PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 152, 170-174 (1976)

Ammonium Sulfate Activation of Phosphodiesterase in Homogenates of Larvae of the European Corn Borer (39353)

D. B. GELMAN, D. K. HAYES, AND M. S. SCHECHTER

Chemical and Biophysical Control Lab., Agricultural Environmental Quality Institute, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Maryland 20705

We first noted activation by high concentrations of ammonium sulfate of phosphodiesterase(s) found in crude and partially purified extracts of larvae of the European corn borer, *Ostrinia nubilalis* (Hübner), after the ammonium sulfate precipitation step when we attempted to purify cyclic adenosine monophosphatase (c-AMP-ase), phosphomonoesterase, and phosphodiesterase by the method of Robison (1). Although neither the ammonium nor the sulfate ions are of major importance in the activation of many enzymes, several workers have reported that ammonium ion enhances the activity of β-D-galactosidase (2) and the deamination of L-serine by the β-subunit of tryptophane synthetase (3). Also, the ammonium ion activates lactose hydrolysis (4), AMP-aminohydrolase (5), and D-β-lysine mutase (6). Moreover, in the absence of potassium ions, β-lysine mutase is activated by ammonium ions; if potassium ions are present, the activity is inhibited (6). Ammonium ions can substitute for one subunit of tryptophane synthetase from *Escherichia coli* (7), and nucleoplasmin, divalent manganese ions, and ammonium sulfate together activate RNA polymerases derived from eukaryotic nuclei (8-13). Kastenschmidt *et al.* (14) demonstrated activation of phosphorylase b from rabbit skeletal muscle by AMP. This activation was enhanced by sulfate and fluoride ions when the reaction mixture was made up in glycylglycine. They suggested that sulfate ion enhanced the interaction of AMP with phosphorylase. Appleman found that inactive phosphorylase from liver is not activated by AMP unless a salt solution such as sodium sulfate is added (15). Sulfate ions also activate sulfatases (16).

Since one goal of our work is to determine differences in metabolism between insect tissues and other invertebrate and mammal tissues, in the present study we determined phosphodiesterase levels in at least one other arthropod (the crayfish), in two ruminants, and in a rat. We believe that ours is the first report describing activation by ammonium sulfate of phosphodiesterase(s) prepared from insect tissue.

Materials and methods. The European corn borer larvae used in the study were supplied by the European Corn Borer Laboratory, ARS, USDA, Ankeney, Iowa. The insects were maintained in our laboratory as described by Odesser *et al.* (17). The adult bovine brain and liver were supplied by Richard W. Miller of our laboratory, the yearling ovine liver, kidney, and brain tissue by the Ruminant Nutrition Laboratory, the rat livers from the Carbohydrate Laboratory and 3-in. Louisiana crayfish, *Cambarus* sp., by the Pesticide Degradation Laboratory (all Beltsville Agricultural Research Center, USDA, Beltsville, Md.)

The phosphodiesterase and phosphomonoesterase were assayed as described by Odesser *et al.* (17) except that phenylthiourea, which prevents browning of insect extracts, was omitted from the extraction fluid. When ammonium sulfate was tested, it was added directly to the incubation tubes before the 1.4 ml of bis-Tris-$MgCl_2$ was added. All tubes were centrifuged at 25,000 rpm for 30 min before colorimetric determination. All reagents were C.P. grade or better.

Results and discussion. Figure 1 shows the effect of the ammonium sulfate concentration on hydrolysis of bis(*p*-nitrophenyl) phosphate. Maximum phosphodiesterase activity was obtained when the reaction mixture was 30% saturated with respect to ammonium sulfate as illustrated in Fig. 1. Precipitation of diesterase was a major factor in the observed decrease of enzyme activity above 30% saturation since ammonium sul-

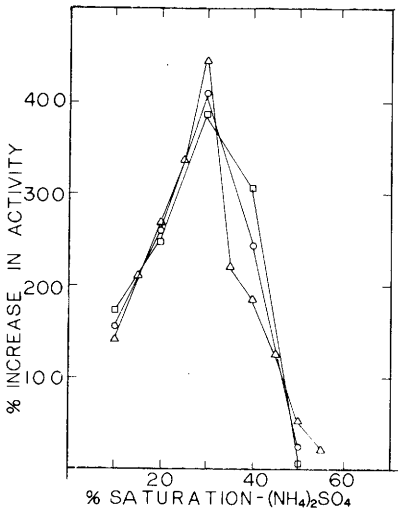

FIG. 1. Activation of crude phosphodiesterase preparation by ammonium sulfate. Reaction vessel contains, in addition to ammonium sulfate as indicated, 0.6 ml of enzyme preparation containing 15–18 mg of tissue (wet wt) in sucrose (0.25 *M*); phenylthiourea (0.1 saturation); bis(*p*-nitrophenyl) phosphate (6 μmole in 0.6 ml of Tris [(hydroxymethyl)amino]-methane buffer (Tris); 0.2 *M*, pH 8.4); 0.6 ml of Tris buffer (0.2 *M*, pH 8.4); 0.2 ml of $MgCl_2$ (1 *M*). □, Trial 1; △, Trial 2; ○, average of two trials.

fate is used to precipitate phosphodiesterase during purification of the enzyme (1).

