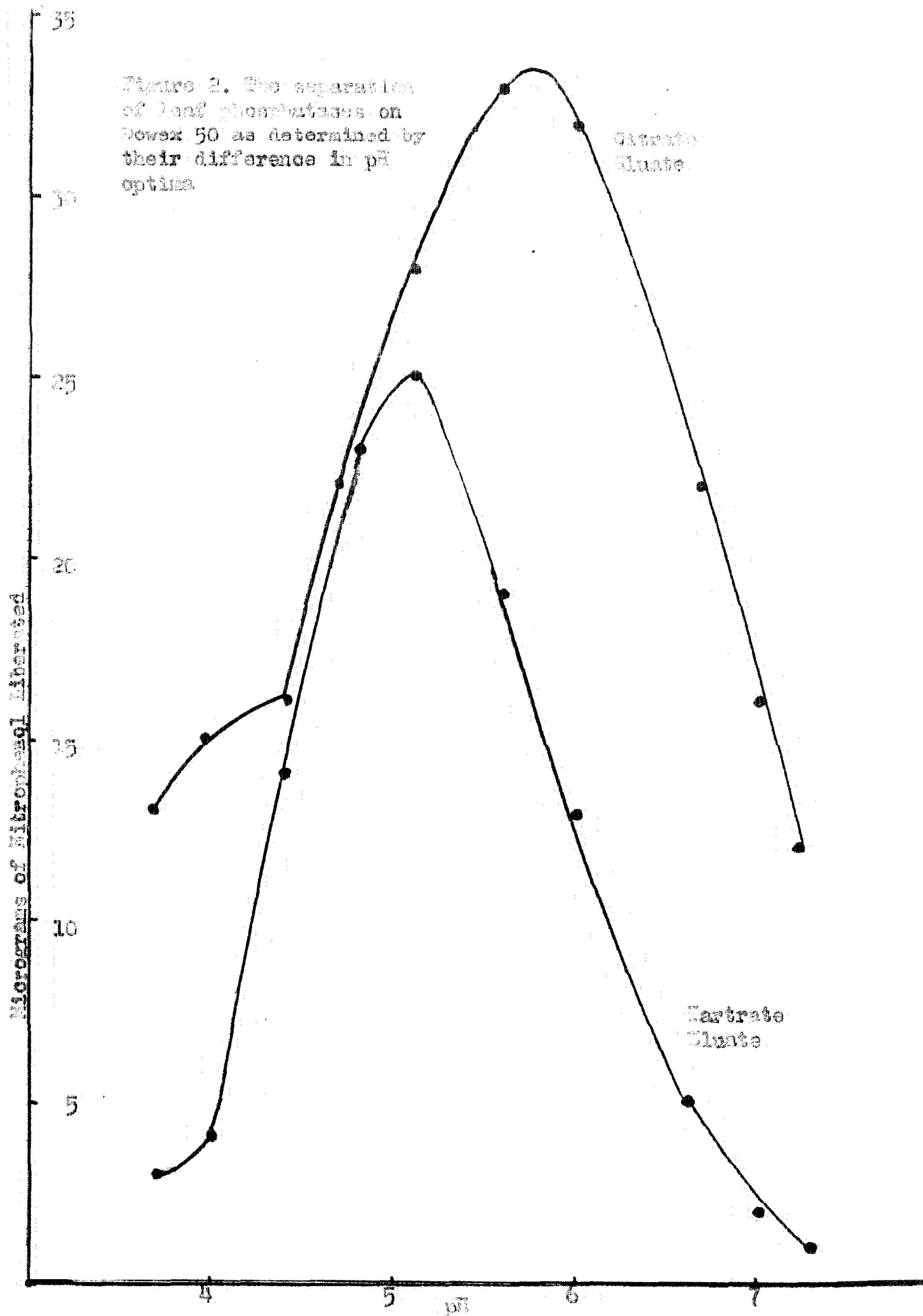

Figure 2. The separation of leaf phosphatases on Dowex 50 as determined by their difference in pH optima

tion of phosphatase at the expense of the bulk protein and other components, namely, by dialysis against acid buffers. Spinach whole cytoplasm was dialyzed against 0.1M citrate buffer of pH 4.0 and 4.8, and against 0.1M potassium maleate buffer of pH 7.0. The aggregated proteins were removed by centrifugation at 20,000g. for one hour, and 0.1 ml. aliquots of the supernatant solutions were incubated at 37°C. with NPP made up in 0.1M potassium maleate buffer from pH 3.5 to 7.2. As aliquots of the hydrolysis mixture were removed to determine the amount of nitrophenol liberated, a duplicate aliquot was removed for pH determination. Figure 3 is a graph of these data obtained.

A very clear separation was obtained on repeated experiments. The original mixture of phosphatases present in whole cytoplasm has an optimum in the vicinity of 5.2, while the sample dialyzed at pH 4.8 has shifted its optimum to the neighborhood of 6.4. The sample dialyzed at 4.0 now has an optimum of about 6.0. Both dialyzed samples show a hump in the activity curves suggesting the presence of other phosphatases not completely separated. Since the optimum of the original mixture is 5.2, components having an optimum on the acid side of 5.2 are to be expected in order to reconstitute the mixture. It is suggested that such components are present in the mixture but have been destroyed by dialysis at 4.8, and that it would be possible to demonstrate their presence by dialysis between 5.0 and 7.0.

The shift in pH optima of the dialyzed samples is not due simply to the removal of an inhibitor since this would not be consonant with the observation that the sample dialyzed at pH 4.8 has

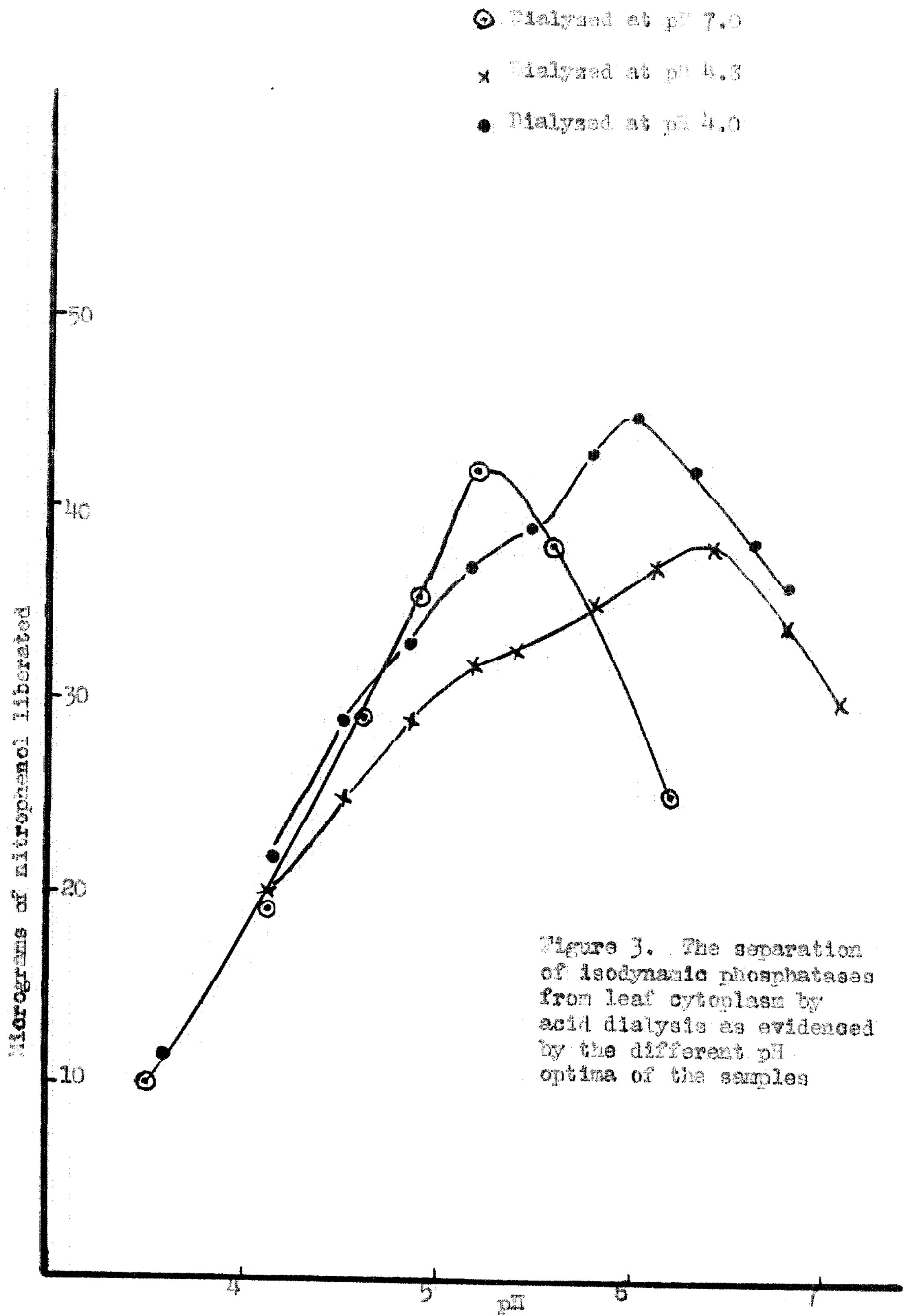

Figure 3. The separation of isodynamic phosphatases from leaf cytoplasm by acid dialysis as evidenced by the different pH optima of the samples

shifted more to the alkaline side than that dialyzed at pH 4.0. One would expect a gradual, uniform shift one way or the other from the optimum of the mixture as was observed by Inouye and reported in the preceding review of the phosphatases.

3) Separation by means of antigen antibody reactions. The third attempt to fractionate leaf phosphatases relied upon the fact that enzymes are frequently capable of producing specific antibody responses in suitable animals. If the antibodies produced in response to the injection of a mixture of phosphatases were to some degree specific for particular components of the mixture, they might precipitate these components while leaving unaffected in the supernatant of a precipitin test the remainder of the phosphatases. It is also possible to assume different stability levels of the phosphatases present in the cytoplasmic mixture, so that the antigen actually effective in promoting specific antibody response would not be the original mixture injected, but one having fewer components. In testing the resulting antiserum with whole cytoplasm, the stable enzymes might be precipitated by their specific antibodies, leaving in solution the remainder of the phosphatases which were not capable of functioning as antibody formers. Differences in the phosphatases might then be detected by an examination of their respective Michaelis constants, and pH optima.

Sugar beet leaves were ground in the usual manner in the colloid mill, and the resulting whole protoplasm was heated at 58°C. for three minutes. The fluid was cooled as rapidly as possible in a salt-ice bath, and the heavy precipitate removed by centrifugation.

The supernatant was adjusted to pH 4.0 by the addition of glacial acetic acid while stirring vigorously. The precipitate was removed by centrifugation, and the supernatant dialyzed against running distilled water for two hours. The protein solution at 4°C. was saturated with ammonium sulfate, and the mixture was allowed to stand overnight. No phosphatase activity remained in the supernatant solution. The salt precipitated protein was dissolved in 0.1M acetate buffer of pH 5.5, and dialyzed against 10 volumes of additional buffer with two changes during 16 hours. An aliquot of this preparation to be used for injection was reduced in volume by fanning in order to raise the concentration of protein, and then dialyzed against physiological saline. The thoroughly dialyzed solution was centrifuged for one hour at 20,000g. The TCA precipitable protein was 11.8 mg./ml.

One intraperitoneal and 7 marginal vein injections were given to a rabbit of about 3 kg. weight on alternate days. A total of 91 mg. was injected. Ten days after the last injection, the rabbit was bled and the serum tested for precipitating antibody. A precipitate was found to occur down to an antigen concentration of 0.18 mg./ml. Normal serum gave no such reaction. Seventeen days after the last injection, the rabbit was given a booster shot intraperitoneally, and two intravenous injections. Five days after the final injection, 40 ml. of blood was removed by cardiac puncture, and the complement was inactivated by heating the serum for 30 minutes at 56°C. The serum obtained from the re-immunization was used in all subsequent experiments.

Dilutions of the original antigen were mixed with the antiserum and allowed to stand at 4°C. for 48 hours. The precipitates were washed three times with cold physiological saline solution. Phosphatase activity was found in the precipitate as well as in the supernatant solution. Approximately 60% of the activity recovered was present in the supernatant, and 40% in the precipitate, in all dilutions of antigen up to 1:8. About 90% of the total original activity tested in normal serum was recovered in the supernatant plus the precipitate. This evidence would indicate that the precipitation was specific and that the enzyme was not merely adsorbed on the precipitate in non-specific fashion. One would expect the per cent of antigen non-specifically adsorbed to be less at higher dilutions, owing to the smaller amount of precipitate. No phosphatase activity was found in the antiserum alone, since this phosphatase was destroyed during the inactivation of complement at 56°C.

In most antigen antibody reactions of the precipitin type, as the amount of antigen added is increased while keeping the antibody constant, a maximum amount of precipitate is formed in a particular zone. Beyond this zone, greater amounts of antigen give less precipitate, probably because of the formation of soluble complexes. The concentrations of purified phosphatase tested did not reach this zone of inhibition. It was felt, therefore, that when using whole cytoplasm as the test antigen, in which the phosphatase was diluted by the non-enzymatic proteins present, equal volumes of antiserum and whole cytoplasm would keep the reaction in the zone of antibody excess, and all the precipitable antigen would be precipitated.

In order to determine the Michaelis constants of the enzymes which might have been fractionated, 1 ml. of sugar beet whole cytoplasm was incubated with 1 ml. of antiserum for two hours at room temperature, and 48 hours at 4°C. The precipitate was washed as described above. The original cytoplasm, the supernatant from the above reaction, and the washed precipitate reconstituted with saline, to the volume of the supernatant, were incubated at 37°C. with NPP of concentrations M/75, M/150, M/300, M/600, and M/1200 in 6 ml. of acetate buffer of pH 5.0. Figure 4 is a graph of these data obtained by plotting the reciprocal of the reaction velocity, 1/v, determined by the quantity of nitrophenol liberated over a ten minute period, against the reciprocal of the substrate concentrations, 1/S. The Michaelis constants of the phosphatases in the cytoplasm and the washed precipitate were approximately 10^{-3} M. in two independent experiments, and the value for the supernatant was found to be about 3×10^{-4} M. These differences suggest that the precipitate contains an enzyme or mixture of enzymes similar in kinetic character to that in the original cytoplasm, but that the supernatant contains a phosphatase or mixture of phosphatases with different characteristics.

Further evidence for this separation was obtained by comparing the pH optimum of the cytoplasmic phosphatase mixture with that of the enzymes remaining in the supernatant. Aliquots were incubated with NPP, and the graph presented in Figure 5 indicates that the cytoplasm mixture has an optimum of about 5.3, while the optimum of the supernatant is closer to 4.9 - 5.0.

