DEPHOSPHORYLATION OF ADENOSINE TRIPHOSPHATE BY TISSUES OF THE AMERICAN COCKROACH, PERIPLANETA AMERICANA (L.)

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The recent demonstration (Albaum and Kletzkin, 1948; Calaby, 1951) of adenosine triphosphate (ATP) in insects has suggested that this compound may have as important a function in the cellular metabolism of insects as it is known to have in mammals. Although, with the exception of muscle, the significance of dephosphorylation of ATP in mammalian metabolism is still obscure, the resolution of the mechanism of ATP breakdown has been furthered by the partial characterization of this process in other tissues. Analogously, the first reports of dephosphorylation of ATP in insects were with muscle. No doubt contributory insight into this process can be obtained in insects also by a study of the action of other tissues towards ATP.

Compared with the large amount of information available for mammalian tissues, little is known of adenine nucleotide breakdown in insects. Gilmour (1948) noted a Mg-activated soluble enzyme, derived from locust myosin extracts, which split both labile phosphates from the ATP molecule. A similar observation was made by Barron and Tahmisian (1948) with American cockroach muscle homogenates. The effect of temperature on an apyrase in cockroach muscle was studied by Chin (1951). Recently, Gilmour and Calaby (1952) have reported further investigations on the soluble apyrase found in the muscle of locusts. In conjunction with the confirmation that this enzyme was Mg-activated and removed both labile phosphates from ATP, they demonstrated that although traces of adenylate kinase (myokinase) activity were present in their preparation, this mechanism was not responsible for the removal of the second phosphate. Further, the same apyrase could utilize inosine triphosphate (ITP), adenosine diphosphate (ADP) and inosine diphosphate (IDP) as substrates, but was without effect on adenosine monophosphate (AMP) and several other organic phosphates and pyrophosphates. In addition to the apyrase, it was noted that the locust also possesses a Ca-activated ATPase which is associated with the myosin fraction of the muscle.

In contrast to the situation in orthopteran muscle, Sacktor (1953) found other mechanisms of ATP breakdown in the indirect flight muscles of the house fly. Here it was shown that mitochondria isolated from these flight muscles possesssed a specific ATPase, in that they cleaved only the terminal phosphate from ATP and that ATP was the only phosphorylated compound which released inorganic phosphate. Although liberation of orthophosphate also occurred when ADP was the substrate, it was proved that this activity was due to the presence of a Mg-activated adenylate kinase, which converted ADP to ATP and AMP, and thus provided

substrate for the specific ATPase. The mitochondrial ATPase, furthermore, was activated by Mg and Mn but not by Ca ions. On the other hand, an ATPase associated with the muscle *fibrils* was Ca-activated. A soluble fraction of whole thoracic homogenates, like the mitochondria, possessed a Mg-activated ATPase; but this could be distinguished from the mitochondrial ATPase by concurrent inorganic pyrophosphatase activity as well as by differences in the effects of several inhibitors.

The contrast in the mechanism of ATP breakdown between the specialized fibrillar muscle of the house fly and locust muscle leads to the question as to the situation in the other tissues of an insect. Moreover, such investigations should afford a better understanding of the relatively uncomprehended metabolic function of the various insect tissues. In this respect, an approach to a related problem in the comparative physiology of insect tissues was previously reported (Sacktor and Bodenstein, 1952). It was found that different tissues of the American cockroach had different cytochrome *c* oxidase activities which were related to their probable metabolic performance. Furthermore, these results corresponded strikingly with the tracheation of these tissues, as reported by Day (1951). Also, it was discovered that the cytochrome *c* oxidase activity of several tissues was significantly influenced by the sex of the animal. This supplemented the previous report by Barron and Tahmisian of enzymatic differences between male and female muscle homogenates. Accordingly, the present communication is concerned with the relative capabilities of the various cockroach tissues, male and female, to dephosphorylate ATP, and with the influence of bivalent cations on this process. From such findings, further investigations on the mechanism of nucleotide breakdown in insects can be logically approached.

