IMMUNOLOGICAL DIFFERENCES OF PHOSPHORYLASES

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A number of reports in the literature indicate that there are immunological variations in enzymes from different sources (1–4), but these studies did not include a comparison of intracellular enzymes from different tissues of an animal. The experiments reported in this paper were undertaken to determine whether a particular enzyme (phosphorylase) differed immunologically in the tissues of a single species, certain phosphorylases from different species or sources being also included. This information may serve as a model to help explain some variations in tissue or organ response to certain closely related chemicals.

Antisera to dog liver phosphorylase (LP) and to dog heart phosphorylase (HP) were prepared. By means of these sera, it was found that phosphorylases differed in their reaction with the antisera, depending on the source of the tissue and on the species.

Materials and Methods

Materials—Glucose-1-phosphate (G-1-P), glycogen, tris(hydroxymethyl)aminomethane (Tris), ammonium sulfate, and calcium phosphate gel were prepared or treated as described by Sutherland and Wosilait (5). Sodium fluoride was obtained from Mallinckrodt, and adenosine-5-phosphate (5-AMP) was purchased from the Nutritional Biochemicals Corporation.

Phosphorylase Preparations—The dog liver phosphorylase used for injection to produce antisera was highly purified and dialyzed, and corresponded to the dialyzed and centrifuged fourth alcohol fraction (Step 7) by Sutherland and Wosilait (5). The specific activities of these preparations were about 24, and were therefore estimated to contain about 80 per cent active phosphorylase. Dog liver phosphorylase used for routine testing of antisera was less pure, corresponding to a dialyzed third alcohol fraction (Step 5). Occasionally crude dog liver phosphorylase was used as described below. Radioactive liver phosphorylase was prepared as described by Rall, Sutherland, and Wosilait (6).

The dog heart phosphorylase for injection was considerably purified and

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corresponded to the fourth alcohol fraction (Step 3) prepared by Rall, Wosilait, and Sutherland (7). The specific activity of this fraction was about 5.

Phosphorylase from other sources was prepared as follows: All the operations were carried out at 3°, unless otherwise noted. Approximately 10 gm. of tissue were sliced into 40 ml. of 0.1 M NaF and 0.005 M K$_2$HPO$_4$. Muscle tissue was frozen and sliced with a chisel, and then homogenized in a Waring blender for 2 minutes. The homogenate was adjusted to pH 6.5 with acetic acid and centrifuged for 10 minutes at 4000 × g to remove coarse material. Calcium phosphate gel equal to one-twelfth of the homogenate volume was then added, and after 15 minutes was removed by centrifugation and discarded. For each 10 ml. of supernatant fluid, 4.6 gm. of (NH$_4$)$_2$SO$_4$ were added; the solution was adjusted to pH 7.2 with KOH and centrifuged, and the precipitate was dissolved in 0.1 M NaF and frozen until used.

Preparation of Serum for Assay—In most cases, the sera used were “albumin-free” fractions prepared by precipitating the serum with an equal volume of saturated (NH$_4$)$_2$SO$_4$ at pH 7.2. The precipitate was dissolved in 0.9 per cent NaCl and dialyzed for 24 hours against 1 liter of H$_2$O, which was changed once during the 24 hour period. The globulin fraction was in solution at the completion of the dialysis, possibly because enough salt remained. It was necessary to treat this fraction for use in experiments in precipitation by adding 0.15 volume of 0.9 per cent NaCl. The precipitate which formed was removed without affecting the activity of the serum. For some experiments, it was necessary to dilute the sera before testing with 0.9 per cent NaCl, since 0.05 ml. of the undiluted antiserum often contained an excess of antibody for the amount of enzyme used in the standard assays.

Standard Assay—Assays for the effect of the sera on phosphorylase were carried out in a total volume of 0.2 ml. Various phosphorylase preparations were diluted, so that the original activity per tube was approximately equal. The serum was incubated with the phosphorylase for 10 minutes at 37° in a solution containing, as a final concentration, 0.033 M Tris, pH 7.2, and 0.075 M NaF in a volume of 0.15 ml. After this incubation, phosphorylase activity was determined by measuring the rate of liberation of inorganic phosphate from G-1-P, essentially as described by Sutherland and Wosilait (5). Phosphorylase reaction mixture (0.05 ml., pH 6.1) was added and resulted in a final concentration of 0.036 M G-1-P, 0.1 M NaF, and 4.03 mg. per ml. of glycogen. Incubation at 37° was continued for 10 minutes; then the reaction was stopped by the addition of 1.8 ml. of cold 5 per cent trichloroacetic acid. After centrifugation, 1.0 ml. of supernatant fluid was used for determination of inorganic phosphate by
the method of Fiske and Subbarow (8), as adapted to the Klett-Summers
son colorimeter.

EXPERIMENTAL

Preparation of Antisera—Young adult albino rabbits and young roosters
(weight, 1.5 to 2.1 kilos) were used. The roosters injected in the first
series were white Leghorns; those in the second series were apparently
hybrids. All injections were given intravenously, in an ear vein in the
rabbits and in a ventral wing vein in the roosters. Prior to injection, the

| Table I |

Antiphosphorylase Activity of Rooster and Rabbit Sera
| after Injection of Dog Liver Phosphorylase |

Highly purified LP was adsorbed on gel and injected intravenously in Amount
A (28.8 mg. of protein per animal during the series) or in Amount 0.1 A. At the
times indicated, blood samples were taken and the sera were assayed for antiphos-
phorylase activity in the standard test system. The results are expressed as the
per cent inhibition of LP by 0.01 or 0.05 ml. of antisera.