4) Conclusions. The selective adsorption of phosphatases

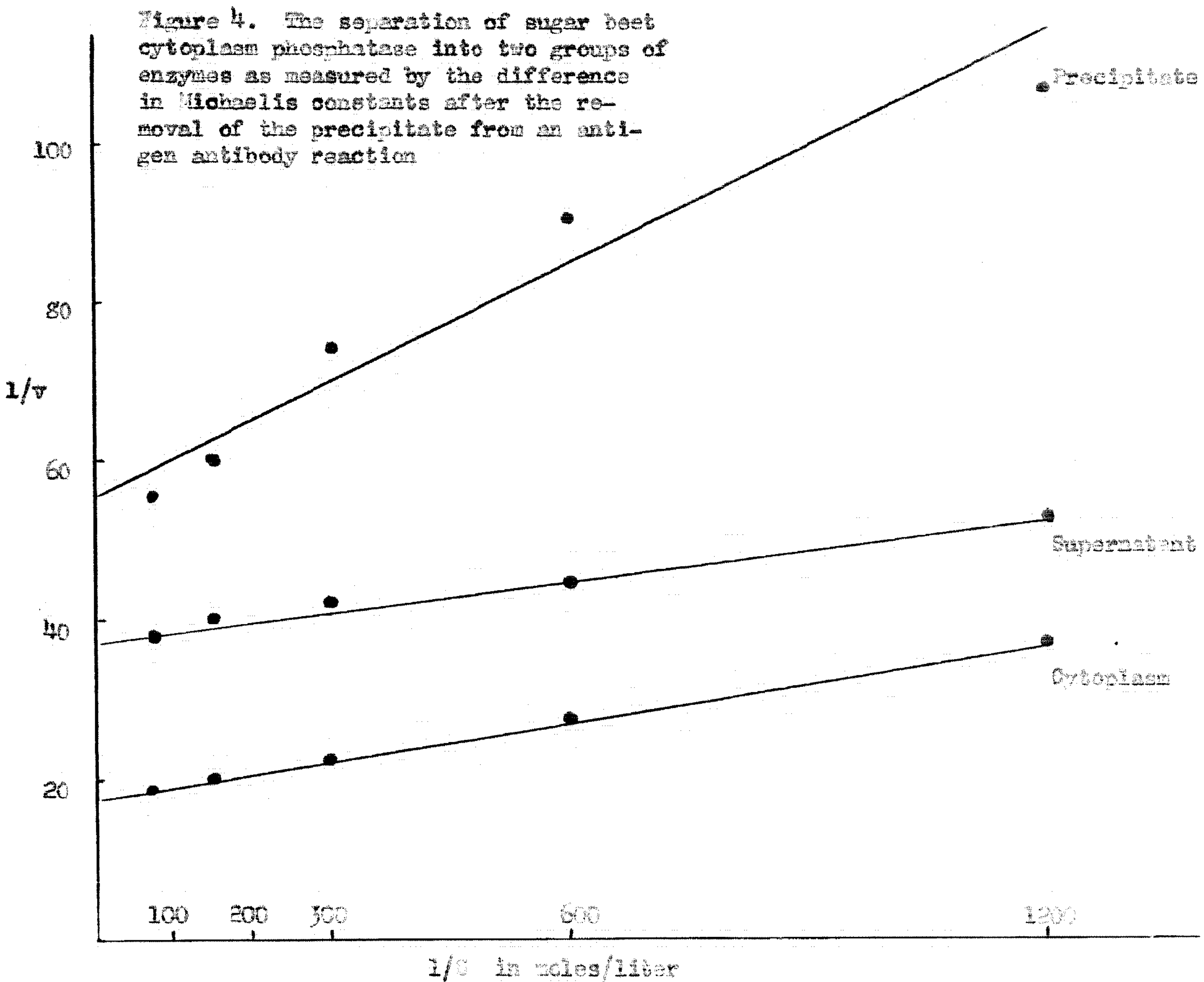

Figure 4. The separation of sugar beet cytoplasm phosphatase into two groups of enzymes as measured by the difference in Michaelis constants after the removal of the precipitate from an antigen antibody reaction

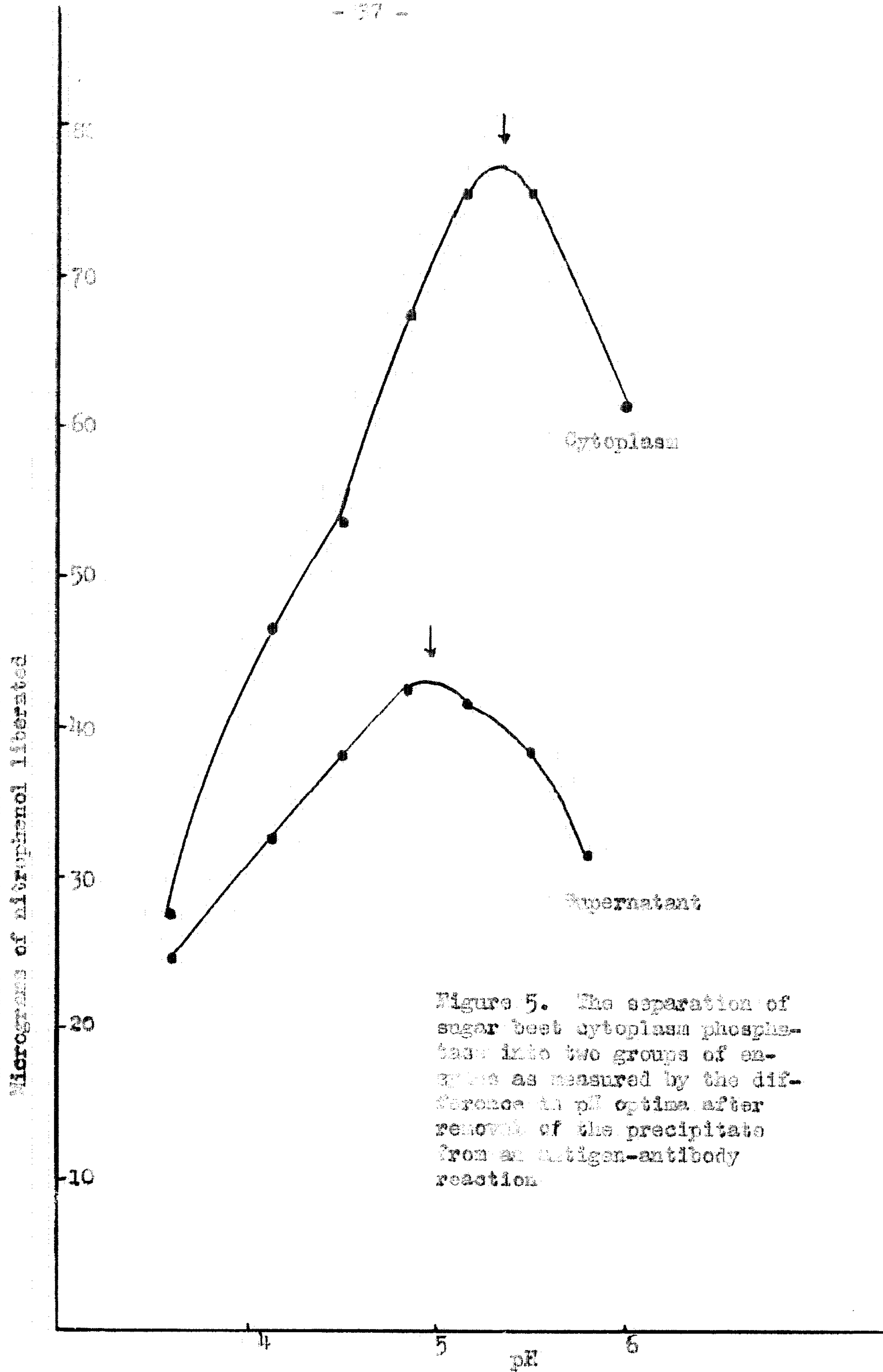

Figure 5. The separation of sugar beet cytoplasm phosphatase into two groups of enzymes as measured by the difference in pH optima after removal of the precipitate from an antigen-antibody reaction.

on Dowex, and the subsequent separate elution by different buffers, coupled with the difference in the pH optima of the eluates, is suggested as evidence for the occurrence of isodynamic phosphatases in the cytoplasm of green leaves. This suggestion is supported by data which indicate some degree of fractionation as the result of dialysis against buffers, and finally, by evidence obtained from antigen antibody reactions, in which differences were shown both in the pH optima and the Michaelis constants of the original cytoplasmic phosphatases and the supernatant remaining after specific precipitation of part of the enzymatic complex. A more rigorous examination of the fractions obtainable from adsorption on resins or C γ alumina gel, or by dialysis might well disclose additional isodynamic phosphatases, perhaps of more limited substrate specificity.

V. The role of metals in acid phosphatase activity.

Although it had been observed that the dialysis of leaf whole cytoplasm against acid buffers resulted in complete inactivation if the acidity were high enough, no attempt was made to reactivate the enzyme except by returning the pH to a more alkaline range, and this procedure failed. During some experiments on the inhibition of darkening of whole cytoplasm, it was noticed that the phosphatase activity decreased in the presence of certain chelating substances. From 20% to 30% inhibition occurred in the presence of diethyldithiocarbamate, sodium versenate, or 8-hydroxyquinoline. While these figures are not impressive, they do suggest that metals might be involved in acid phosphatase activity, even though Roche's recent review claims the contrary.

Metals may function in enzyme systems (48) by being the catalytic center, as in the heme enzymes. They may function as a binder between substrate and protein, as is thought to be the case with the peptidases (49). They may also act by maintaining an ionic balance with a second metal, as in the potassium-calcium antagonistic control of cell permeability.

1) Inactivation by dialysis and reactivation by metals. In order to test the hypothesis that metals might reactivate acid dialyzed phosphatase, spinach whole cytoplasm was dialyzed against 0.1M citrate buffer, pH 2, for 22 hours at 4°C. The aggregated proteins were removed by centrifugation at 20,000g. for one hour. The dialyzed enzyme was incubated at pH 5.0 with NPP and the addition of cupric chloride, magnesium sulfate, manganese chloride, or zinc sulfate. The salts were dissolved in acetate buffer and re-adjusted to pH 5 when necessary. The final concentration of the metals was 0.01M. Table 7 indicates that the copper without enzyme does not act catalytically in this system to hydrolyze the substrate, nor does copper plus boiled enzyme. However, with the aid of copper, a 400% to 500% increase over the control was obtained from the acid dialyzed enzyme. Magnesium was less effective, and zinc gave the same values as the control.

With a second series of metals tested, it was necessary to measure the amount of phosphate split off, rather than the nitrophenol, since the hydroxides of the metals would be precipitated by the NaOH used to develop the nitrophenol color. The following solutions were made up in 0.1M acetate buffer and re-adjusted to pH 5

TABLE 7

The reactivation by metals of leaf acid phosphatase inactivated by acid dialysis.

Treatment	µg of NP liberated after minutes:	
	10	20
Original cytoplasm	13	26
Dialyzed cytoplasm	2	3.5
" " + Cu	8	16
" " + Mg	6	11
" " + Zn	2	3.5
Cu alone	0	0
Cu + boiled enzyme	0	0

when required: cobalt chloride, ferric chloride, ferrous sulfate, calcium chloride, cupric chloride, and manganese chloride. The final concentration of the metal was 0.01M in each case.

It was necessary to test for the possible interference of these metals in the Allen method for the determination of orthophosphate (50). Twenty micrograms of phosphorus in the form of potassium phosphate were added to the solutions of each metal, and in each case 100% recovery of the phosphorus was obtained.

Since the dialyzed enzyme retained a small amount of activity, the solution was adjusted to pH 2.0 at which point it was completely inactive as a phosphatase. This completely inactivated enzyme was then incubated with NPP and the above metals. The phosphate liberated was determined by measuring the increase in optical density in a Coleman spectrophotometer at 660 mu.

Table 8 shows that copper is again the most effective metal, while cobalt is only about one half as efficient. Neither calcium or ferric iron caused reactivation, but manganese and ferrous iron caused a slight activity. Thus the reactivity of the metals tested in order of their decreasing ability is copper, magnesium, cobalt, manganese, and ferrous iron.

2) The optimum concentration of copper for reactivation. The optimum concentration for the action of copper was determined by incubating the partially inactivated enzyme with NPP and various concentrations of copper. The results of this experiment are shown in Figure 6. The optimum for this preparation is approximately 5×10^{-4} M.

TABLE 8

The reactivation by metals of leaf acid phosphatase inactivated by acid dialysis.