Experimental Procedures

Preparation of tissues. Adult male and female roaches were dissected in 0.9% KCl and the desired tissues were removed as described previously (Sacktor and Bodenstein, 1952). The tissues, immediately prior to enzymatic assay, were homogenized for 30 seconds in 1.0 ml. KCl solution with a Potter-Elvehjem homogenizer. Separate determinations were made on the tissues from each of 10 roaches. Because of the small amount of tissue available from one roach, the separate tissues of two roaches were pooled for the experiments with brain and Malpighian tubes. Thus, 20 roaches were used for the 10 determinations of the enzymatic activity of these tissues.

Determination of enzyme activity. The dephosphorylation of ATP was determined by assaying for inorganic phosphate after 15 minutes of incubation at room temperature (22 to 25 degrees C.). The final concentration of ingredients was: tris (hydroxymethyl) aminomethane buffer, pH 7.4, 0.03 *M*; ATP (Na salt from Pabst Laboratories) 0.2%; $MgCl_2$, or $CaCl_2$, 10^{-3} *M*; 0.2 ml. of tissue homogenate (except muscle, where but 0.1 ml. was used); and 0.9% KCl to a final volume of 1.0 ml. Inorganic phosphate was measured by the method of Sumner (1944) in a Klett-Summerson photoelectric colorimeter with a No. 66 filter. Zero time was at the instant of addition of enzyme, and the value determined at this time was subtracted from the final value. With this procedure for enzymatic assay, the rate of dephosphorylation of ATP is directly proportional to the amount of enzyme

present and is linear with time during the 15-minute incubation period. A representative set of data is depicted in Figure 1. Furthermore, no inorganic phosphate is liberated either in the absence of homogenate or in the presence of tissue but in the absence of ATP.

Determination of protein. Protein was determined by the method of Lowry *et al.* (1951). Due to the presence in the fat body of urates, which interfere with the color reaction, aliquots of this tissue homogenate were first extracted with

