THE PHOSPHORYLATION OF ALKALINE PHOSPHATASE*

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The phosphorylation of alkaline phosphatase from *E. coli* by orthophosphate has the characteristics expected of a reaction at the active center of the enzyme. Phosphate is fixed to the hydroxyl group of a particular serine residue in the enzyme.[1, 2] The amino acid sequence of a tetradecapeptide containing the reactive serine residue has been reported.[3] This phosphorylation requires enzymatically active phosphatase; phosphate esters, as well as inhibitors of phosphomonoesterase activity, interfere with the fixation of orthophosphate by the enzyme,[1, 2] and ester phosphate and orthophosphate both phosphorylate the same serine residue in the enzyme.[2] This paper describes experiments bearing on the mechanism of action of alkaline phosphatase.

Experimental.—Alkaline phosphatase was purified from *E. coli* and assayed as previously described.[1, 4] Measurements of activity were used to derive amounts of enzyme, using the turnover number reported by Garen and Levinthal.[4] Orthophosphate containing P^{32} (Oak Ridge National Laboratory, preparation P-1) was boiled before use for 30 min at 100°. Inorganic phosphate was estimated by the method of Fiske and SubbaRow.[5] Radioactivity was determined in a windowless gas-flow counter (Nuclear Chicago); KH_2PO_4 with 2.66 atoms per cent excess O^{18} was obtained from Mildred Cohn. The O^{18} content of phosphate was determined by mass spectrometry after reaction of KH_2PO_4 with guanidine-HCl.[6]

Experiments with P^{32}-phosphate: The specific activity of the phosphate was approximately 10^7 cpm/μmole. The concentration of enzyme in all incubations was 3 mg/ml (38 μM). Tris-acetate and Tris-formate buffers were used at a concentration of 0.1 *M* with respect to Tris; Tris-citric acid buffers were 0.1 *M* with respect to citric acid. The enzyme was inactivated after incubation with phosphate by the rapid addition of 5.7 *N* HCl to a final pH of 0.5, and was precipitated with 4 vol of cold acetone. The precipitates were washed once with the same amount of acetone containing 0.02 *N* HCl.[2]

After residual acetone had been removed *in vacuo* at room temperature, the dried precipitates were hydrolyzed in 0.3 ml of 2 *N* HCl on a steam bath in sealed tubes. After 12 hr the tubes were opened and HCl was removed *in vacuo*. One-tenth ml of water was added; 10 μl samples were electrophorized at pH 3.1.[1] The radioactivity in serine phosphate was located on the electropherogram by radioautography[1] and cut out for mounting on metal planchets. Radioactivity was counted on the paper; counting in this manner was reproducible and 80% as efficient as counting the same amount of radioactivity directly on the metal planchet. The measurements of radioactivity in serine phosphate-P^{32} were used to calculate the amount of phosphorus originally fixed to protein, multiplying by an experimentally determined correction factor of 2.8 to compensate for the decomposition of serine phosphate during acid hydrolysis. The mole ratio of phosphate to enzyme was calculated from the specific activity of the phosphate initially put into the phosphorylating mixture and the amount of enzyme as estimated by its activity. The value of the correction factor does not alter rapidly between 10 and 20 hr of hydrolysis. At 10 hr the radioactivity present in the hydrolysate as free serine phosphate represented 40% of the radioactivity originally fixed to protein. This fraction decreases linearly with time, and at 20 hr is 30%. The rest of the radioactivity is present as inorganic phosphate and phosphopeptides.

Experiments with O^{18}-phosphate: Either 1.5 mg or 15 mg (0.19 μmoles) of alkaline phosphatase were incubated in 3 ml of 0.1 *M* Tris-acetate buffer at pH 4.6, 5.8, or 9 with 0.01 *M* potassium phosphate enriched with O^{18}. After incubation at 30° for 30 min or 300 min, the enzyme was inactivated by the addition of 0.2 ml of 5.7 *N* HCl. All of the following operations were carried out at 0° or 4°. Enzyme was precipitated when 1 ml of 20% trichloroacetic acid was added, and the supernatants were saved. Precipitates were washed once with 1 ml of 5% trichloroacetic acid, the su-