Treatment	µg of NP liberated after minutes:	
	10	20
Original cytoplasm	13	26
Dialyzed enzyme	0	0
" " + Ca^{++}	0	0
" " + Co^{++}	3.5	7.5
" " + Cu^{++}	7	14.5
" " + Fe^{++}	1.5	2.5
" " + Fe^{+++}	0	0
" " + Mn^{++}	1.5	3.0

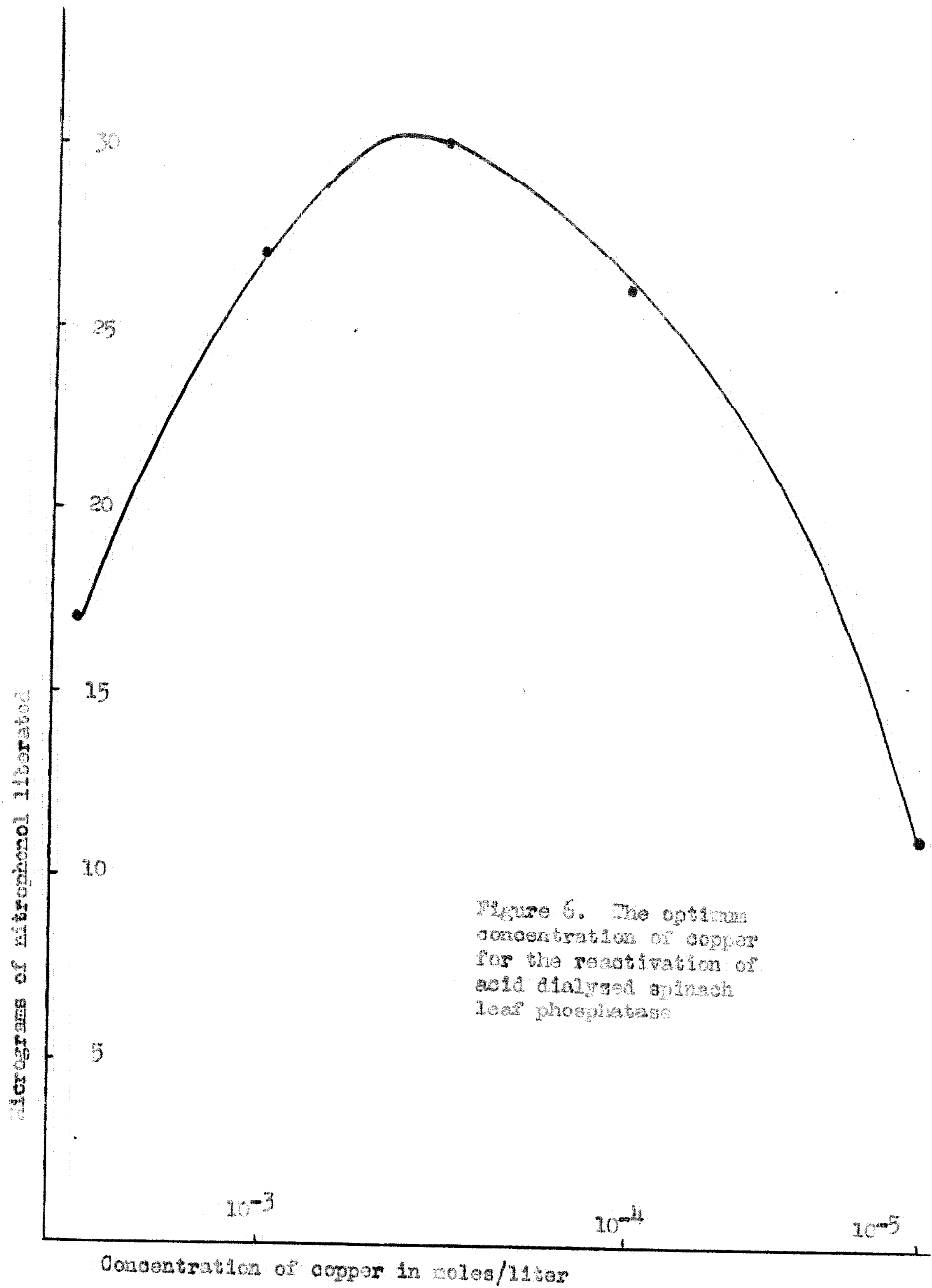

Figure 6. The optimum concentration of copper for the reactivation of acid dialyzed spinach leaf phosphatase

3) The rate of reactivation by copper. It is known that the peptidases exhibit a time lag during activation by metal ions (49). Several hours of incubation of the metal and enzyme prior to the addition of substrate are required for optimal activity. Partially inactivated phosphatase was tested for a time lag by incubating the enzyme with copper at 10^{-2}, 5×10^{-3}, and 10^{-5} M. These mixtures were allowed to stand at room temperature for 30 minutes, one hour, 20 or 23 hours at 4°C. before adding the substrate. In all cases, the rate of hydrolysis was the same. Since the rates of hydrolysis of NPP by enzyme activated with copper are linear over at least a 20 minute period and show no lag phase at all, it may be assumed that the reactivation is instantaneous.

4) Spectrochemical analysis of leaf protoplasm ash. Although the metals mentioned above are capable of varying degrees of reactivation of acid dialyzed phosphatase, it is not possible to conclude from this fact that any particular one of them is the required cofactor for the native enzyme. In order to approach this problem more closely, an attempt was made to correlate the degree of enzymatic activity with the actual amount of the various metals present, as the enzyme was dialyzed against buffers of different acidity. Those metals which are not correlated with the activity are least likely to be cofactors, while a direct relation between the particular metal content and enzymatic activity would favor the concept of that metal being involved with the activated enzyme.

These possibilities were tested by dialyzing spinach whole

cytoplasm against water to reduce the content of the maleate buffer used during the grinding operation. 100 ml. lots were then dialyzed under the following conditions:

A	pH 7.0	0.1M	potassium maleate buffer
B	4.8	"	sodium citrate "
C	4.0	"	" " "
D	3.0	"	" " "
E	2.0	"	" " "

The buffers were changed twice over a period of two days.

The contents of all the dialysis sacs were then centrifuged to remove the aggregated proteins. Duplicate aliquots were used to determine the TCA precipitable proteins, and the degree of enzymatic activity. Seventy ml. of the remaining cytoplasm of each sample were then evaporated to a small volume over a water bath, transferred to quartz crucibles, and ashed. The total amount of ash recovered was weighed, and these samples were analyzed spectrographically for the amount of metal present[1]. These results are shown in Table 9.

5) The correlation between the metal content and enzymatic activity. The content of manganese, iron, copper, and calcium is well correlated with the degree of enzymatic activity. An example of this can be seen in Figure 7, which shows the relation of manganese present to enzymatic activity. The specific activity, that is, micrograms of nitrophenol liberated per milligram of protein per 10 minutes of hydrolysis, is plotted against the pH of the cytoplasm after dialysis.

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1. The spectrographic analyses were made by the Pacific Spectrochemical Laboratory, Los Angeles, California.

TABLE 9

Spectrographic analysis of ashed spinach leaf cytoplasm dialyzed against acid buffers.

Percentage of metal in cytoplasmic ash

	A	B	C	D	E
Potassium	65.0%	0.62%	0.74%	1.14%	0.96%
Sodium	0.66	62.0	62.0	67.0	60.0
Aluminum	0.10	0.68	0.49	0.28	0.015
Boron	0.021	----	----	----	----
Calcium	0.10	0.15	0.21	0.12	0.049
Chromium	0.015	0.0012	0.00048	0.012	0.00078
Copper	0.036	0.011	0.016	0.023	0.011
Iron	0.092	0.022	0.033	0.035	0.018
Lead	0.063	----	----	----	----
Magnesium	0.70	0.66	0.52	0.58	0.76
Manganese	0.015	0.0074	0.008	0.006	0.004
Nickel	0.011	0.021	0.0018	0.0035	0.0075
Silicon	1.2	2.5	1.7	0.88	0.25
Titanium	----	0.013	0.016	0.0045	----
Cobalt	----	----	----	----	----
Zirconium	0.036	0.10	0.062	0.052	----

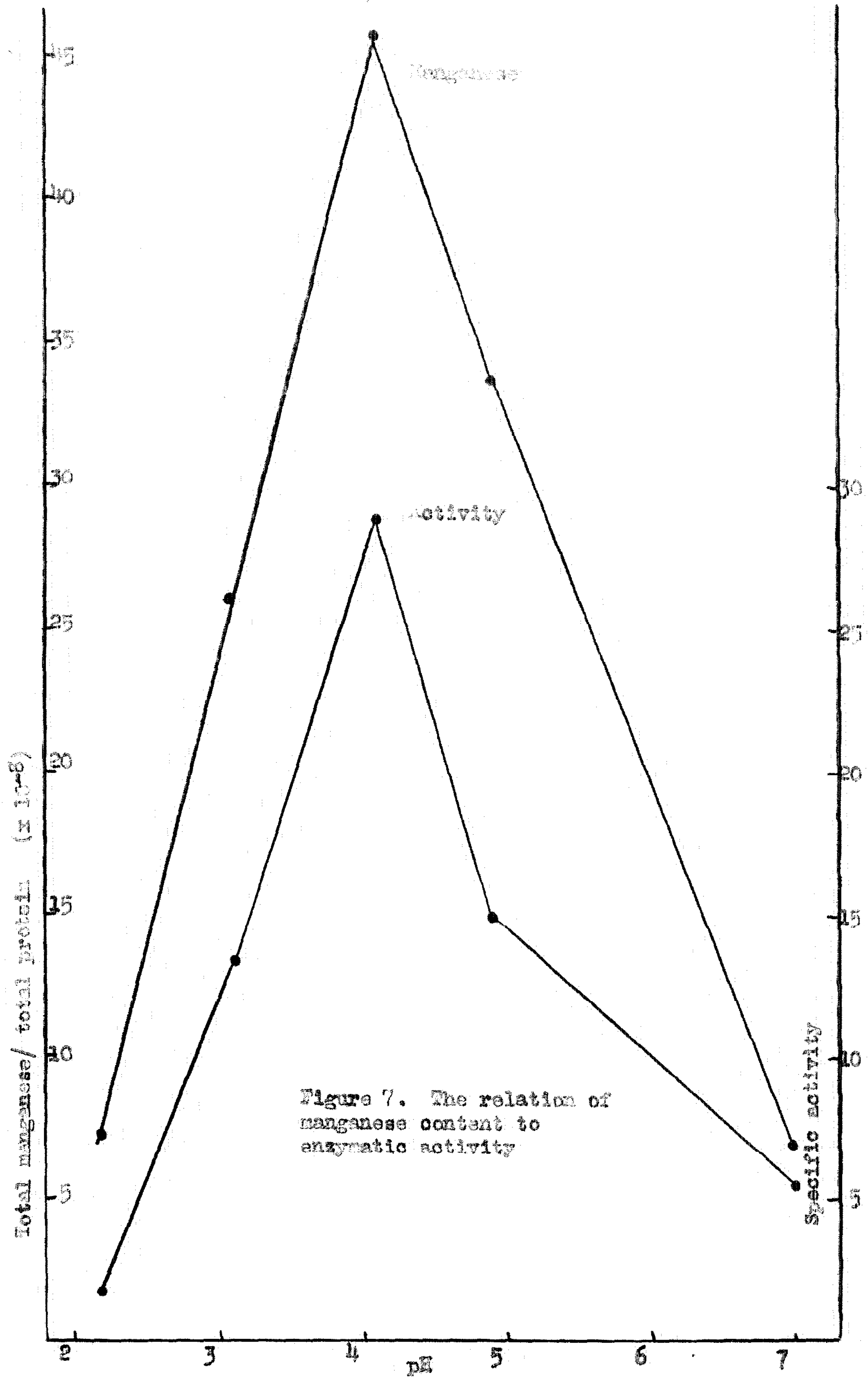


Figure 7. The relation of manganese content to enzymatic activity

On the same abscissa, but on the ordinate to the left, is plotted the total amount of metal divided by the total protein. It is considered that a good agreement between these two functions is demonstrated, in view of the over-all accuracy of the experiment.

The curves for the other metals mentioned are similar. Figure 8 shows the magnesium content, and it can be seen that the agreement between metal and enzyme activity is less direct. Figure 9 shows that the amount of chromium has very little bearing on enzyme activity. It is interesting to observe that cobalt is absent from all the samples. Plants are not known to require cobalt in their nutrition, and therefore cobalt is not likely to be a natural activator.

We are still faced with the problem of deciding which of these metals is the preferential cofactor for the enzyme, and it is a difficult problem to resolve. In systems such as laccase, copper alone is able to reactivate the inactive form of the enzyme. Catecholase, sometimes called tyrosinase, since it is a polyphenol oxidase, can also be reactivated by the addition of copper, cobalt, manganese, or ferric iron after inactivation with cyanide, diethyldithiocarbamate, or potassium xanthate (51). Although the effectiveness of each metal changes with the pH of the system, copper is always the strongest activator. A similar phenomenon occurs with the acid phosphatase system.

6) The change in the effectiveness of metal reactivators with pH shift. Enzyme partially inactivated by dialysis was incubated with NPP and 10^{-4}M. metals at pH 6.0. The following amounts of nitro-