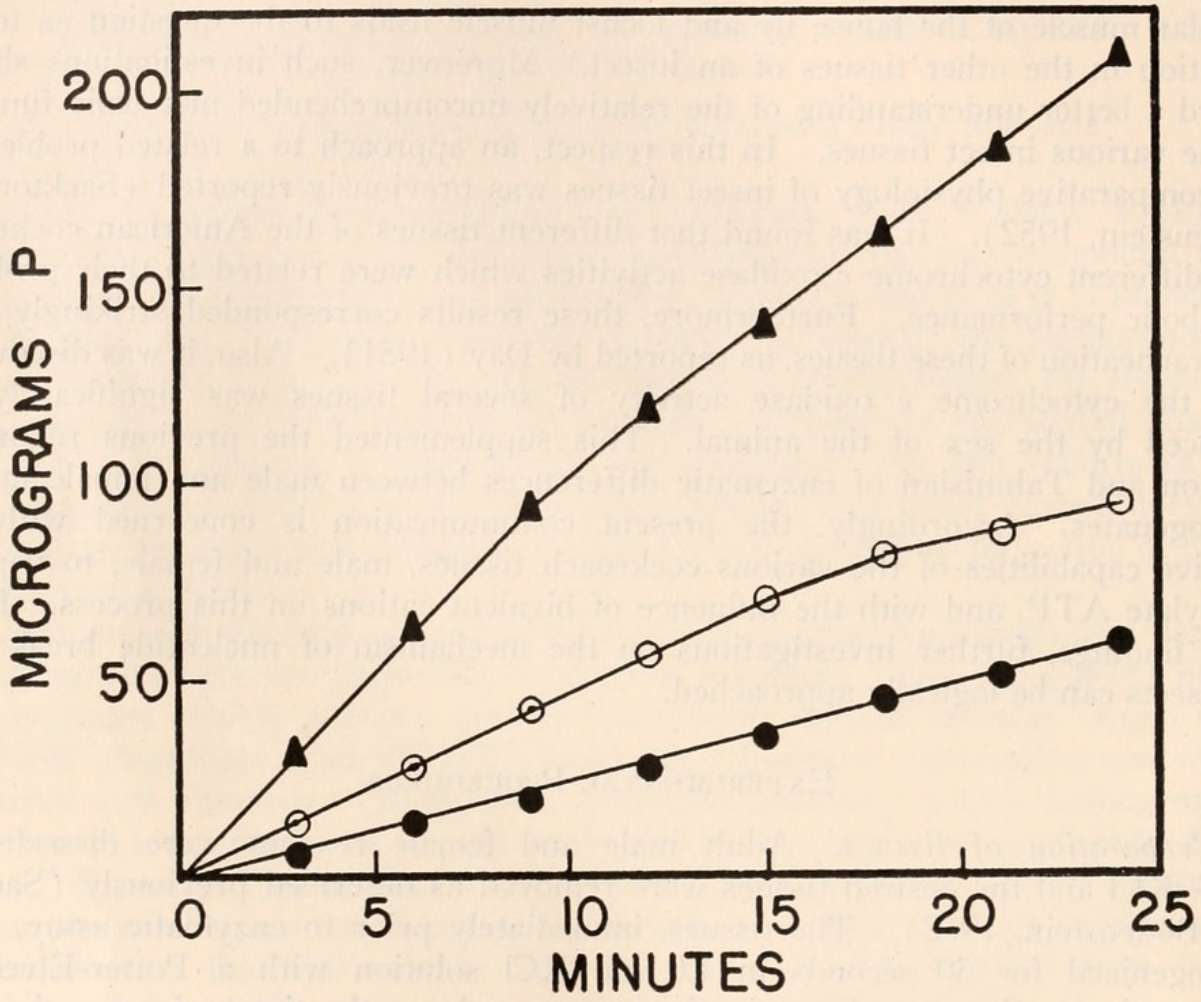

FIGURE 1. The effect of varying concentrations of muscle homogenate on the course of dephosphorylation of ATP. The following symbols: ▲, ○ and ● represent the liberation of inorganic phosphate in the presence of 0.2 ml., 0.1 ml. and 0.05 ml., respectively.

acetone and subsequently the protein was precipitated twice with trichloroacetic acid. Crystalline bovine serum albumin was used as the standard protein.

RESULTS

The relative capabilities of various cockroach tissues, male and female, in dephosphorylating ATP, and the influence of bivalent cations on this reaction are shown in Tables I and II.

Dephosphorylation by tissues. The data in Tables I and II reveal that insect tissues differ in their ability to dephosphorylate ATP. In general, based on their activity, they can be grouped into three categories. These are (1) those with greatest activity: muscle, fat body and Malpighian tubes; (2) those with moderate

TABLE I

Dephosphorylation of ATP
Males

Tissue	Control	Calcium	Magnesium
	micrograms P/hour/mg. protein		
	Mean ± S.E.	Mean ± S.E	Mean ± S.E.
Muscle	119 ± 11	237 ± 13	516 ± 39
Midgut	17 5	29 6	59 10
Foregut	33 6	59 11	77 8
Hindgut	25 5	44 8	103 14
Malpighian tubes	94 21	109 23	145 27
Fat body	140 33	179 44	353 38
Brain	60 10	67 24	113 16
Nerve cord	45 13	54 9	161 25

Control values represent micrograms P liberated in the absence of added bivalent ions. Ca and Mg ion concentration was 10^{-3} *M*. Each datum is the average of 10 determinations.

activity: brain and nerve cord; (3) those of low activity, namely, the three portions of the alimentary canal: hindgut, foregut and midgut. Muscle, as might have been predicted, is the most active. The rate of dephosphorylation by the fat body suggests that this organ may be the site of more extensive cellular metabolism than heretofore realized. This supports recent observations by Bodenstein (1953) on the metabolic role of the fat body. He found that the intermediate metabolism of this tissue responded considerably to changes in the hormonal situation. Although for the most part the gut portions possess relatively little dephosphorylating activity, a notable exception is the hindgut of the female roach.