pernatants were pooled, made alkaline to phenolphthalein with a freshly prepared solution of KOH, and 0.1 ml of 1 *M* $BaCl_2$ was added to precipitate the phosphate. The precipitates were washed once with 2 ml of 0.04 *M* $BaCl_2$ made 0.05 *M* in KOH, after standing for 1 hr, and were dissolved in 0.2 ml of 2 *N* HCl to be precipitated again by adding KOH. Newly precipitated samples were washed and dissolved in acid as before. Enough KOH was added so that samples became yellowish-green on testing with Brom Cresol Green, indicating a pH just above 4. Barium was removed by ion exchange using 0.5 ml of Dowex 50-X8 (200–400 mesh) in the potassium form, suspended in water (approximately 0.16 gm of dry resin), and added with stirring. Suspensions were filtered through small sintered glass funnels, and the filtrates containing KH_2PO_4 were collected and exhaustively dried.

Results.—Experiments with P^{32}-labeled phosphate: The equilibrium amount of phosphate fixed by alkaline phosphatase during incubations at 0° with saturating orthophosphate is shown in Figure 1. Included in the figure for comparison is

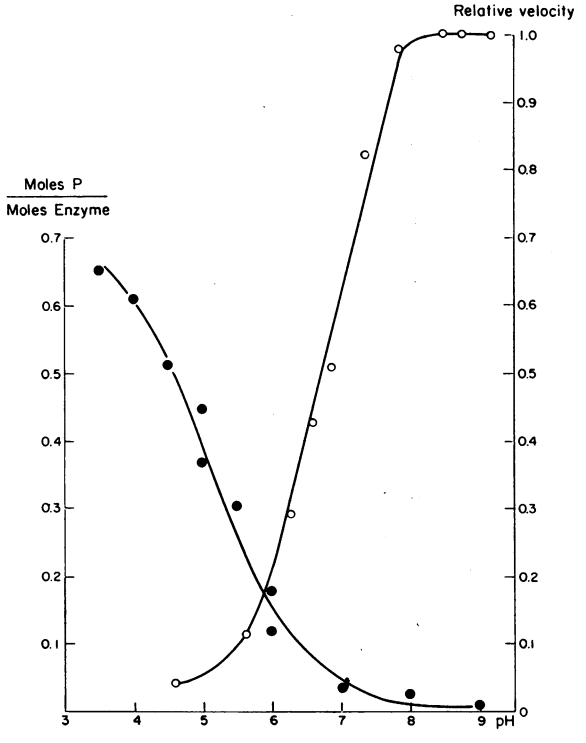

FIG. 1.—Phosphate fixation and enzymatic activity of alkaline phosphatase as a function of pH. *Phosphate fixation* (—●—●—) is the mole ratio of phosphate to enzyme after incubation with 1 mM P^{32}-orthophosphate at 0° for 15 min in Tris-acetate buffers pH 4.6 to pH 9, and Tris-formate buffers pH 3.5 to 0H 4.2. *Relative velocity:* the initial rate of hydrolysis of 0.01 *M* β-glycerol phosphate in 0.1 *M* Tris-acetate buffers was followed by the appearance of inorganic phosphate at 30°. The ratio of the maximal velocities obtained at the various values of pH to the velocity in the optimal pH range are plotted (—○—○—).

the pH-dependence of the maximal rate of hydrolysis (V_{max}) of β-glycerol phosphate at 30°. Under alkaline conditions, optimal for hydrolysis, little phosphate was fixed. With decreasing pH, increasing amounts of phosphoryl enzyme were formed, reaching an optimum around pH 4. The proportion of phosphate fixed to enzyme varied from 0.5 to 1.2 moles of phosphate per mole of enzyme. Phos-