Figure 8. The relation of magnesium content to enzymatic activity

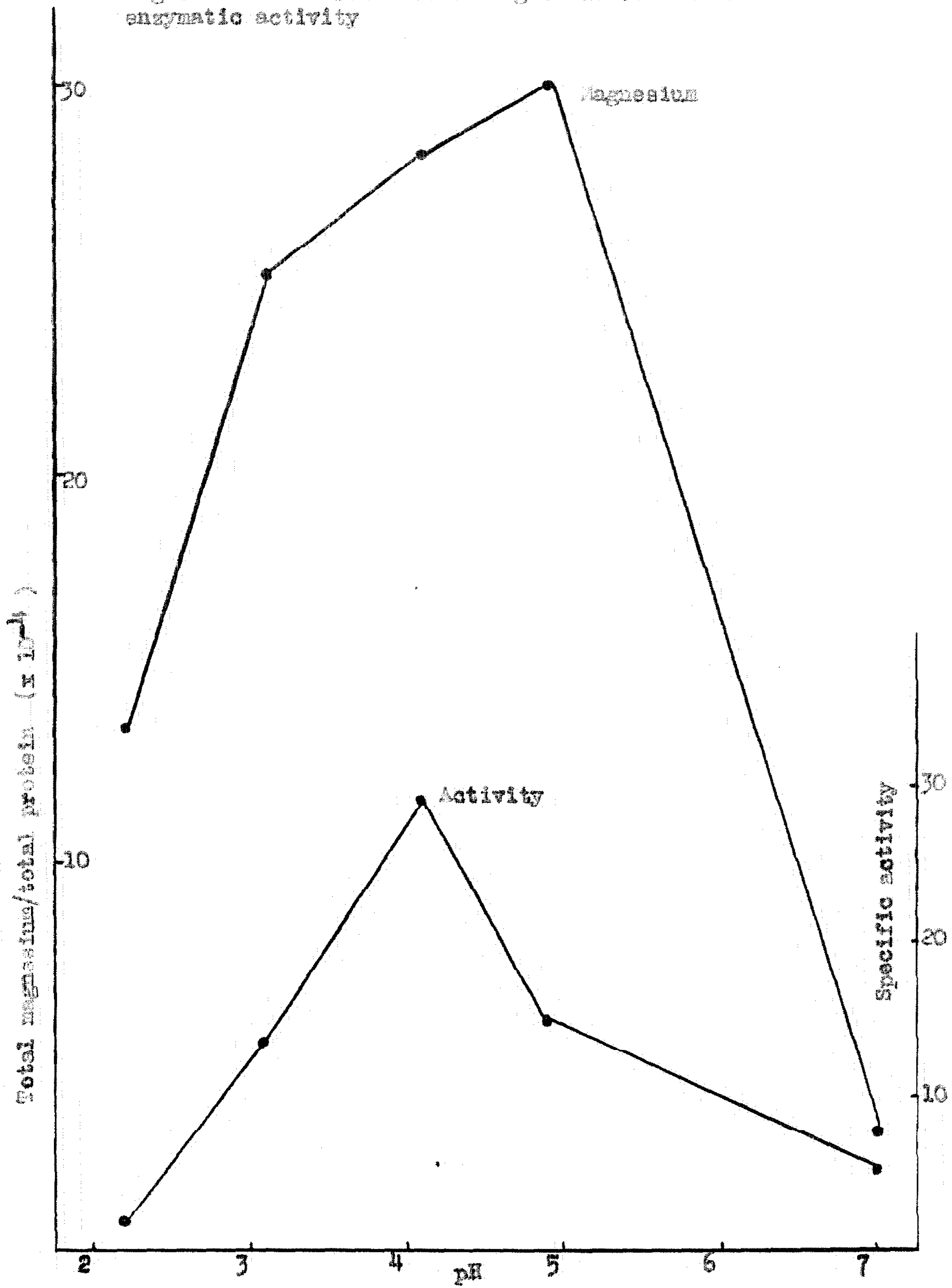
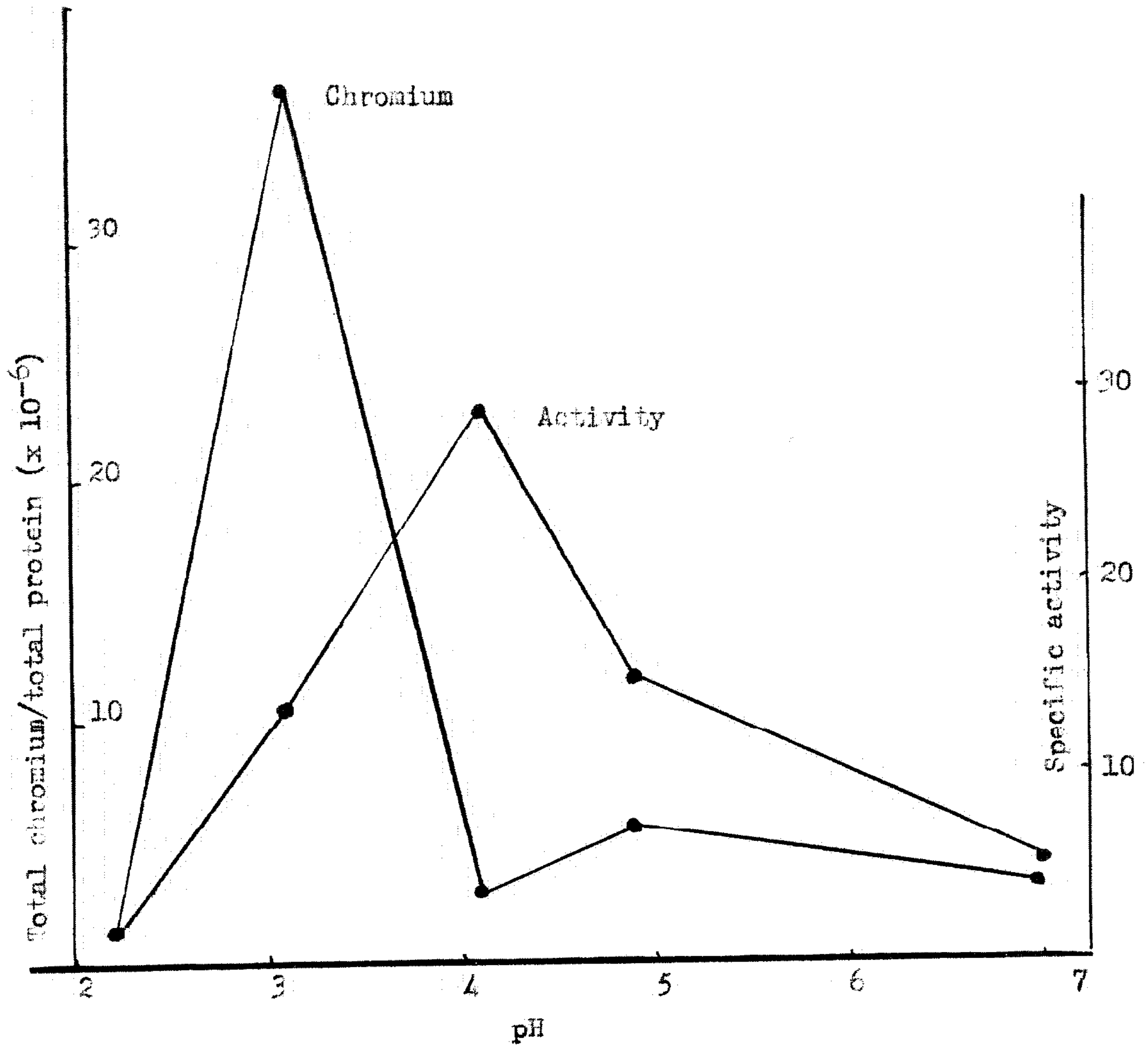


Figure 9. The relation of chromium to enzymatic activity.



phenol were liberated after 40 minutes hydrolysis: control 4, magnesium 8, cobalt 8, and copper 10. These levels are higher for both magnesium and cobalt in comparison with copper in the system at pH 5.0.

Although copper is the most effective activator under all the conditions tested, the accumulated evidence presented here is hardly sufficient to justify the conclusion that copper is in fact the native cofactor for the acid phosphatase. It is suggested that within the cell the architecture of the enzyme may be remodified in a manner more gentle than acid dialysis, and that different phosphatases could be reconstructed with different physiological roles, depending upon the relative concentrations of the metals in the vicinity of the enzyme molecule. Such a hypothesis is admittedly difficult to test, but an approach could be made, once we are in possession of more details about the phosphatases, by studying these enzymes in plants grown with various mineral deficiencies, and examining their pH optima, substrate specificity, and other characteristics.

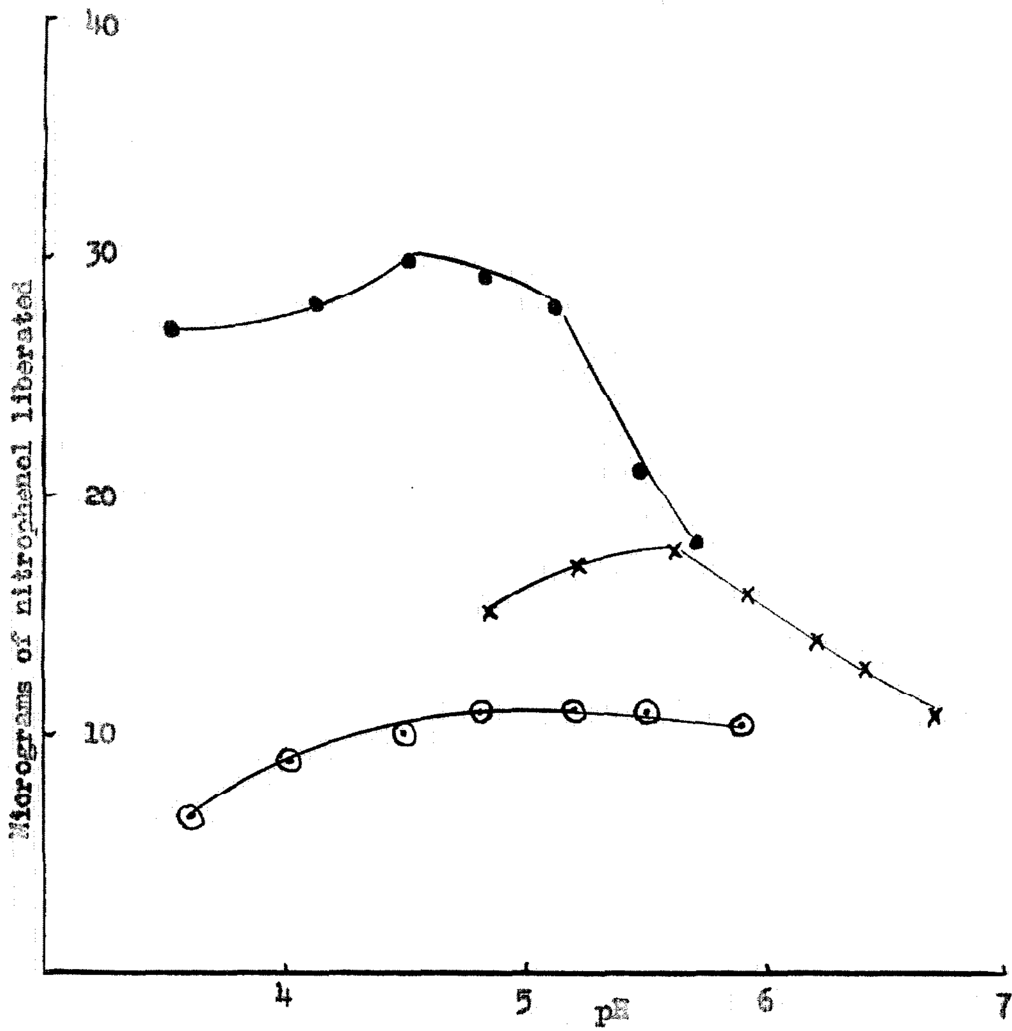
7) The shift in pH optima of metal reactivated enzyme. That phosphatases with different metal prosthetic groups may have such altered characteristics may be inferred from the pH optima curves shown in Figure 10. Partially inactivated acid dialyzed phosphatase was incubated with NPP in 0.1M maleate buffer of pH 3.6 to 6.7. Copper and cobalt were added at 10^{-4}M and magnesium at 10^{-3}M. Instead of a fairly sharp peak in the optimum region, the enzyme reconstructed with metal has a much broader optimum. Since pH optimum may be a function of the dissociation of substrate or enzyme, the plateau

Figure 10. The pH optima of inactivated leaf phosphatase restored by means of metals

● Copper

× Magnesium

⊙ Cobalt



type of optimum must mean that the surface charges on the reconstructed enzyme have been considerably altered, and very likely do not approach the configuration shown in the native state.

8) Conclusion. These data from this section indicate for the first time the possibility that copper may function as the prosthetic group of a non-oxidative enzyme. In any case, it has been demonstrated that the acid phosphatases of green leaves can be inactivated and restored to activity with a variety of divalent metal ions, although copper seems to be the most effective.

VI. The comparative enzymology of acid phosphatases.

There are numerous aspects of the phosphatases which have a comparative as well as an intrinsic interest. A few experiments will be reported here for the purpose of characterizing the acid phosphatases of green leaves in terms of similar experiments performed with other phosphatases.

1) The action of zinc. Cohn (32) reported that zinc inhibited an alkaline plasma phosphatase which was activated by cadmium. It was found that zinc at 10^{-2} M was completely inhibitory to the leaf phosphatases, and that zinc at 2×10^{-3} M still inhibited by 70%.

2) The action of cadmium. The effect of cadmium on leaf phosphatase was tested by incubating whole cytoplasm with cadmium at 10^{-3} M. About 25% inhibition was obtained. A highly active preparation purified by dialysis at pH 3 was inhibited by about 45%. The whole cytoplasm may be inhibited less because of the presence of substances such as amino acids which are capable of blocking the

effective concentration of metals by chelation. Since the acid phosphatase was inhibited by both cadmium and zinc, no congruence between this enzyme and the alkaline phosphatases can be found in this experiment.

3) The action of beryllium. Beryllium is known to be a powerful inhibitor of alkaline phosphatases and of the adenosine triphosphatases (52). It is relatively ineffective on the acid phosphatases. DuBois <u>et al</u>. (53) state that the inhibition of alkaline phosphatases can be reversed by manganese but not by magnesium, while Aldridge (54) found reversal with magnesium. Beryllium is thought to inhibit by preventing the attachment of the magnesium to the enzyme. One would not expect beryllium to inhibit the acid phosphatases unless they are also magnesium activated enzymes. The action of beryllium on leaf acid phosphatases was tested by dissolving $BeCO_3$ in concentrated HCl and diluting the solution to give the required concentrations. Spinach whole cytoplasm phosphatase was incubated with NPP and beryllium at 10^{-3} M, 10^{-4} M, and 10^{-5} M. The latter two concentrations gave no inhibition, but at 10^{-3} M an inhibition of 25% to 30% was obtained. These values do not compare with the 50% inhibition at approximately 10^{-6} M found with alkaline serum phosphatase, and may be taken as evidence for the lack of participation of magnesium in the activity of acid phosphatases of green leaves.

4) The action of ascorbic acid. In view of the controversy about the action of ascorbic acid on both acid and alkaline phosphatases, it was thought to be of interest to see how the leaf phosphatases responded to this substance. Tobacco cytoplasm (undialyzed) and a

sample dialyzed at pH 3 against 0.1M citrate buffer were incubated with NPP with and without ascorbic acid made up to 10^{-2} M in acetate buffer adjusted to pH 5. The ascorbic acid was found to have no effect on either the dialyzed or undialyzed sample of enzyme.

5) The action of fluoride and cyanide. Massart and Vandendriessche (55) have classified some alkaline phosphatases on the basis of their inhibition by fluoride and cyanide. They find, for example, that liver phosphatase is inhibited by fluoride, but not by cyanide, while kidney, bone, and intestinal phosphatases show the reverse characteristics. Sugar beet leaf phosphatase was inhibited 50% to 55% by 10^{-3} M fluoride, while the same concentration of cyanide caused no immediate inhibition. Cytoplasm dialyzed 24 hours against 10^{-3} M cyanide, however, is about 30% inhibited. This is a rather surprising result in view of the commonly accepted belief that cyanide inhibits metal enzymes, and one would certainly expect a copper enzyme to be readily inhibited. However, cytochrome c is not inhibited by cyanide, although it belongs to a class of porphyrin enzymes which generally are. It may be that the metal group in the phosphatase molecule is somehow protected by steric factors which prevent the cyanide from acting. The relatively small amount of inhibition produced by chelating agents may be accounted for in the same manner.

6) The action of amino acids. In view of the claim made by Roche (56) that dialyzed alkaline phosphatases are more strongly activated by alanine and a metal than by a metal alone, the possibility of a similar effect with the acid phosphatases was investigated. Spinach phosphatase partially inactivated by acid dialysis was incubated

with NPP at pH 5.0 with the addition of the metals copper and cobalt at 10^{-3} M and the amino acids at 10^{-2} M. These data reported in Table 10 indicate that alanine is neither an inhibitor nor activator of dialyzed acid phosphatase. Cysteine, which inhibits alkaline phosphatases is not an inhibitor of undialyzed leaf acid phosphatases, but in the experiment reported here the inhibition may be due to the ability of cysteine to form complexes with metals, so that the apo-enzyme remains without a metal cofactor.

7) Phosphotransferase ability. The last aspect of the acid phosphatases to be discussed is their ability to transphosphorylate without mediation by nucleotides. Axelrod (57) first reported the transfer of phosphate from a donor such as NPP to various alcohol acceptors without the appearance of free phosphate in the medium. This reaction was catalyzed by citrus juice phosphatase. Appleyard (58) reported a similar phenomenon with prostate acid phosphatase using phenolphthalein phosphate as the donor. Meyerhof (59) found an alkaline phosphatase to have a similar function.

Although no physiological role of this phosphotransferase activity has been shown, it is conceivable that some phosphatases are able to effect the transfer of phosphate within living tissues. It was of interest, therefore, to see if the mixture of phosphatases present in green leaves was capable of carrying out this type of reaction. The phosphate donor used was NPP, and the acceptor methanol. The procedure followed was that of Axelrod (57). The enzyme was prepared from spinach cytoplasm by heating at 58°C. for three minutes and bringing the cooled supernatant to pH 4.0 with acetic acid. The

TABLE 10

The lack of synergistic action of metals and amino acids on inactivated acid leaf phosphatase.