TABLE II

Dephosphorylation of ATP
Females

Tissue	Control	Calcium	Magnesium
	micrograms P/hour/mg. protein		
	Mean ± S.E.	Mean ± S.E.	Mean ± S.E.
Muscle	231 ± 31	396 ± 33	556 ± 71
Midgut	20 5	26 6	65 12
Foregut	45 10	68 13	99 22
Hindgut	99 18	111 21	148 27
Malpighian tubes	118 23	143 21	120 19
Fat body	95 26	127 42	159 54
Brain	47 10	64 10	99 17
Nerve cord	74 16	74 17	140 27

Control values represent micrograms P liberated in the absence of added bivalent ions. Ca and Mg ion concentration was 10^{-3} *M*. Each datum is the average of 10 determinations.

Effect of bivalent ions. The data in Tables I and II demonstrate the influence of bivalent cations on ATP dephosphorylation. It is apparent that both Ca and Mg ions stimulate the release of inorganic phosphate from ATP, but that Mg^{++} is more effective. There are, however, differences in the magnitude of response to these ions with the different tissues. Thus, muscle is activated almost 100% by Ca^{++} whereas the slight stimulation observed with brain, nerve cord, Malpighian tubes and fat body was not statistically significant. The Ca-activation noted with the gut parts may represent the effect of this cation on the intrinsic musculature. Mg ions do not have a considerable effect on dephosphorylation of ATP by the Malpighian tubes but, in contrast, do activate the other tissues notably.

Effect of sex. A comparison of Tables I and II indicates that the dephosphorylation of ATP by a given tissue may be influenced by the sex of the roach. Thus, the enzymatic activity of the muscle in the female is significantly (beyond the 0.01 level) greater than that in the male, either in the absence of bivalent cations or in the presence of Ca ions, but not in the presence of Mg ions. The failure to

TABLE III

Inhibition of dephosphorylation of ATP by p-chloromercuribenzoate

Inhibitor conc. (*M*)	Per cent inhibition		
	Control	Calcium	Magnesium
5×10^{-6}	7	13	6
1×10^{-5}	40	28	31
5×10^{-5}	82	73	41
1×10^{-4}	89	85	54
5×10^{-4}	96	96	84
1×10^{-3}	97	97	92
5×10^{-3}	100	100	98
1×10^{-2}	100	100	100

Muscle of the male roach was used in these experiments. Control values represent the per cent inhibition in the absence of added bivalent ions. Ca and Mg ion concentration was 10^{-3} *M*.

observe a sex difference with muscle activated by Mg ions is in agreement with the results of Gilmour and Calaby, who found that the sex of the locust had no influence on the activity of the Mg-activated soluble apyrase. As in muscle, the hindgut of the female is more active than that of the male in the absence of bivalent cations or when stimulated by Ca ions. On the other hand, the fat body of the male, in the presence of Mg ions, is more active than that of the female. The differences, due to sex, exhibited by the other tissues are not statistically significant.

Effect of inhibitors. The effect of several known inhibitors of dephosphorylation on the activity of male roach muscle homogenates was ascertained since, in a previous study on the dephosphorylation of ATP by house fly flight muscle (Sacktor, 1953), it was found that azide and p-chloromercuribenzoate inhibited mitochondrial ATPase whereas fluoride did not. Fluoride, however, effected considerable inhibition of a soluble ATPase. In contrast with these observations on house fly flight muscle, it was found in the present study that NaN_3 and NaF at concentrations as high as 10^{-2} *M* are without appreciable effect on the dephos-

phorylation of ATP by roach muscle. However, p-chloromercuribenzoate inhibits dephosphorylation here also, as shown in Table III. A similar effect of this -SH inhibitor was noted by Gilmour and Calaby with their enzyme preparation from the locust.