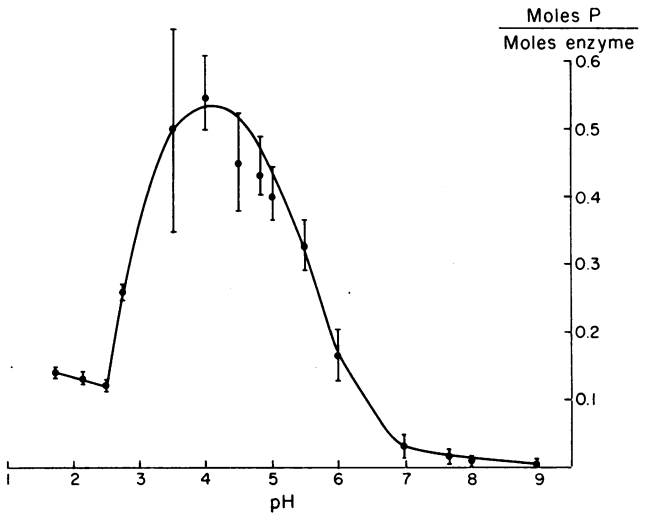

FIG. 2.—Phosphate fixation as a function of pH. Enzyme was incubated at 0° for 15 min in 0.1 *M* buffers with 1 mM P^{32}-orthophosphate. Tris-citrate was used as buffer from pH 1.8 to pH 6, Tris-formate from pH 3.5 to pH 4.2, and Tris-acetate from pH 4.6 to pH 9. No significant differences were found in the results when different buffers were used at the same pH. Brackets indicate the absolute variation in 5 to 6 determinations.

phate fixation studied over a wider range of pH is shown in Figure 2. Measurements made between pH 3 and pH 5 were the least reproducible, probably because the enzyme is partly out of solution in this range due to isoelectric precipitation,[4] and because it is inactivated by dissociation into subunits under the influence of acid.[7] For these reasons, the following experiments were carried out above pH 5 even though phosphate fixation was not optimal.

With the quantity of enzyme protein convenient for isolation by precipitation (1 mg), the formation of phosphoryl enzyme was almost instantaneous (Fig. 3).

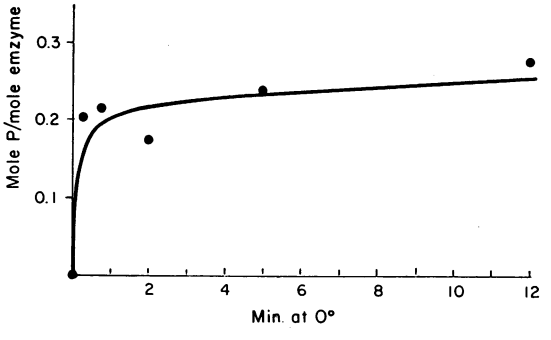

FIG. 3.—Time course of phosphate fixation at pH 5.5. Enzyme was incubated at 0° in Tris-acetate buffer pH 5.5, with 1 mM P^{32}-orthophosphate. Samples were removed and inactivated at the times indicated.

Fixation of phosphate by the enzyme depended on the concentration of orthophosphate, as shown in Table 1. The enzyme was saturated between 1 and 10 mM. At a given pH, the equilibrium amount of phosphoryl enzyme was not markedly increased by increasing the phosphate concentration above 1 mM, as shown in Table 2. About 70 per cent saturation was obtained at 1 mM phosphate. A 10-fold increase in the phosphate concentration did not increase the amount

TABLE 1

DEPENDENCE OF PHOSPHATE FIXATION ON CONCENTRATION OF ORTHOPHOSPHATE

P_i(mM)	Mole P/mole enzyme
0.1	0.19
1.0	0.52
10	0.66
20	0.69

Enzyme was incubated at 0° in Tris-citrate buffer pH 5.2 for 15 min with the concentrations of P^{32}-orthophosphate indicated.

TABLE 2

CONCENTRATION DEPENDENCE AND pH

pH	P_i(mM)	Moles P/moles enzyme 1	10
4.2		0.40	0.53
5.6		0.35	0.50
7.1		0.023	0.036

Enzyme was incubated at 0° with 1 and 10 mM P^{32}-orthophosphate for 15 min in Tris-citrate buffers at the values of pH indicated.

TABLE 3

EQUILIBRATION OF ENZYME-BOUND P^{32}- WITH P^{31}-ORTHOPHOSPHATE AT 30°

Time after addition of P_i^{31} (min)	cpm in serine phosphate
0	2,400
1/4	285
1/2	209
1	184
3	156
9	196
30	192
Calculated ∞	228

See text. Enzyme was incubated at 30° in 1 ml of Tris-acetate buffer pH 5.5 with 1 mM P^{32}-orthophosphate. After 15 min, an equal volume of buffered P^{31}-orthophosphate at 30° was added to bring the concentration of orthophosphate in the mixture to 10.5 mM. Samples were removed at the times indicated. Infinity was calculated by dividing the number of counts in serine phosphate at zero time by 10.5.