The reason for the 400-fold activation of phosphodiesterase could possibly be one or more of the following:

1. Precipitation of magnesium ammonium phosphate could drive the reaction as follows: [a] bis(*p*-nitrophenyl) phosphate→*p*-nitriphenyl phosphate + *p*-nitrophenol; [b] *p*-nitrophenyl phosphate → P_i + *p*-nitrophenol; [c] $P_i + Mg^{+2} + NH_4^+ \rightarrow$ magnesium ammonium phosphate.
2. Precipitation of a high molecular weight inhibitor.
3. Action as a cation and anion activator of the enzyme.
4. Alteration of the tertiary or quaternary structure of the enzyme so that a more active form was obtained.
5. Action resulting from some combination of the preceding possibilities.

The results of a test of the first possibility were negative. Addition of magnesium chloride made no difference in the activation by ammonium sulfate; with magnesium present activity was 47.8 μmoles nitrophenol released, and with magnesium absent activity was 49.8 μmoles nitrophenol released in a whole homogenate; in a 105,000 *g* supernatant values with and without magnesium were, respectively, 9.0 and 10.1 μmoles liberated.

A decrease in magnesium concentration should have resulted in a decrease in diesterase activity. Also, the addition of ammonium sulfate should have resulted in increased monoesterase activity, since it is this reaction which generates the inorganic phosphate ion (see Eq. [b]). In two trials phosphomonoesterase activity was inhibited by ammonium sulfate (30% satd.); from 13.1 to 8.6 and 13.9 to 11.0 μmoles nitrophenol liberated/g wet wt corresponding to 35 and 21% inhibition, respectively.

Another test was made to determine whether a permanent change occurred in the configuration of the enzyme or whether an inhibitor was precipitated. Thus we added ammonium sulfate to the larval extract at 30% saturation, centrifuged half the mixture at 5000 *g* for 20 min at room temperature and held the other half at room temperature for the same length of time. Both samples were then dialyzed at 40° overnight against the homogenizing fluid (sucrose, 0.25 *M*) which in this case was 0.1 saturated with phenylthiourea. After dialysis the diesterase activity of each sample was determined. The activities of the two samples were not different from each other nor were they different from the activity of untreated homogenates. We therefore concluded that an inhibitor had not been precipitated and that no permanent change had occurred in the configuration of the enzyme. Figure 2 shows that another ammonium salt, ammonium chloride and another sulfate, sodium sulfate, did activate the enzyme although not to the same extent as ammonium sulfate. These data suggest that ammonium and sulfate together are more effective than either ion alone in activating the enzyme.

The possibility that activation might have been the result of increased salt concentration was tested. The ionic strength of 10% sodium sulfate is 2.10; the ionic strength of 30% saturated ammonium sulfate is 4.05.

The ionic strength of 30% ammonium chloride was 6. These concentrations had been chosen on a somewhat arbitrary basis; for example, proteins are more readily precipitated in sodium sulfate than in ammonium sulfate and this had been considered in designing the tests. A test to determine the effects of sodium chloride and ammonium sulfate on enzyme activity when ionic strengths were approximately equivalent is illustrated in Table III. Here the salt effects of NaCl appear to increase activity about 50%, similar to effects obtained with ammonium chloride, i.e., about 50%. The effect was distinctly less than the effect observed in ammonium sulfate in which activity was increased either 248 or 463%, depending upon the concentration.

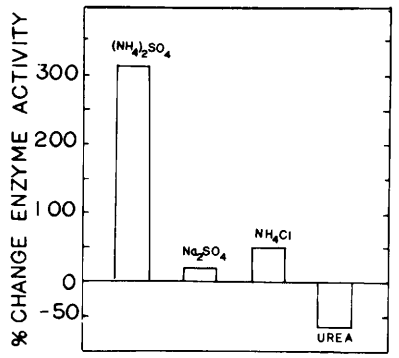

FIG. 2. Activation and inhibition of phosphodiesterase by inorganic salts and by urea. The reaction mixture consisted of 0.6 ml of enzyme preparation containing 15–18 mg of tissue (wet wt) bis(*p*-nitrophenyl) phosphate 6 μmole in 0.6 ml of Tris (0.2 *M*), pH 8.4, 0.6 ml of Tris (0.2 *M*), pH 8.4, 0.2 ml of $MgCl_2$ (1 *M*). Concentration of additives in reaction mixture: $(NH_4)_2SO_4$, 30% saturation; Na_2SO_4, 10%; NH_4Cl, 30%; urea, 8 *M*. Results with $(NH_4)_2SO_4$ are the average of 11 trials ± SE of mean. A 105,000 *g* supernatant was used in the test with ammonium chloride.