A comparison of the present results with those obtained with the house fly (Sacktor, 1953) indicates that p-chloromercuribenzoate is approximately 100 times as effective an inhibitor in the roach. The data suggest further that inhibition in the roach is somewhat lessened in the presence of Mg ions. With rat liver mitochondrial ATPase, however, Novikoff *et al.* (1952) found no difference in the inhibition by p-chloromercuribenzoate (5×10^{-4} *M*) in the presence of Mg or Ca ions.

Discussion

The data presented show that various cockroach tissues differ in their ability to dephosphorylate ATP and that both Ca and Mg ions activate dephosphorylation. The magnitude of stimulation by these cations depends on the particular tissue. It should be emphasized, however, that these results were obtained with the use of a standard experimental procedure for all tissues. This procedure, which is based on optimal conditions for muscle, need not represent the ideal situation for the other tissues.

Complete characterization of the dephosphorylating mechanisms with the various tissues must await further experimentation; nevertheless, an interesting comparison between the enzymatic activities found in roach muscle and in vertebrate muscle is now possible. In a recent study of the biochemical differences between red and white muscle, Lawrie (1952) found that red muscle had more myoglobin and a higher activity of cytochrome *c* oxidase, succinic dehydrogenase and succinoxidase than did white muscle, but, conversely, a lower ATPase activivty. The coloration of red muscle was attributed to the greater content of myoglobin. In the roach, however, the muscle of the male is reddish in color, whereas that of the female is much lighter, almost white (Sacktor and Bodenstein, 1952; Edwards, 1953). It was found, too, that these color variations were reflected in the cytochrome *c* oxidase (Sacktor and Bodenstein, 1952) as well as succinic-cytochrome *c* reductase activities (Sacktor, unpublished data); for, muscles with more coloration had a higher activity; and, as with vertebrate muscle, the dephosphorylation of ATP in female (white) muscle was greater. Furthermore, Barron and Tahmisian (1948) reported more cytochrome *c* and Fe in the male roach muscle. Contrary to the situation in vertebrates, there is no evidence for the presence of myoglobin in roach muscle. These facts indicate that in insects, at least, the color differences may be due to differences in the cytochrome content, and suggest that cytochromes as well as myoglobin are possibly concerned in the color of vertebrate muscle.

Despite many similarities between insect and vertebrate muscle, distinctions can be made even between the various insect species. Differences in the mechanism of ATP breakdown by house fly flight muscle and locust muscle have been described above. In addition, dissimilarities in the action of several inhibitors of dephosphorylation were found between roach muscle homogenates and house fly flight muscle preparations. In the roach muscle, p-chloromercuribenzoate inhibited dephosphorylation whereas azide and fluoride did not. In contrast, with mitochon-

dria isolated from the specialized flight muscles of the fly, ATPase was inhibited by azide as well as by p-chloromercuribenzoate (Sacktor, 1953). Furthermore, fluoride prevented orthophosphate liberation by a soluble fraction obtained from these specialized fibrillar muscles. Other species-specific enzymatic characteristics have been noted recently in other insects. Metcalf and March (1949) found that certain organic phosphates inhibited fly brain cholinesterase but had little effect on bee brain cholinesterase. Supplemental evidence on species variations was obtained by Rockstein and Levine (1951) in their studies on acid phosphatases in five insect species. From these numerous heterogeneities, it is becoming increasingly evident that generalizations on the enzymatic properties of all insects are no longer valid. Careful examination of such parallels and differences in insects may well yield fruitful leads for investigation in the tissues of other animals.