Treatment	µg NP liberated after minutes:	
	10	20
Control 1	3	6
" " + alanine	2	4
" " + cobalt	8	17
" " + cobalt and alanine	8	16
" " + cysteine	2.5	5
" " + copper	22	41
" " + cysteine and copper	3	6
Control 2	4	8
" " + alanine	4	8
" " + copper	14	29
" " + copper and alanine	14	29

enzyme remaining after acidification was dialyzed against 0.1M maleate buffer at pH 7.0. The nitrophenol split off was measured as usual, and the free phosphate estimated by the Allen method (50). In the control system, water was used in place of the methanol acceptor.

The percent hydrolysis of the organic phosphate is calculated as follows: in the control, (0.97 x 100)/0.92 = 105% of the phosphate was liberated, while in the presence of methanol (0.58 x 100)/1.06 = 54.6% was liberated. This means that about 45% of the phosphate was transferred.

Although phosphotransferase action has been demonstrated, the question remains: is this action accomplished by the phosphatase itself or by a separate enzyme which has not yet been distinguished from the phosphatase? Appleyard (60) is of the opinion that two enzymes are involved, one a phosphatase, the other a transphosphorylase, since she was able to obtain differential enrichment of the two activities during fractionation of tissue extracts with ammonium sulfate. Axelrod (61), on the other hand, considers that the phosphotransferase activity is due to a phosphatase, but that in the mixtures generally used for assay, more than one type of phosphatase is present. Some phosphatases are capable of phosphotransferase activity and some are not. Axelrod found no differences in the ratio of phosphatase to phosphotransferase activity during salt fractionation, partial inactivation by heat, or inhibition by arsenate. The evidence presented in this thesis on the multiplicity of phosphatases would lend support to Axelrod's theory, and it should now be possible to test the various leaf phosphatases for phosphotransferase ability.

TABLE 11

The transfer of phosphate from NPP to methanol with the aid of green leaf acid phosphatases.

Minutes of hydrolysis	µM P/ml. hydrolyzed		µM NP/ml. hydrolyzed	
	No acceptor	Acceptor	No acceptor	Acceptor
0	0	0	0.086	0.115
15	0.97	0.58	0.92	1.06
30	1.23	0.71	1.06	1.12

VII. Summary.

It has been found that the bulk protein common to leaves of dicotyledenous plants is separable from the phosphatase enzyme which had been found associated with it. This separation was accomplished both by differential centrifugation and by salt precipitation. The two proteins were further distinguished on the basis of their stability toward heat and acid. The enzyme is quite stable under the conditions used, while the bulk protein is completely denatured.

This enzyme was further shown to be a mixture of isodynamic acid phosphatases with characteristically different pH optima. Fractionation was obtained by adsorbtion and elution from a Dowex column, by dialysis against buffers of different pH, and by specific antibody absorbtion. The latter process revealed enzymes of different K_M values.

It was possible to inactivate the enzyme completely by dialysis against citrate buffer about pH 2. This inactive preparation was re-activated by means of various divalent metals, among which copper was the most efficient. A spectrographic analysis of the ash of cytoplasm dialyzed against increasingly acid buffers revealed a direct relation between the degree of enzymatic activity and the quantity of copper, iron, magnesium, calcium and manganese present. Cobalt, which is also capable of reactivating the enzyme, was absent from the ash.

The enzyme was found to be inhibited by zinc and cadmium, but beryllium had very little effect. Neither ascorbic acid or alanine proved to be cofactors, as has been claimed for some alkaline phosphatases. The enzyme is inhibited by fluoride, but cyanide inhibits

only after dialysis. The enzyme was also shown to have phosphotransferase activity as has been reported for both acid and alkaline phosphatases from other sources.

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Part II. Studies on the role of indoleacetic acid in cell elongation.

I. The problem.

Auxins are organic substances which in low concentrations are capable of inducing in plant tissues a variety of characteristic responses of which cellular elongation is the most typical. Auxins have been recognized as definite chemical entities for slightly more than 20 years, although the concept was stated by Sachs in the last century.

Studies on the mechanism of auxin action have been divided into four categories in a recent review by Bonner and Bandurski (1). These are:

1. studies of the effects on individual enzymes;
2. studies of the effects on the chemical composition of the plant;
3. studies of the effects on respiration;
4. studies of the effects on water uptake.

In the review section which follows, these effects will be discussed in some detail, but for the present it may be stated that no clear interpretation of the actions of auxins has as yet been generally accepted.

The influence of auxin was sought in a number of different metabolic pathways, including the formation of soluble components and constituents of the cell wall itself. C^{14} labeled sucrose, acetate, and amino acids were used for this purpose. The C^{14} does not remain in the metabolite, but appears in a host of other compounds.

It is the intent of this study to determine the rate of incorporation of this radioactive label into the various plant constit-

uents under the influence of exogenous auxin. Thus the rate of incorporation of carboxyl C^{14} from glycine and leucine into the proteins of corn and Avena coleoptiles was studied. A second group of experiments traced the label of carboxyl C^{14} acetate into the plant lipid constituents. A third series of experiments with both acetate and uniformly labeled sucrose was designed to trace qualitative or quantitative differences which might appear in cell wall components fractionated into various solubility classes. These differences were looked for by means of chromatography and radioautography. Finally, the components obtained after the above solubility fractionation were oxidized to CO_2, and the amount of radioactivity recovered was determined.

The study presented here offers a new approach to the problem of the mechanisms by which auxin induces cell elongation. By using radioactive isotopes, it has been possible to study the rate of incorporation of the label from a particular metabolite into various constituents of growing cells, and to examine these rates in the presence and absence of indoleacetic acid (IAA).

II. A review of the literature.

The recent appearance of several excellent reviews (1, 2, 3, 4, 5) on the subject of auxins makes a detailed summary of the literature seem unwarranted. It will be necessary for clarity, however, to present the background of the problem.

The review will consider the four categories of Bonner and Bandurski referred to above. Of the 19 enzymes listed in that review as having been tested for sensitivity to auxin *in vitro*, auxins were either inhibitory or had no effect. On the other hand, it has been

possible to demonstrate increases in activity of certain enzyme systems within the tissue studied, after the application of physiological concentrations of auxins. For example, tobacco pith grown in vitro with IAA may have more ascorbic acid oxidase than similar tissue grown without IAA.(6) Olsen (7) has shown that corn roots grown in the presence of 2,4-dichlorophenoxyacetic acid (2,4-D) have more phosphatase activity than those grown without it. Galston, however, (8) has shown a decrease in catalase content in Jerusalem artichoke grown in vitro with either IAA or 2,4-D. Thus the effects of auxins on individual enzyme systems are variable, and these effects cannot account for the mechanism by which auxin influences the process of cell elongation.

Christiansen and Thimann have in the past few years made a rather complete analysis of the constituents of pea stems as affected by IAA (9-12). When they compared IAA treated sections with suitable controls, they found a greater loss of sucrose, fats, organic acids, and amino acids in the presence of IAA, and concluded that a greater synthesis of proteins concomitant with growth was of importance. Examination of these data, gathered from this series of papers in Table 1, shows that these differences are, however, very small. While striking changes occurred in cell elongation, auxin induced changes in comparison are small, except for the increased utilization of sucrose which these authors suggest is used in part by increased respiration and in the deposition of cell wall material. Their conclusions that "cell elongation does involve considerable synthesis of protein", and "organic acid metabolism which is primarily responsible for growth" seem unwarranted (13).

TABLE 1

Auxin induced changes in pea stems. Data from Christiansen and Thimann (9-12).

	Percent of initial dry weight		
Fraction	Initial	Control	Treated
Sucrose	5.59	2.41	1.61
Total phosphorus	.27	.28	.29
Cell wall			
Pectin	5.2	5.1	5.5
Hemicellulose	10.7	11.8	12.6
Cellulose	7.0	8.0	9.1
Ether soluble	.3	.3	.2
Protein	3.4	4.4	4.3
Cell wall deposited	——	3.0	5.1
Organic acids	6.2	5.0	4.7
Total lipids	8.95	6.64	6.18
Amino acids	13.7	1.9	.6
Protein	16.7	23.3	24.4

Burström, on the other hand, finds that the elongation of wheat roots results in only a small amount of protein synthesis, and concludes that there can be no causal relation between the large amount of growth and the little protein formed (14).

The relation between growth and respiration is more readily assessed. Mitochondria are responsible for most of the pyruvate oxidation in mung bean seedlings, but the rate of this oxidation *in vitro* is unaffected by added IAA (15). Growth may be partially or completely inhibited while respiration remains the same, as for example with arsenate. Dinitrophenol (DNP) inhibits growth by 88% while respiration is increased 38% (16). The uncoupling of energy-rich phosphorylations by DNP suggests that growth is in some way dependent on the availability of adenosine triphosphate.

The process of water uptake is believed to be aerobic according to the results of Reinders, Rosene, and others (17) since it is inhibited by anaerobiosis, cyanide, or azide. van Overbeek found (18) that tomato roots grown in hypertonic mannitol took up water, and that this process is inhibited by cyanide. In these conditions, water uptake can hardly be interpreted as being due to osmotic pressure differences, and one must invoke the concept of metabolically controlled water uptake.

That auxin induces water uptake above control values has been established by a number of workers (17). Commoner *et al*. (19) concluded that auxin stimulated salt absorption which in turn is reflected by an increased water uptake, but van Overbeek (20) showed that auxin increased water uptake of potato discs in distilled water, a result which also suggests the possibility of "active" water uptake.

III. <u>The rate of incorporation of C^{14} from carboxyl labeled amino acids into the proteins of Avena and corn coleoptiles as influenced by auxin.</u>

1) Procedure. It has been pointed out that Christiansen and Thimann considered protein metabolism to be greatly influenced by the presence of auxin. A study of the rate of incorporation of C^{14} from carboxyl labeled amino acids offers a more dynamic approach to the problem of auxin induced changes during cell elongation.

Avena or corn was planted in washed vermiculite and grown at 25°C. in a room with low intensity red light. On the fourth day, the leaves were removed, and 10 mm. sections were cut beginning 5 mm. back from the tip. Sections were floated on 3% sucrose in 0.03 M potassium maleate buffer of pH 4.6. IAA (3 mg/l.) was added or withheld from a total of 20 ml. of solution. C^{14} carboxyl labeled glycine or leucine was added in a concentration of 0.002 M. The sections were kept in the dark at 25°C., and after various time intervals they were rinsed with buffer and their length was measured. The sections were then ground in a mortar and homogenized with a lucite pestle in a fitted test tube. To the slurry, trichloracetic acid was added to give a final concentration of 5%. This mixture was heated for 5 minutes at 90°C., cooled, and allowed to stand overnight in the refrigerator. The precipitate was washed 10 times with TCA, twice with acetone, and twice with ether.

In the experiments with Avena, the washed precipitates were oxidized to CO_2 by the mixture of Van Slyke-Folch (21). The CO_2 was trapped in 20 ml. of 0.7N NaOH which was held in two towers packed

with glass beads. The 0.7N NaOH solution was made up in CO_2 free water from a saturated solution of NaOH. This procedure removes almost all traces of carbonate.

After the oxidation was completed, the towers were thoroughly rinsed with CO_2 free water. The two samples were combined, and 2.5 ml. of saturated $BaCl_2$ was added to a total of 60 ml. The $BaCO_3$ precipitate was allowed to settle overnight.

Plaques for counting the radioactivity in the $BaCO_3$ samples were prepared by filtering the precipitate through tared, hardened 2 cm. filter paper discs held on a sintered glass funnel. The precipitates were washed with absolute ethanol, and dried for 30 minutes at 95°C. They were then cooled in a desiccator, re-weighed, and mounted on aluminum holders preparatory to being counted with a thin mica end window Geiger Müller counter built at this Institute. All counts except background were in excess of 2000 in order to reduce the counting error, and were corrected for self absorption according to a table prepared by Professor Borsook.

In some instances, when enough $BaCO_3$ was available for more than two duplicates, all the excess was weighed on a single filter paper, but not counted. The sum of the entire $BaCO_3$ recovered was used to determine the total activity.

2) The incorporation of C^{14} from leucine into Avena coleoptile proteins. The use of Avena was discontinued owing to the small amount of protein which could be recovered from a limited number of sections, and the subsequent experiments in this group were done with corn

coleoptiles. The results from a typical experiment are shown in Figure 1. The specific activity, counts/minute/mg. $BaCO_3$, is plotted against the duration of incubation in hours. The increase of incorporation of C^{14} with time is quite small, but in general, the rate is the same with or without IAA. During the 17 1/2 hours, the control sections grew from 10 to 10.7 mm., while those treated with IAA grew to 13.6 mm.

3) The incorporation of C^{14} from glycine into corn coleoptile proteins. A very much clearer picture was obtained with corn coleoptiles which were treated exactly as described above, up to the point of precipitation of the proteins with TCA and subsequent washing. The precipitates, instead of being converted to CO_2 at this point, were transferred to round bottom flasks with 20 ml. of hydrochloric acid and diluted with an equal volume of water. The protein was then hydrolyzed by refluxing for 20-24 hours at 140°C. The hydrolysate was evaporated to dryness under vacuum, and redissolved in distilled water. Aliquots were removed for the determination of amino nitrogen by the method of Moore and Stein (22). The carboxyl carbons of the remaining amino acids were converted to CO_2 by the ninhydrin method of Van Slyke _et al_. (23), and the CO_2 was trapped in NaOH as has been described above. The preparation of $BaCO_3$ plaques, and the counting procedure was also the same as that already described.

In the experiment reported in Table 2, carboxyl labeled glycine was used in the manner described. The sections without IAA grew from 10 to 12 mm. in 6 hours, and to 17.6 mm. in 20.5 hours. Sections grown

Figure 1. The similarity of the incorporation of C^{14} from carboxyl labeled leucine into the proteins of Avena coleoptile in the presence and absence of IAA.