TABLE 4

COMPARISON OF THE RATES OF PO_4^{18} TURNOVER WITH V_{max} OF HYDROLYSIS AT VARIOUS VALUES OF pH

pH	% V	N/Vmax 1.5 mg	15 mg
9	100	0.035	0.013
5.8	14	0.67	0.17
4.6	4.4	0.56	0.30

The enzymatic activity at the indicated values of pH relative to the maximal velocity of hydrolysis of β-glycerol phosphate at the pH optimum (% V) was determined from the measurements of activity presented in Fig. 1 (relative velocity). The ratios of the rates of O^{18}-phosphate turnover to the maximal velocities of hydrolysis at a given pH (N/V_{max}) were calculated from the values of N presented in Fig. 4 for the incubations with 1.5 mg of enzyme for 30 min and for the incubations with 15 mg of enzyme for 300 min. The hydrolytic activity of these amounts of enzyme at the various values of pH was determined from measurements of activity carried out in the same way as those presented in Fig. 1.

fixed, and this was true even at pH 7.1 where this amount was much less than one phosphate per mole enzyme.

Phosphorylation of the enzyme was reversible on dilution, as has already been shown,[1] and the greater quantity of phosphate fixed at a lower pH was released at higher pH. The P^{32}-phosphate fixed by the enzyme was exchangeable with unlabeled orthophosphate, as shown in Table 3. After a preliminary incubation for 15 min at pH 5.5 with 1 mM P^{32}-phosphate, unlabeled phosphate was added to dilute 10.5-fold the specific radioactivity of the phosphate already present. Samples were taken at intervals, hydrolyzed, and assayed for radioactivity in serine phosphate. Equilibration was complete within 15 seconds after the addition of the unlabeled phosphate.

Experiments with O^{18}-labeled phosphate: Phosphorylation of the enzyme was best at low pH and, as estimated by P^{32}-phosphate fixation, hardly occurred at all under conditions optimal for enzyme activity. The use of orthophosphate enriched with O^{18} permits detection of the phosphorylation reaction under alkaline conditions where P^{32}-phosphate fixation is poor. Greater sensitivity is achieved when O^{18} is used as an isotopic label, since phosphate turnover rather than phosphate fixation is measured, and because changes are measured in the large orthophosphate pool rather than in the much smaller amount of phosphorylated enzyme.

Alkaline phosphatase has been shown previously to catalyze a relatively slow incorporation of the oxygen atoms of H_2O^{18} into orthophosphate.[1, 8] In the earlier

studies, small quantities of the enzyme were used, and the reaction was measured only under alkaline conditions optimal for enzymatic hydrolyses. In the present study, this exchange reaction was examined using large quantities of enzyme at three different values of pH. As shown in Figure 4, these amounts of enzyme not

Enzyme (mg)	1.5		1.5		15	
Time (min.)	30		300		30	
pH	PO_4^{18}	N	PO_4^{18}	N	PO_4^{18}	N
9	2.26	0.7	-	-	1.26	2.7
5.8	1.70	1.9	0.7	5.5	0.77	4.9
4.6	2.36	0.5	1.7	1.8	1.24	2.7

FIG. 4.—For conditions of incubation, see *Experimental.* In the columns headed by PO_4^{18} are tabulated the atoms per cent excess of O^{18} in KH_2PO_4 reisolated after incubations with the indicated quantities of enzyme. *N* is the number of cycles of phosphate turnover calculated from the formula of Cohn and Drysdale.[18] The initial content of O^{18} in KH_2PO_4 was 2.66 atoms per cent excess. The KH_2PO_4 reisolated after addition to 15 mg of acid inactivated enzyme contained 2.36 atoms per cent excess. Data from experiments with 15 mg of enzyme were corrected accordingly by subtracting 0.3 atoms per cent excess. Since it was assumed that the lowering of the specific activity of the KH_2PO_4, reisolated after it had been mixed with the large amount of inactivated enzyme, was caused by contamination with organic material, data obtained in experiments with the smaller amount of enzyme were corrected by subtracting only $^1/_{10}$ of the blank, i.e., 0.03 atoms per cent excess.

only catalyzed loss of O^{18} from O^{18}-orthophosphate, but also catalyzed a number of complete cycles of phosphate turnover. The variation in rate with the time of incubation and with the concentration of enzyme apparent in Figure 4 was not due to a loss of enzymatic activity during incubation. No more than 12 per cent of the activity was lost, and that only at pH 4.6.