Summary

1. The dephosphorylation of ATP by various tissues of the American cockroach was investigated. It was found that these tissues differ in their dephosphorylating activity and may be rated, in decreasing order, approximately as follows: muscle, fat body, Malpighian tubes, nerve cord, brain, hindgut, foregut and midgut.

2. In general, both Mg and Ca ions activated the breakdown of ATP, although Mg was more effective. The magnitude of activation by these bivalent cations depended on the tissue.

3. Some differences due to sex were found. The dephosphorylating activity of the muscle and hindgut of the female was significantly greater than that in the male either in the absence of bivalent cations or in the presence of Ca ions. The fat body of the male, in the presence of Mg ions, was more active than that of the female.

4. The dephosphorylation of ATP by roach muscle was inhibited by p-chloromercuribenzoate, but not by azide and fluoride. Since these three compounds were effective inhibitors of house fly flight muscle ATPase, this distinction further emphasizes the heterogeneity of ATP breakdown mechanisms in insects.

LITERATURE CITED

Albaum, H., and M. Kletzkin, 1948. Adenosine triphosphate from *Drosophila melanogaster*. *Arch. Biochem.*, **16**: 333–337.

Barron, E. S. G., and T. N. Tahmisian, 1948. The metabolism of cockroach muscle (*Periplaneta americana*). *J. Cell. Comp. Physiol.*, **32**: 57–76.

Bodenstein, D., 1953. Studies on the humoral mechanisms in growth and metamorphosis of the cockroach, *Periplaneta americana*. III. Humoral effects on metabolism. *J. Exp. Zool.* (In press.)

Calaby, J. H., 1951. Adenosine triphosphate from insect muscle. *Arch. Biochem. Biophys.*, **31**: 294–299.

Chin, C. T., 1951. High temperature coefficient of enzymic activity of the muscle of the American cockroach (*Periplaneta americana*). *Arch. Biochem. Biophys.*, **31**: 333–335.

Day, M. F., 1951. Studies on the digestion of wool by insects. III. A comparison between the trachea of the midgut of *Tineola* larvae and that of other insect tissues. *Austral. J. Scientific Res.*, **B4**: 64–74.

Edwards, G. A., 1953. *In:* Insect physiology. Edited by K. D. Roeder. John Wiley and Sons, Inc., New York: 96–146.

Gilmour, D., 1948. Myosin and adenylpyrophosphatase in insect muscle. *J. Biol. Chem.*, **175**: 477–478.

GILMOUR, D., AND J. H. CALABY, 1952. The magnesium-activated apyrase of insect muscle. *Arch. Biochem. Biophys.*, **41**: 83–103.

LAWRIE, R. A., 1952. Biochemical differences in red and white muscle. *Nature*, **170**: 122–123.

LOWRY, C. H., N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**: 265–275.

METCALF, R. L., AND R. B. MARCH, 1949. Studies on the mode of action of parathion and its derivatives and their toxicity to insects. *J. Econ. Entom.*, **42**: 721–728.

NOVIKOFF, A. S., L. HECHT, E. PODBER AND J. RYAN, 1952. Phosphatases of rat liver. I. The dephosphorylation of adenosine triphosphate. *J. Biol. Chem.*, **194**: 153–170.

ROCKSTEIN, M., AND L. LEVINE, 1951. Enzymes in insects. Acid phosphatase. *Ann. Entom. Soc. Amer.*, **44**: 469–472.

SACKTOR, B., 1953. Investigations on the mitochondria of the house fly (*Musca domestica*). I. Adenosine triphosphatase. *J. Gen. Physiol.*, **36**: 371–387.

SACKTOR, B., AND D. BODENSTEIN, 1952. Cytochrome *c* oxidase activity of various tissues of the American cockroach, (*Periplaneta americana* L.). *J. Cell. Comp. Physiol.*, **40**: 157–160.

SUMNER, J. B., 1944. Method of colorimetric determination of P. *Science*, **100**: 413–414.