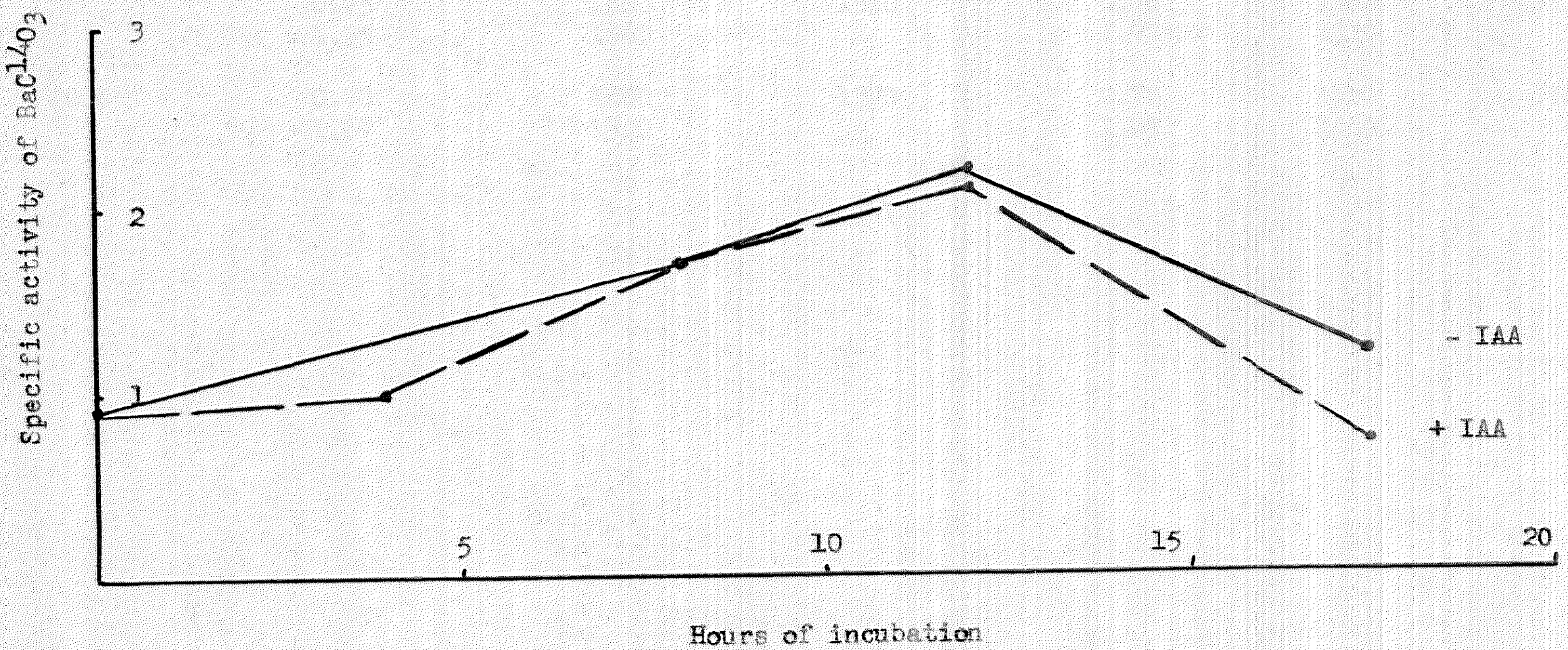


TABLE 2

The constancy of protein bound amino nitrogen of corn coleoptiles grown in the presence and absence of IAA.

Time (hours)	Without IAA			With IAA		
	µg. NH_2-N in aliquot	µg. NH_2-N total	Average	µg. NH_2-N in aliquot	µg. NH_2-N total	Average
0	0.72	1440	1470			
	1.87	1500				
6	0.75	1500	1530	0.70	1400	1410
	1.95	1560		1.77	1420	
20.5	0.70	1400	1370	0.78	1560	1560
	1.67	1340		1.95	1560	

in the presence of IAA increased to 14.5 and 20.5 mm. respectively. Thus there was a definite increase in length as a result of IAA treatment.

After hydrolysis of the samples, amino nitrogen was determined on a 0.2 and a 0.5 ml. aliquot. These data are reported in Table 2. The extreme difference in amino nitrogen as between all the samples is 190 micrograms or 12% of the average. The duplicates obtained from the two different aliquots vary by as much as 60 micrograms. Since a large multiplication factor was used, it is considered that the actual differences in amino nitrogen among the samples is very small, and that very likely no net gain or loss of protein occurred during the period of the experiment. We cannot yet say on the basis of these data above if there was any protein synthesis or degradation.

For an answer to this question, it is necessary to examine the results with respect to the amount of radioactivity incorporated into the proteins and determined as $BaC^{14}O_3$. These results are shown in Table 3. It can be seen that both the specific activity and the total activity of the samples with or without IAA are very similar, and although no statistical analysis has been made, it is evident that no significance can be attributed to the differences.

We may conclude that the C^{14} label from the glycine appeared in the protein residues, and that protein synthesis therefore occurred. The course of this synthesis is apparent from Figure 2, which shows the increase in specific activity of the C^{14} label in the proteins over a period of time. Since the rate of incorporation of the C^{14} is the same in both cases, and the specific activity and total amino nitrogen is

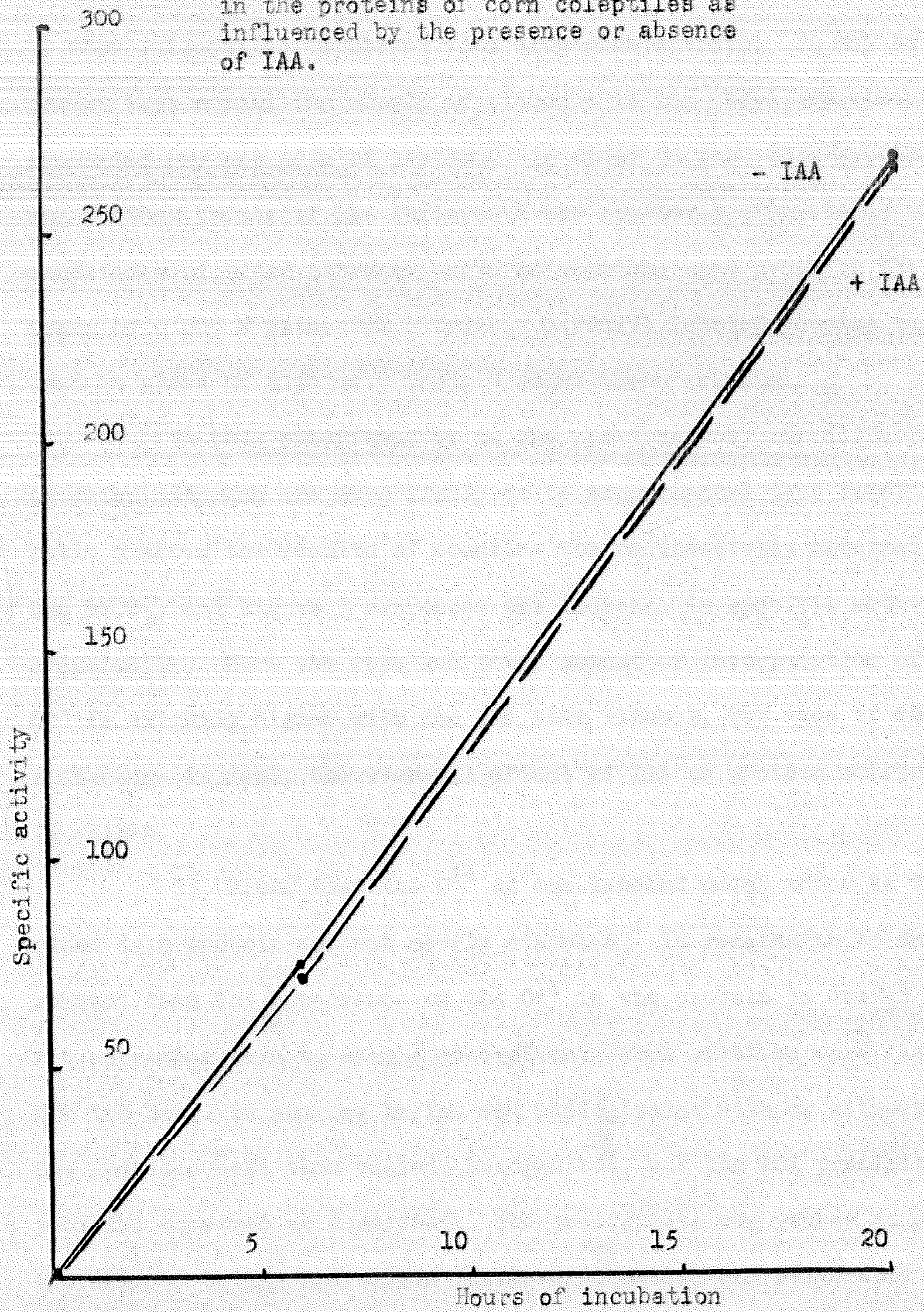
TABLE 3

The lack of difference of incorporation of C^{14} from glycine into the proteins of corn coleoptiles in the presence and absence of IAA.

Time (hours)	Treatment	Mg. $BaCO_3$	Counts/minute	Specific[1] activity	Average	Total counts/minute
0		13.1	0			
6	-IAA	7.8	546	78.8	75.6	3100
		10.7	640	72.4		
	+IAA	9.1	529	67.4	71.5	3400
		11.0	683	75.6		
20.5	-IAA	8.0	2084	294.	268.	11000
		9.5	1882	243.		
	+IAA	8.7	1844	244.	266.	11800
		11.1	2583	288.		

1. Specific activity: counts/minute/mg.

Figure 2. The similarity of increase in the appearance of C^{14} from glycine in the proteins of corn coleptiles as influenced by the presence or absence of IAA.



also the same, we may conclude that during cell elongation, IAA does not influence the protein metabolism to any significant extent.

4) The incorporation of C^{14} from leucine into the proteins of corn coleoptiles incubated with exogenous nitrate. It may be objected that a limiting supply of nitrogen in the above experiments prevented any net gain of protein. In order to test this hypothesis, and further to see if IAA influenced the synthesis of proteins under conditions of added nitrogen, corn coleoptiles were grown in the presence of 0.005 M potassium nitrate. Carboxyl labeled leucine was used in place of glycine. Table 4 shows these results.

In this experiment as in the previous one, the differences in amino nitrogen are more likely to be experimental than intrinsic. Table 5 shows the results of counting the radioactivity obtained in the $BaCO_3$, and Figure 3 expresses the increase in specific activity graphically. Here the rate and total amount of incorporation of the C^{14} is slightly higher with the IAA than without, but even if the difference is real, the over-all effect of IAA on protein metabolism is slight.

5) Proof that the C^{14} of the labeled amino acids is incorporated into protein and not merely adsorbed. It remains to be demonstrated that the appearance of the C^{14} in the protein is due to incorporation rather than to simple adsorption. Corn sections were floated for two hours in sucrose buffer and radioglycine with or without IAA. The sections were then washed, homogenized, and the TCA precipitable proteins obtained as described. The precipitate was washed as usual, transferred to the hydrolysis flasks with water, and evaporated

TABLE 4

The constancy of protein bound amino nitrogen of corn coleoptiles grown with potassium nitrate in the presence and absence of IAA.

	Without IAA			With IAA		
Time (hours)	µg. NH_2-N in aliquot	µg. NH_2-N total	Average	µg. NH_2-N in aliquot	µg. NH_2-N total	Average
0	0.60 1.42	1200 1140	1170			
5	0.57 1.42	1140 1140	1140	0.57 1.53	1140 1220	1180
16.5	0.65 1.62	1300 1300	1300	0.53 1.42	1060 1140	1100
24	0.65 lost	1300	1300	0.65 1.57	1300 1260	1280

TABLE 5

The lack of difference of incorporation of C^{14} from leucine into the proteins of corn incubated with nitrate in the presence and absence of IAA.

Time (hours)	Treatment	Mg. $BaCO_3$	Counts/minute	Specific activity	Average	Total counts/minute
5	-IAA	7.0	227	35.8	39.8	1190
		11.8	406	43.8		
	+IAA	11.0	364	40.2	37.9	1125
		7.5	239	35.6		
16.5	-IAA	9.7	943	114.4	112.0	4670
		14.5	1190	109.5		
	+IAA	10.7	1110	125.0	122.3	4340
		8.8	913	119.6		
24	-IAA	10.9	1472	164.0	166.7	6400
		14.2	1819	169.5		
	+IAA	11.6	1698	181.0	180.0	6940
		12.6	1775	179.0		

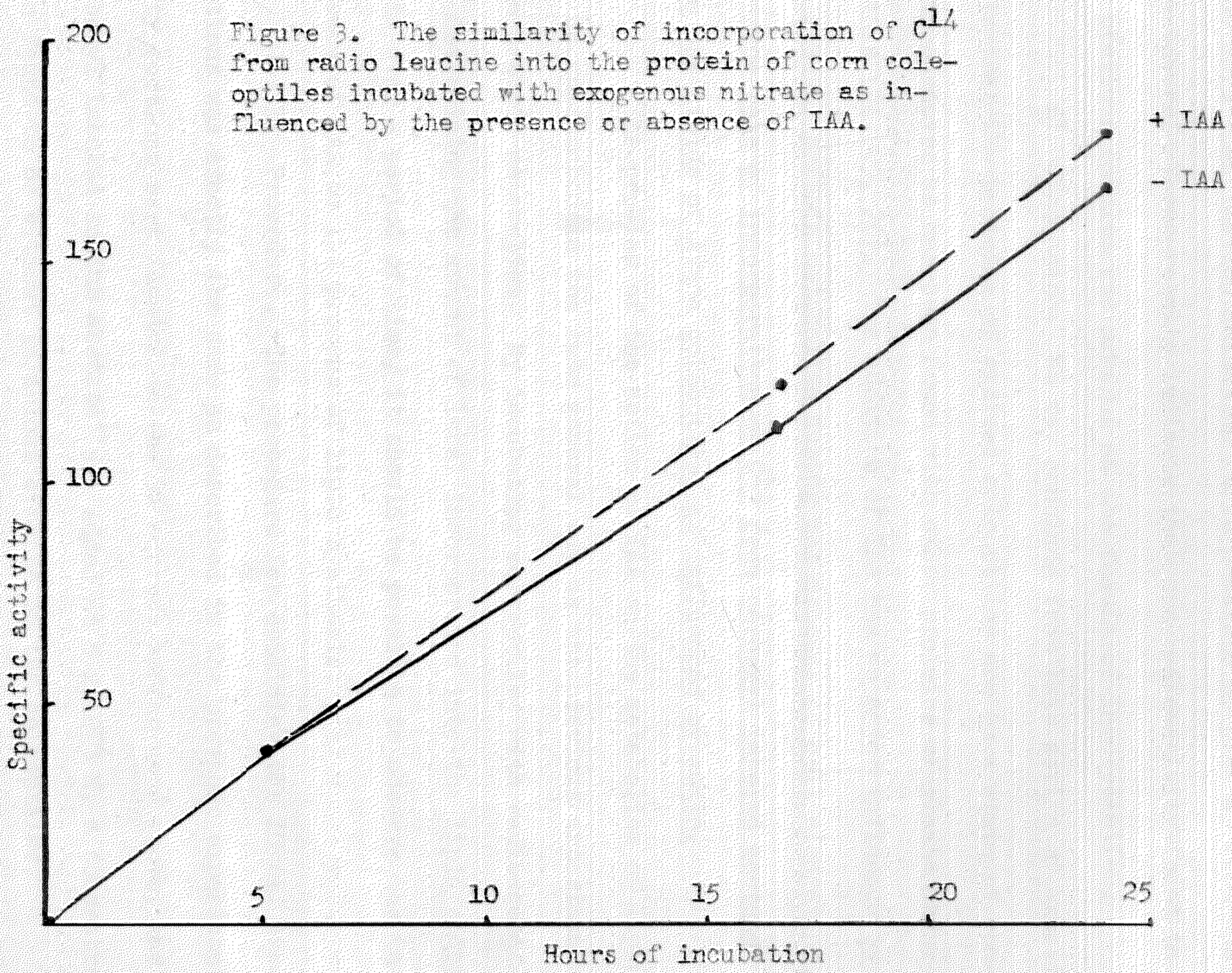


Figure 3. The similarity of incorporation of C^{14} from radio leucine into the protein of corn coleoptiles incubated with exogenous nitrate as influenced by the presence or absence of IAA.

to dryness without being hydrolyzed. The carboxyl carbon was liberated as usual with ninhydrin, and the $BaCO_3$ obtained was counted for radioactivity. With both samples the counts above background were 1.2. This is no more than the contamination it is possible to pick up from the wash towers. These results indicate that the radioactivity found in the previous samples was present as the result of incorporation.