We anticipated that 8 *M* urea might activate the enzyme since it disrupts tertiary structures. If high concentrations of ammonium sulfate were required, 30% saturation could produce alteration in the tertiary or quaternary structure. This was not the case as shown in Fig. 2. The 8 *M* urea solution inhibited activity 67%, probably by disrupting tertiary structure. Since Winstead and Wold (18) observed that 0.5 *M* ammonium sulfate in 20% aqueous dioxane inhibited enolase by causing dissociation into subunits, the activation we observed could possibly have depended upon dissociation of our enzyme into subunits that were more active than a larger enzyme molecule.

Our most reasonable explanation of the ammonium sulfate effect is that there was activation by both ammonium and sulfate ions. However, further work with purified enzyme will be required to determine whether this interpretation is correct.

Subsequently the addition of ammonium sulfate to 30% saturation was found to decrease the pH of our incubation mixture to 7.6. Phosphodiesterase activity at pH 7.6 and 8.4 was not different in the crude preparations for in six samples run at each pH value the average activity was 11.5 ± 5.3 (pH 8.4) and 11.5 ± 4.9 (pH 7.6).

In our previous paper (17) we demonstrated that preparations from insect sources exhibited phosphodiesterase activities quite different from the phosphodiesterase found in snake venoms. The results shown in Tables I and II indicate that this is true in other species as well, since ammonium sulfate inhibited or did not affect rather than activated the phosphodiesterase of all samples tested (Table I). Also, Table II shows that the inhibitors, caffeine and thiophylline, inhibited some mammalian phosphodiester-

TABLE I. INHIBITION OR ACTIVATION OF PHOSPHODIESTERASE ACTIVITY OF HOMOGENATES OF SELECTED VERTEBRATE AND INVERTEBRATE TISSUE BY $(NH_4)_2SO_4$ AT 30% SATURATION.

Organ	*p*-Nitrophenol released		Change (%)
	μmole/g wet wt + $(NH_4)_2SO_4$	μmole/g wet wt − $(NH_4)_2SO_4$	
Rat liver (1)[a]	7.8	6.8	−13
Bovine liver (1)	11.4	5.3	−54
Bovine kidney (1)	8.5	7.8	−8
Crayfish digestive gland (2)	37.6	32.6	−13
Whole European corn borer larvae (2)	8.3	46.8	+463

[a] Number of determinations in parenthesis.

TABLE II. THE EFFECT OF CAFFEINE (0.008 *M*)[a] AND THEOPHYLLINE (0.008 *M*)[a] ON PHOSPHODIESTERASE ACTIVITY OF SELECTED VERTEBRATE AND INVERTEBRATE TISSUES.

Organ	Untreated homogenate	Percentage difference in level of enzyme activity after addition to reaction mixture of:	
	p-Nitrophenol released (μmole/g wet wt.)	Caffeine[c]	Theophylline[c]
Rat liver (1)[b]	13.4	−4.5	−15.7
Bovine liver (1)	33.0	−7.6	−23.1
Bovine brain (1)	1.6	−25.0	−39.3
Ovine liver (2)	16.6	−15.8	−23.6
Ovine kidney (2)	27.6	−11.3	−46.2
Ovine brain (2)	1.8	−16.8	−22.3
Crayfish digestive gland (2)	21.6	−4.0	+11.8
Crayfish green gland (1)	28.6	−6.3	−17.9

[a] Final concentration in reaction mixture.

[b] Number in parentheses indicates number of trials.

[c] Odesser *et al.* (14) found 51 and 60% inhibition by caffeine (0.118 *M*) and theophylline (0.008 *M*), respectively, of phosphodiesterase in homogenates prepared from fifth instar European corn borer larvae.

TABLE III. EFFECT EQUIVALENT OSMOLAR CONCENTRATIONS OF NaCl AND $(NH_4)_2SO_4$ ON PHOSPHODIESTERASE ACTIVITY[a] IN HOMOGENATES OF EUROPEAN CORN BORER LARVAE.