The rates of phosphate turnover, measured with O^{18}-orthophosphate, are compared in Table 4 with the maximal rates of enzymatic hydrolysis of β-glycerol phosphate at various values of pH. At pH 9, the rate of turnover was less than 4 per cent of the rate of ester hydrolysis. With decreasing pH, the two velocities approached each other.

The rate of phosphate turnover measured with O^{18}-orthophosphate can also be compared to the rate of turnover estimated from the equilibration experiment already presented in Table 3. For example, the number of cycles at pH 5.8 (Fig. 4, last column) represents the turnover of 147 μmoles of O^{18}-phosphate in a half time of 15 min, so that the half time for 0.19 μmoles of phosphorylated enzyme, the quantity of enzyme used in this experiment, would be approximately 1 second; this time is consistent with the longest time for a full turnover of P^{32}-phosphate at pH 5.5, that is, less than 15 seconds.

Discussion.—It has been presumed for a long while that ester hydrolyses catalyzed by nonspecific phosphatases occur in two catalytic steps. The first step is the phosphorylation of the enzyme by substrate with the loss of the alcohol group; the second is the hydrolysis of the phosphoryl enzyme. This mechanism was advanced by R. K. Morton[9] to explain the phosphoryl transferase activity

of the enzymes, and has been supported by the observation that substrate is hydrolyzed by alkaline phosphatase at the P-O bond,[10] which is to be expected if, indeed, the phosphoryl group is transferred. For the bacterial enzyme, the similarity in the maximal rates of hydrolysis of different substrates with a wide variety of substituents[11] also supports a mechanism in which the rate-limiting reaction is the hydrolysis of the common intermediate, phosphoryl enzyme.

It seems reasonable to suppose that the serine residue, which is phosphorylated by orthophosphate, is directly involved in the catalytic mechanism of the enzyme, and that its hydroxyl group acts as a nucleophilic agent. The assumption that this serine participates in catalysis is not proved, since the information that implicates it relies solely on degradative methods. However, it is supported by analogy with a group of hydrolases that are inhibited by diisopropylfluorophosphate (DFP).[12] The analogy seems particularly appropriate since the sequence of amino acids around the reactive serine in alkaline phosphatase has been shown to resemble closely the sequence around the active sites of those hydrolases.[3] The phosphorylation of alkaline phosphatase by orthophosphate is favored at low pH, as is the acylation of the reactive serine in chymotrypsin by acyl esters. pH affects the V_{max} of the two hydrolases in the same way,[13] and both enzymes catalyze virtual reactions.[14] The stability of acyl chymotrypsin in dilute acid results from the sluggishness of its hydrolysis relative to the ease of its formation.[13] Similarly, alkaline phosphatase appears to be phosphorylated in a pH-dependent equilibrium.

Phosphorylation of enzyme under alkaline conditions was found to be far from complete, and was not increased by increasing the orthophosphate concentration above saturation. Finding that enzyme can be saturated for phosphorylation below optimal yield suggests that orthophosphate is bound to enzyme before the serine residue is phosphorylated, and that the binding site is different from the site of phosphorylation. Suggestion that the binding step is distinct from the phosphorylation step is to be found in the fact that orthophosphate profoundly inhibits enzymatic activity at alkaline pH,[4] where phosphorylation hardly occurs; moreover, under these conditions, orthophosphate is firmly bound to the enzyme in equilibrium dialysis experiments.[15] The enzyme is known to contain zinc,[16] which is a likely binding site. By coordination with zinc, both ester phosphate and orthophosphate could be activated toward the nucleophilic attack of the hydroxyl group of serine.