6) Conclusion. We may conclude from this series of experiments that the proteins of excised coleoptiles are in a state of constant turnover as has been found with other proteins of plants and animals (24, 25, 26). The addition of IAA has no major influence on the general protein metabolism during the process of cell elongation.

IV. The rate of incorporation of C^{14} from carboxyl labeled acetate into the lipids of Avena coleoptiles as influenced by auxin.

The problem of the action of IAA on the metabolism of lipids can be approached in the same general way as the previous experiments with protein metabolism. By incubating coleoptiles with radioacetate and determining the amount of label incorporated into the lipid constituents over a period of time, it should be possible to observe the influence of IAA on the process of lipid synthesis.

1) Procedure. The procedure for obtaining the 10 mm. Avena sections was exactly the same as described in the previous experiment. Fifty sections were floated on the 3% sucrose buffer used above, and carboxyl labeled sodium acetate[1.] was added at 5×10^{-4} M, a concentration found to be without injurious effects on the coleoptiles.

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1. The acetate was obtained from Tracerlab, Boston. The activity was 0.5 mc./mM.

The sections were kept in the above solutions in the dark at 25°C. for various periods of time in the presence or absence of IAA (3 mg./l.). The sections were rinsed with water, and dried at 70°C. for 48-72 hours. The dried tissue was extracted in a Soxhlet apparatus with redistilled ligroin of B.P. 65°C. The extract and rinsings were washed three times with 15 ml. of aqueous sodium acetate (10g./l.) in order to dilute out any water soluble acetate which may have been trapped in the ligroin during the extraction process.

In a test experiment, a known quantity of radioacetate in water was added to ligroin, and the mixture shaken with carrier acetate. No radioactivity appeared in the ligroin after the first extract, yet complete recovery of all the radioactivity was obtained only after three washings. This is because very small droplets of water containing the labeled acetate stick to the sides of the vessels.

The washed ligroin was then transferred to a small digestion flask and oxidized to CO_2 by the method already described. The CO_2 evolved was trapped in NaOH as has also been described.

2) Incorporation of C^{14} from carboxyl labeled acetate. This procedure, when applied to lipid quantities of less than one milligram as in these experiments, is unfortunately not uniformly reproducible. Data from a typical experiment is shown in Figure 4. It can be seen that the incorporation of the label increases almost linearly with time in the case of the coleoptiles incubated with IAA. On the other hand, the sections incubated without IAA show an apparent marked discontinuity at 17 hours. This point is held to be an experimental error, and in no single experiment was it possible to obtain a series of consistent curves

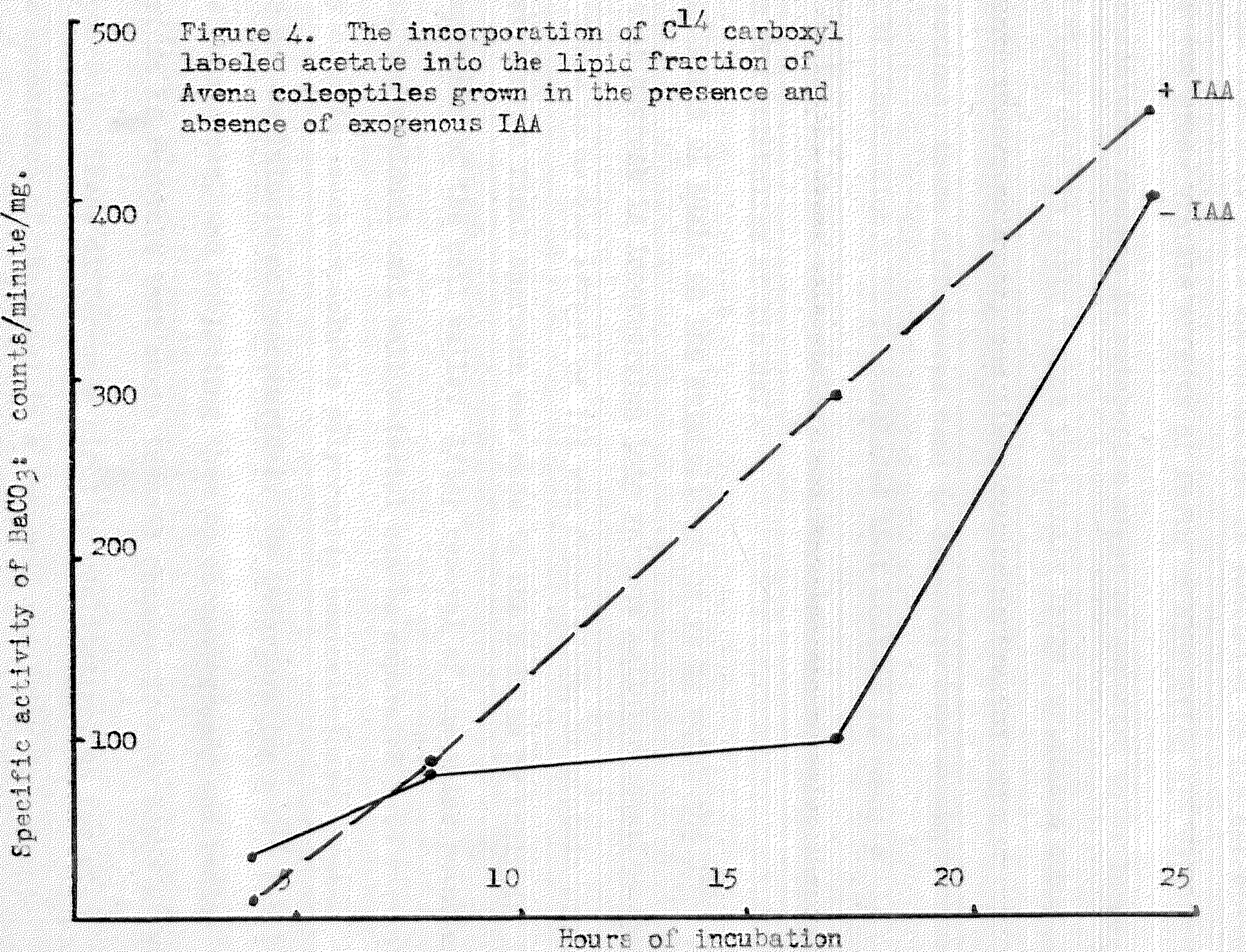

Figure 4. The incorporation of C^{14} carboxyl labeled acetate into the lipid fraction of Avena coleoptiles grown in the presence and absence of exogenous IAA

for both treated and untreated samples.

As an approximation, therefore, it is proposed to present a table of data from various experiments giving the total number of counts at each time interval. This approximation is justified on the assumption that since the gross visible effects of auxin on elongation are very marked, concomitant changes in metabolism might be expected to be large as well. These data in Table 6 show that while large variations occur in the counts recovered at any one time, comparing treated and untreated sections, these differences tend to disappear when summed.

3) Conclusion. It is concluded that acetate is readily converted to fat in Avena coleoptiles, and that IAA has no major influence on the metabolism of lipids.

V. <u>The influence of auxin on the metabolic pathways by which the C^{14} of acetate or sucrose is incorporated into various cellular constituents.</u>

Since no marked effect of IAA was found either in protein or lipid metabolism, a final approach was made to the problem by analysis of the components of the cell walls. After removal of the water soluble substances, the residual cell wall material can be separated into various solubility classes, such as soluble polyuronide hemicelluloses, protopectin, or non-cellulosic polysaccharides. By incorporating a C^{14} label into these substances, and subjecting them to chromatography and radioautography, it is possible that auxin induced changes if they occur might be made apparent. These differences might be qualitative, as for example, the appearance of the label in a compound resulting from treatment or lack of treatment with IAA. Quantitative differences in the amount of labeling as a result of IAA treatment might be inferred from

TABLE 6

The effect of IAA on the amount of radioactivity present in the lipids of Avena incubated with radioacetate. Data pooled from four experiments.

Hours of incubation		Total number of counts	
		Without IAA	With IAA
4		226	330
6		322	570
8		5060	3580
12		3250	3390
16		3260	3890
17		8200	9400
24		9800	10800
	Total	30118	31960

the comparative density of the spots appearing on the radioautograph.

Avena sections were obtained as described in the previous chapter, and floated on 0.03 M potassium maleate buffer of pH 4.6 containing carboxyl labeled acetate at 5×10^{-4} M, or uniformly labeled sucrose[1] at 10^{-3} M. IAA (3 mg./l.) was added to the test samples and withheld from the controls. The sections were kept in darkness at 25°C. for 3 hours, drained, rinsed with water, and ground in a mortar.

1) Fractionation procedure. The fractionation procedure was adapted from those described by Bonner (27) and McColloch (28). The resulting brei was filtered with suction through hardened filter paper on a sintered glass funnel. The brei was washed with hot water, and the control and test sample filtrates made to equal volume. In this fraction one would expect to find the soluble sugars, amino acids, organic acids, and various other compounds.

The residue was divided in half. One part was extracted with 0.2% ammonium oxalate at room temperature for one hour, and the extraction repeated. The solution contains the soluble pectates. The residue was extracted twice with 0.05N HCl at about 85°C. for one hour. This solution contains the protopectins, now solubilized.

The other half of the original residue was extracted with cold 4% NaOH for four hours. The solution contains the soluble polyuronide hemicelluloses. The residue from this treatment was extracted with cold 17.5% NaOH for four hours. The solution contains the non-cellulosic polysaccharides, and the final residue is alpha cellulose. A scheme

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1. The labeled sucrose was obtained from the Nuclear Instrument and Chemical Co., Chicago. The activity was 0.5 uc./mg.

summarizing the above operations is shown in Figure 5. It should be emphasized that this procedure does not result in the preparation of well defined substances, but in all cases a certain amount of overlapping occurs. Thus, some non-cellulosic polysaccharides including some araban, galactan, etc. are removed in the 4% NaOH.

2) Chromatography and radioautography of water soluble and cell wall components. Equal quantities (0.05-0.1 ml.) of each solution was spotted on Schleicher and Schuell No. 589 Blue Ribbon paper 9 inches square. The spots were applied 1 1/2 inches from a corner, and dried with a stream of warm air. The papers were held in a glass trough and equilibrated in an aquarium tank at room temperature. The tank was fitted with a rubber gasket and sealed with plasticene after putting in the chromatograms. Solvents were introduced through a stoppered hole in a glass cover, and the chromatograms developed by descending flow. The paper was air dried, turned through 90°, and developed with a second solvent.

The sheets after development in two dimensions were air dried and enclosed in an 8 x 10 inch Eastman cardboard X-ray film holder with Eastman or Ansco non-screen X-ray film. These films were exposed from one to three weeks. Radioactivity was not detectable in the protopectins, pectates, or non-cellulosic polysaccharides by this method.

Although it was possible to detect the C^{14} label in as many as five clearly distinct spots on a single chromatogram from the water soluble components, as can be seen from Table 7, in no case was any significant difference apparent between the IAA treated and the non-treated coleoptiles. Representative data are shown in Table 7 which

Figure 5. A scheme for the fractionation of cell walls of Avena coleoptiles based on Bonner (27) and McColloch (28).

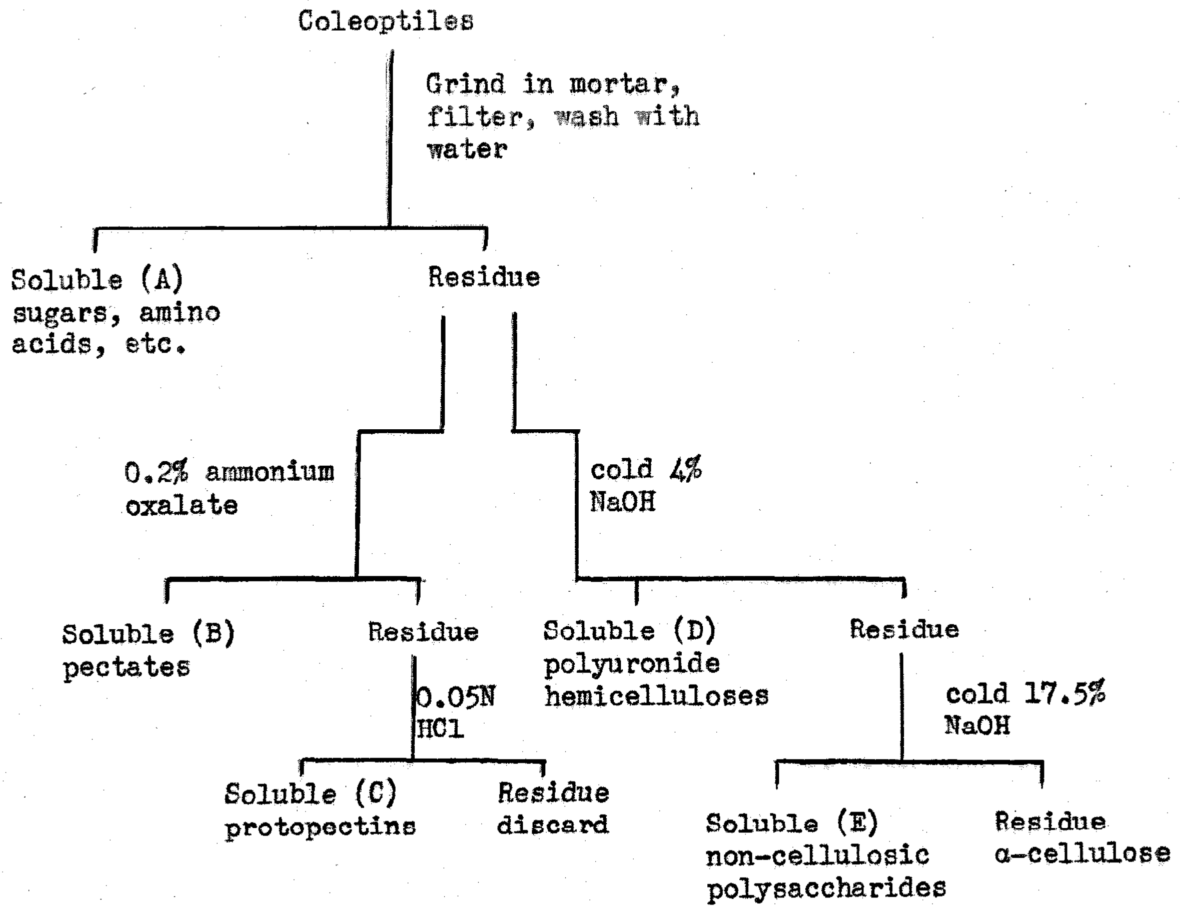


TABLE 7

The chromatographic separation of soluble and cell wall components of Avena coleoptiles after incubation with radioactive acetate or sucrose.