Salt added	Osmoles of salt added	*p*-Nitrophenol released/g wet wt (μmole)	Increase in activity (%)
None	0	8.3	–
NaCl	1.95	12.7	53
$(NH_4)_2SO_4$	1.95	28.9	248
NaCl	3.90	13.0	57
$(NH_4)_2SO_4$	3.90	46.8	463

[a] The reaction mixture contained 0.6 ml of enzyme preparation containing 15–18 mg of tissue (wet wt); bis (*p*-nitrophenol) phosphate, 6 μmole in 0.6 ml of Tris (0.2 *M*), pH 8.4; 0.6 ml of Tris (0.2 *M*), pH 8.4; 0.2 ml of $MgCl_2$ (1 *M*). Either NaCl or $(NH_4)_2SO_4$ was added so that final solution contained 1.95 or 3.90 osm of the salt.

ases slightly, but the levels of inhibition were far lower than the 51% inhibition (caffeine) and 60% (theophylline) reported for European corn borer diesterase (17). Also, another arthropod, the crayfish, had diesterases that behaved differently from our enzyme, since the phosphodiesterase of the digestive gland (hepatopancreas) was activated by caffeine in the concentrations used. Also, the phosphodiesterase of the green gland (antennal gland, renal-type function) is inhibited 6.3% in the presence of caffeine and 17.9% in the presence of theophylline (Table II). This suggested that the phosphodiesterases of the European corn borer were qualitatively different from those in other species examined in this study.

Activation of the corn borer enzyme by ammonium sulfate may provide an explanation for an earlier, previously unreported observation made in our laboratory. We found that when 0.03 *M* ammonium chloride was injected into European corn borer larvae, it caused death, while topical treatment of pupae of another species of Lepidoptera, the tobacco budworm, *Heliothis virescens* (F.), with ammonium chloride also resulted in high mortality. The toxicity could be a result of activation of the phosphodiesterase(s) that attack diphosphates *in vivo*. Further studies may suggest ways in which activation of phosphodiesterases could be used to manipulate harmful pest populations.

Summary. European corn borer phosphodiesterase is highly activated by $(NH_4)_2SO_4$ and moderately activated by NH_4Cl (pH 7.6, 33°). Vertebrate and crayfish diesterases, on the other hand, are inhibited by $(NH_4)_2SO_4$. It is likely that $(NH_4)_2SO_4$ causes some configurational change in the European corn borer phosphodiesterase molecule which results in the exposure of more active sites and hence greater enzyme activity. In *in vitro* tests caffeine (0.008 *M*) and theophylline (0.008 *M*) inhibit phosphodiesterase more effectively in European corn borer larvae than in crayfish, ovine, bovine, or rat tissue.

1. Robinson, G. A., Butcher, R. W., and Sutherland, E. W., *in* "Cyclic AMP," p. 473. Academic Press, New York (1971).
2. Wallenfels, K., and Well, R., *in* "The Enzymes" (P. D. Boyer, Ed.) Vol. 7, p. 645. Academic Press, New York (1972).
3. Crawford, I. P., and Ito, J., Proc. Nat. Acad. Sci. USA **51,** 390 (1964).
4. Cohn, M., and Monod, J., Biochem. Biophys. Acta **7,** 153 (1951).
5. Turner, D. H., and Turner, J. F., Biochem. J. **79,** 143 (1961).
6. Bray, R. C., and Stadtman, T. C., J. Biol. Chem. **243,** 381 (1968).
7. Goldberg, M. E., York, S., and Stryer, L. Biochemistry **6,** 2113 (1967).
8. Jacob, S. T., Sajdel, E. M., and Munro, H. N., Nature (London) **225,** 61 (1970).
9. Kedinger, C., Gniadzowskim, M., Mandel, J. L., Gissinger, F., and Chambon, P., Biochem. Biophys. Res. Commun. **38,** 165 (1970).
10. Lindell, T. J., Weinberg, F., Morris, P., Raeder, R. G., and Rutter, W. J., Science **170,** 447 (1970).
11. Roeder, R. G., and Ritter, W. J., Proc. Nat. Acad. Sci. USA **65,** 675 (1970).
12. Stirpe, F., and Flume, L., Biochemistry J. **105,** 779 (1967).
13. Tata, J. R., Hamilton, M. J., and Shields, D., Nature New Biology **238,** 161 (1972).
14. Kastenschmidt, L. L., Kastenschmidt, J., and Helmreich E., Biochemistry **7,** 4543-4556 (1968).
15. Nicholls, R. G., and A. B. Roy, *in* "The Enzymes" (P. D. Boyer, Ed.), Vol. 5, p. 31-33. Academic Press, New York (1971).
16. Appleman, M. M., Krebs, E. G., and Fischer, E. H. Biochemistry **5,** 2101 (1966).
17. Odesser, D. B., Hayes, D. K., and Schechter, M. S., J. Insect Physiol. **18,** 1097 (1972).
18. Winstead, J. A., and Wold, F., Biochemistry **4,** 2145 (1965).

Received September 6, 1974. P.S.E.B.M. 1976, Vol. 152.