Orthophosphate behaves as a substrate when it phosphorylates the enzyme.[8] Since it has been shown to bind at least as well as ester phosphate at alkaline pH,[4] and since the enzyme lacks specificity for substituent groups,[11] it is surprising that the rate of phosphate turnover at pH 9, determined in experiments presented here, was so much slower than the optimal rate of ester hydrolysis. Although little charge difference exists between ester phosphate and orthophosphate below pH 10 when they are free, there might be a difference between the two when they are bound to the enzyme. The difference could arise by the creation of a new ionizing group when orthophosphate combines with the enzyme. Coordination of phosphate to a positive group in the enzyme might be expected to lower the pK of the third hydroxyl group of phosphate. A negative charge at the phosphorus atom would inhibit the nucleophilic attack by the serine; hence, above the pK of the

new ionizing group, phosphorylation of the enzyme would be less rapid than ester hydrolyses since the analogous complex of ester phosphate with enzyme could not ionize. Consistent with this idea, the rate of phosphate turnover was seen to be greater at pH 5.8 than at pH 9, despite the fact that only 10 per cent of the enzyme is in the catalytically active form.

Evidently ester phosphate and orthophosphate are bound to the enzyme at the same site, and form similar complexes. The phosphate in both complexes phosphorylates the same serine residue in the enzyme, and both ester phosphate and orthophosphate behave as substrates. These considerations suggest that the processes of phosphorylation either by ester phosphate or by orthophosphate, and of hydrolysis of the resulting phosphoryl enzyme, are similar in mechanism and involve the same catalytic groups in the enzyme. Hydrolysis is precisely the reversal of phosphorylation when the enzyme is phosphorylated by orthophosphate. When ester phosphate phosphorylates the enzyme, dephosphorylation also proceeds by a reversal in mechanism of the phosphorylation reaction; but water, ordinarily present in high concentration, replaces alcohol, and this results in the hydrolysis of the phosphoryl enzyme. Evidence that the hydrolysis of acyl chymotrypsin proceeds similarly by a reversal in mechanism of the acylation reaction has been summarized by Bender.[17]

In summary, a plausible mechanism of action of alkaline phosphatase embodying the elements discussed is presented in Figure 5. After binding the sub-

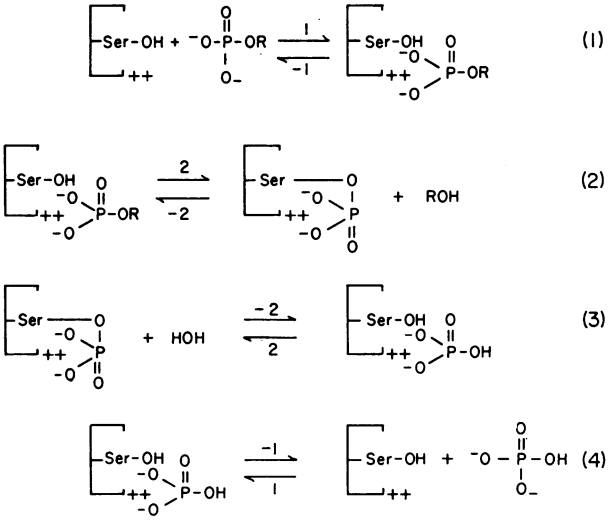

FIG. 5.—A plausible mechanism for the hydrolysis of phosphate esters by alkaline phosphatase.

strate by reaction 1 (1), the enzyme is phosphorylated at serine with the loss of the substituent alcoholic group by reaction 2 (2); the resulting phosphoryl enzyme is then hydrolyzed by reversal of reaction 2 (3) to yield bound orthophosphate. This complex decomposes by reversal of reaction 1 (4) to release free enzyme and orthophosphate as product.

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*GROWTH AND CELLULAR CONSTITUENTS**

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More than a decade ago attempts were begun at this laboratory to demonstrate the possible presence of biologically active substances in the thymus gland which might have had a hormonal activity.[1] After exploring several blind alleys, malignant growth was applied as a test material.[2] These experiments led to the isolation of two fractions, one of which had a strong inhibitory action on malignant growth, while the other showed a strong promoting effect.[3] The hypothetic substance responsible for the retardation was called "retine," while the promoting substance was called "promine." The fractions showing the latter effect also sterilized mice, male and female. Later, the growth-promoting and sterilizing activity were separated[4, 5] and the substance responsible for the latter was termed "infertine."

In our first report,[3] we left the problem of specificity open. Later,[6] however, we could demonstrate that a substance showing identical activity and chemical properties, such as retine, was present also in other tissues. Retine thus had to be a general tissue constituent. The same was demonstrated lately for promine. Earlier negative results on this line could be ascribed to the great experimental difficulties connected with this work and the ease with which the two substances, retine and promine, compensate each other's activity. Attempts to demonstrate