Labeled metabolite applied	Compounds	Solvent	R_f	Solvent	R_f
Sucrose	Water soluble	Butanol-propionic acid	.30 .20 .25	Water saturated phenol	.25 .30 .45
Acetate	Water soluble	Same	.20 .15 .15 .50 .95	Same	.20 .30 .55 .75 .85
Sucrose	Polyuronide hemicelluloses	Butanol 10 ethanol 1 water 2	.65	Tetrahydro fur-furyl alcohol 8 water 2	.10
Acetate	Polyuronide hemicelluloses	Butanol-propionic acid	.50	Water saturated phenol	.6

give the R_f values for various spots. Not only were the spots obtained from the sections with or without IAA superimposable, but so far as could be judged by the eye, the intensities were also alike. No differences were apparent in intensity or R_f values in the spots obtained from the soluble polyuronide hemicelluloses. The two radioautograms made from the water soluble components whose R_f values are given in Table 7 have been copied and represented in Figures 6 and 7. Figure 6 represents the radioautogram of the water soluble components obtained from Avena sections incubated with labeled sucrose. The chromatogram of the sections incubated in the presence of auxin was sprayed with ninhydrin to locate the amino acids, and the chromatogram made from Avena incubated without auxin was sprayed with p-anisidine to detect sugars (30). Of the three spots containing enough C^{14} to be detectable on the film, one was found to be an amino acid, and one a ketose sugar. The third spot remains uncharacterized. Figure 7 represents a similar experiment using the water soluble components from Avena sections incubated with labeled acetate. In this instance although five spots were detectable, only one was found to be an amino acid, and one a ketose sugar. The nature of the remaining spots is unknown.

3) Oxidation of cell wall components and the determination of the radioactivity in the recovered $BaC^{14}O_3$. Since the general procedure of chromatography did not seem to be particularly well adapted to the solution of this problem, the amount of radioactivity incorporated into the fractions outlined in Figure 5 was determined by the method of wet combustion previously used.

The coleoptiles were thoroughly washed to remove water soluble

Figure 6. The appearance of the C^{14} label of sucrose in an amino acid and ketose sugar in the water soluble components of Avena coleoptiles.

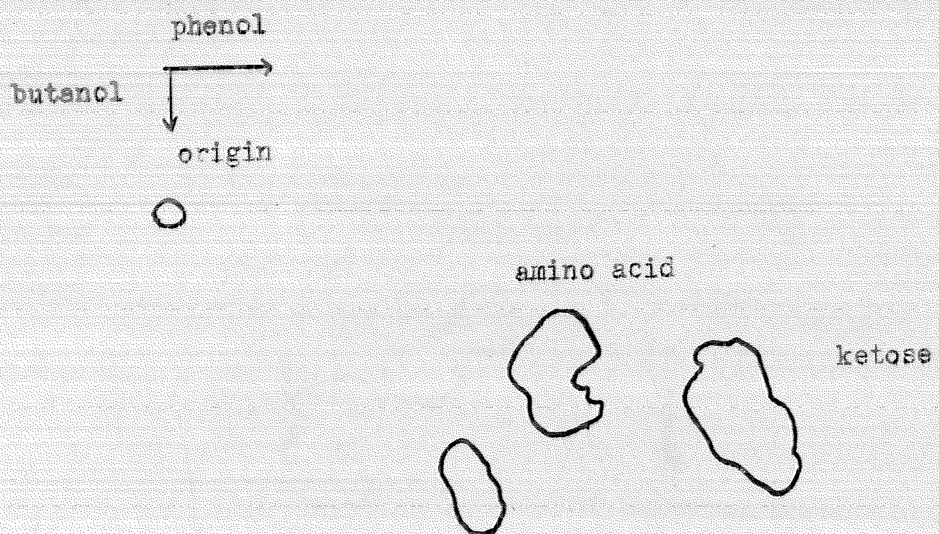
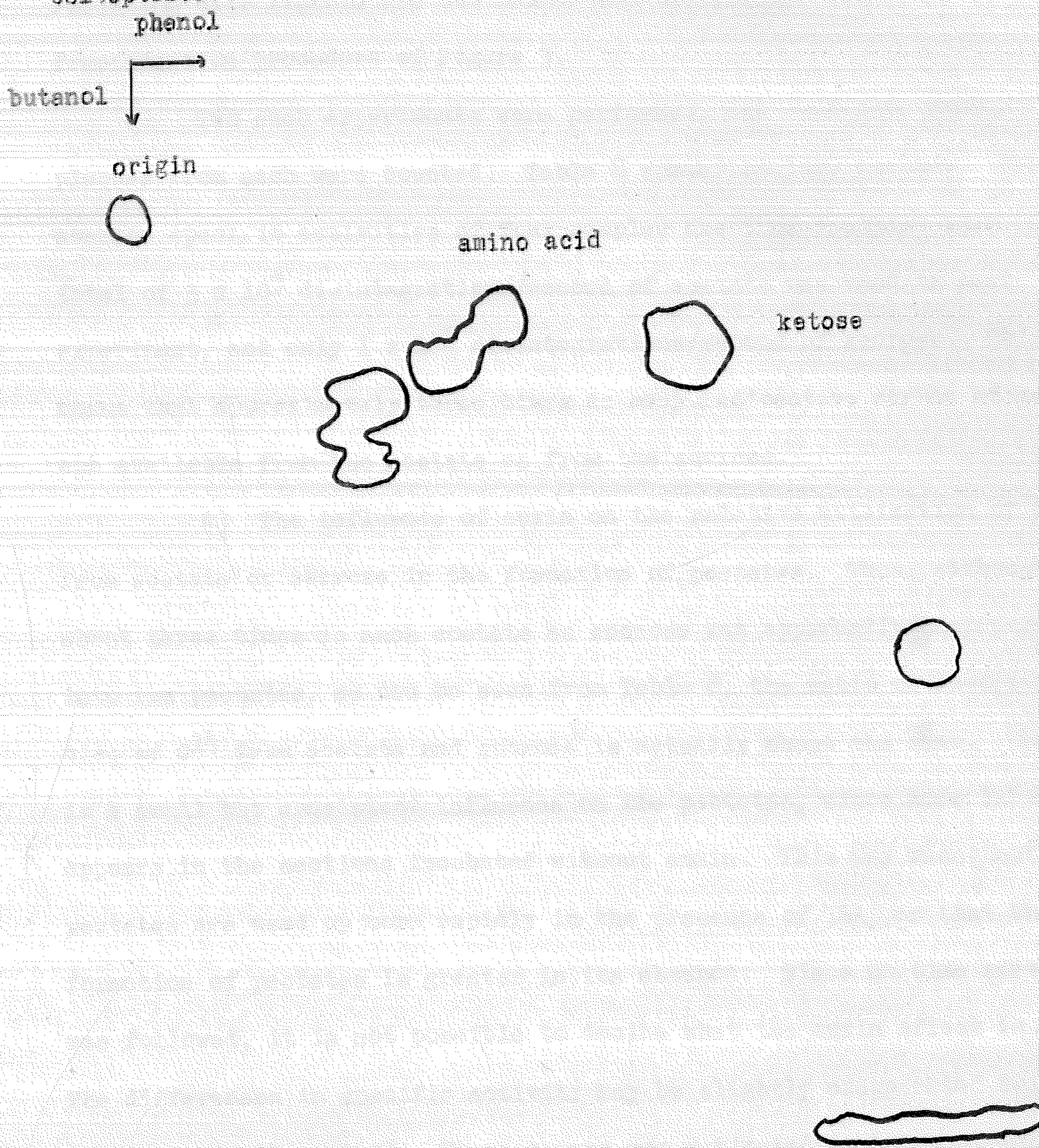


Figure 7. The appearance of the C^{14} label of acetate in an amino acid and ketose sugar in the water soluble components of Avena coleoptiles.



substances by infiltrating them with water and filtering with suction. This procedure was repeated four times. The fats were then removed by refluxing with ligroin for two hours, and the residue subjected to the fractionation procedure of Figure 5.

Two such experiments were performed, and duplicate $BaCO_3$ plaques from each were counted. Table 8 summarizes the results. The average specific activities of four samples are thus recorded here. A total of 3×10^5 disintegrations/second of acetate was used in each experiment, and only 1×10^5 disintegrations/second of sucrose. This means that approximately three times as many radioactive carbon atoms are available from the acetate as from the sucrose.

4) The influence of auxin on the relative utilization of C^{14} from acetate or sucrose in the formation of pectates. Thus, although about three times as much acetate as sucrose was apparently incorporated into the pectates, as can be seen from Table 8, the ratio of incorporation of C^{14} from acetate and sucrose is actually about the same. There is a small but consistent influence on the pectates, since more label appears in the sections incubated without auxin. This may mean that the pectates are used up more rapidly in the presence of IAA, or that the formation of pectates is greater in its absence. Since no time curve was followed, it is not possible to decide what the auxin effect is. The differences in specific activity may be slightly exaggerated owing to errors in the method. These errors are estimated as being of the order of 10%, but in samples of low specific activity, the errors may be as large as 20%.

5) The influence of auxin on the relative utilization of C^{14} from acetate or sucrose in the formation of protopectins. No effect

TABLE 8

The incorporation of C^{14} from acetate and sucrose into the cell wall components of Avena under the influence of IAA.

Components	Average specific activity			
	Without auxin		With auxin	
	Acetate	Sucrose	Acetate	Sucrose
Pectates	58.8	19.6	40.0	15.3
Protopectins	6.3	8.3	14.1	8.8
Soluble polyuronide hemicelluloses	101.8	10.1	84.1	8.2
Non-cellulosic polysaccharides	61.4	3.2	84.2	6.4
Alpha cellulose	16.6	9.2	18.5	10.2

of IAA is apparent on the incorporation of the sucrose C^{14} into the solubilized protopectins, but more than twice as much C^{14} from acetate has been incorporated into the protopectins in the presence of IAA as in its absence. One would hardly expect the source of the label to make such a difference, and the discrepancy is relatively large. Since the matter cannot be explained, it is felt that no definite conclusion can be reached as regards the effect of auxin on the protopectins. It is clear, however, that in the synthesis of protopectin, sucrose is more effectively utilized than acetate for the making of galacturonic acid residues. This is not surprising, for it is known that interconversion of hexoses occurs within plants (29).

6) The influence of auxin on the relative utilization of C^{14} from acetate or sucrose in the formation of soluble polyuronide hemicelluloses. The soluble polyuronide hemicelluloses also show a small auxin effect in that more label appears in the absence of auxin when either acetate or sucrose is used as substrate. It is difficult to know what this might mean in terms of cellular elongation, since little is known about the biochemistry of hemicelluloses. They contain in general xylose and glucuronic acid units. D-xylose has the configuration of D-glucose except that the number six carbon atom is lacking. One would expect that this simple transformation could be readily accomplished in the plant, but it is known in fact that D-xylose is not utilized by starving barley leaves, while most hexoses are. Acetate is at least three times as effective a precursor for polyuronide hemicelluloses as sucrose, judging from the values in Table 8. We may thus infer that the hemicelluloses are more readily made from a carbon pool to which

acetate contributes, than from the interconversion of sugars directly.

7) The influence of auxin on the relative utilization of C^{14} from acetate or sucrose in the formation of non-cellulosic polysaccharides. Another instance of an auxin effect can be seen from the specific activities of the non-cellulosic polysaccharides. More label appears in the auxin treated tissue with either acetate or sucrose as metabolite. The nature of the substances in Avena which are soluble in cold 17.5% NaOH is unknown, so it is not possible to postulate any particular compound as being responsible for the increase in labeling. Approximately three to four times as many C^{14} atoms from acetate are utilized for the synthesis of these substances as from sucrose.

8) The influence of auxin on the relative utilization of C^{14} from acetate or sucrose in the formation of alpha cellulose. An examination of the specific activities obtained from the alpha cellulose residues shows no auxin effect at all. The label from sucrose enters the cellulose slightly less than twice as rapidly as the acetate. It is perhaps surprising that more than a trace of radioactivity is detectable in the cellulose of sections treated with either acetate or sucrose. Although cellulose constitutes approximately one half of the cell walls of Avena (27), once it has been laid down, presumably little change in composition occurs (27). While the data above are not capable of indicating turnover, they show that metabolism of cellulose is going on, and that even in so short a time as three hours, a considerable amount of synthesis is possible.

9) Summary. It can be concluded that there is no effect of

exogenous auxin on the rate of incorporation of a C^{14} label from glycine or leucine into the proteins of Avena or corn coleoptiles. Similar experiments on the metabolism of the lipids of Avena using carboxyl labeled acetate were obscured by experimental difficulties, but disclosed no marked effects of IAA. In all probability, the importance of IAA in growth does not reside in an effect on lipid metabolism.

Chromatography of the substances present in the various solubility classes shown in Figure 5 disclosed no qualitative or quantitative differences in the number of compounds which become labeled from acetate or sucrose as a result of added IAA. The R_f values obtained from the radioautograms were substantially alike in both instances.

The oxidation of compounds present in the cell walls, extracted by means of the fractionation scheme of Figure 5, disclosed that auxin did in some instances influence the incorporation of C^{14} from the metabolites used. Thus the pectates and soluble polyuronide hemicelluloses were found to be more labeled without auxin, but the non-cellulosic polysaccharides were more labeled in the presence of auxin. No influence of IAA was apparent on the alpha cellulose fraction, and it is preferred to draw no conclusion on the effect of IAA on the soluble protopectins. In general, the auxin effects are small, and very likely cannot explain the process of cell elongation.

Sucrose was found to be more available than acetate as a precursor of the protopectins and alpha cellulose. Both substrates were equally utilized by the pectic substances, and the label from acetate was incorporated to a greater extent than that from sucrose by the

soluble polyuronide hemicelluloses and the non-cellulosic polysaccharides.

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