<table>
<thead>
<tr>
<th>Time of assay</th>
<th>Rooster antisera</th>
<th>Rabbit antisera</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>To Amount A</td>
<td>To Amount 0.1 A</td>
</tr>
<tr>
<td>days</td>
<td>0.01 ml. 0.05 ml.</td>
<td>0.01 ml. 0.05 ml.</td>
</tr>
<tr>
<td>After 6th injection...</td>
<td>5</td>
<td>36</td>
</tr>
<tr>
<td>&quot; 6th &quot;... 49</td>
<td>13</td>
<td>19</td>
</tr>
<tr>
<td>&quot; 1st booster...</td>
<td>4</td>
<td>50</td>
</tr>
<tr>
<td>&quot; 1st &quot;... 7</td>
<td>26</td>
<td>20</td>
</tr>
<tr>
<td>&quot; 2nd &quot;... 7</td>
<td>82</td>
<td>99</td>
</tr>
<tr>
<td>&quot; 3rd &quot;... 7</td>
<td>38</td>
<td>99</td>
</tr>
</tbody>
</table>

enzyme preparation was diluted with cold H₂O, so that it contained ap-
proximately 3 to 4 units of enzyme per ml. The enzyme was then ad-
sorbed on 0.1 volume of calcium phosphate gel. The gel-enzyme precipi-
tate was resuspended in 0.1 M NaCl, so that the volume for injection was
3 ml.

In the first series (Table I), two rabbits and two roosters served as con-
trols, and a like number were injected with each of the two different
amounts of antigen. Two rabbits and two roosters were each given 4.1
mg. of protein per injection, and a similar number of animals were given
0.41 mg. of protein per injection. Each series of animals was given a total
of six injections, equally spaced over a period of 18 days. After the com-
pletion of this schedule, no further injections were given for a period of
7 weeks. Each group of animals was then given three booster doses of
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the gel-adsorbed enzyme. The animals which received the larger dose of antigen were given 1.2 mg. of protein per booster injection, whereas those receiving the smaller dose were given 0.12 mg. of protein per booster injection. Thus, one series of animals received a total of 28.8 mg. of protein and the other one-tenth of this amount. Control animals were given injections of calcium phosphate gel suspended in 0.1 M NaCl.

It may be seen (Table I) that the response of the roosters was significantly better than that of the rabbits in terms of total antiphosphorylase activity, and also that the smaller amount of antigen (Amount 0.1 A) produced essentially the same final result as the larger amount (Amount A). The amounts of antiphosphorylase activity in the sera of the two animals in each group were similar; the average of the two is shown in Table I as a single result. The lower antibody titers after a 7 week rest period and the response to "booster" injections are responses typical of immunological phenomena.

A second series of animals, all roosters, were given intravenous injections of dog liver phosphorylase and dog heart phosphorylase adsorbed on gel (Table II). In addition, two roosters were given dog liver phosphorylase which was not adsorbed on gel. The roosters injected with the gel-adsorbed liver phosphorylase received a total of 8.8 mg. of protein in eleven injections. Eight injections were given at 3 day intervals, then, after a 4 week rest period, three more injections were given at 10 day intervals.

The roosters receiving heart phosphorylase were injected according to the same schedule, but were given a total of 5.5 mg. of protein in their eleven injections. The roosters injected with liver phosphorylase without gel were given a total of eight injections at 3 day intervals, each injection consisting of 0.8 mg. of protein.

The responses (Table II) of the two animals injected with the same preparation were similar, but the results summarized in Table II refer to one animal only, since the antiphosphorylase activities of sera of the duplicate animals were not measured at all the times included. It can be seen that the injection of gel-adsorbed enzyme resulted in higher levels of antibody than when the enzyme alone was injected. As injections were continued, the antibody titers fell in all cases, for reasons which have not been clarified. After a 4 week rest-period, the levels did not respond to booster injections as in the first series. The roosters in the second series showed signs of anaphylactoid reactions, such as blanching of the comb and staggering, whereas the first series of roosters showed no ill effects.

Properties of Antisera—The antibody was found to be stable when stored under a variety of conditions and treated in a variety of ways. The sera retained the same amount of antiphosphorylase activity, whether stored at room temperature for several days, frozen for months, or re-
peatedly thawed and frozen. The antiphosphorylase activity was fully retained after the preparation was heated at 56° for 30 minutes. Thus it seems likely that a complement is not necessary for the antiphosphorylase activity as tested in the standard assay system.

It was observed that the serum exerted its inhibitory effect in a very short time. When the serum was combined with the enzyme, the same quantitative effect was noted in 5 minutes at 3° as in 10 minutes at 37°. When enzymatic activity was determined immediately after the antiserum and enzyme were added simultaneously with no preincubation, the inhibitory effect was only about 10 per cent less than when the two were preincubated for 10 minutes at 37°.

Unfractionated control serum displayed a small but significant (5 to 10 per cent) inhibitory effect on phosphorylase activity. The “albumin-free” control serum exerted essentially no inhibitory effect on the enzyme. For this reason, all sera used in subsequent experiments were first fractionated with (NH₄)₂SO₄ to remove most of the albumin component.

Effect of Substrates, Etc.—Several investigators have found that an enzyme may be protected against its antibody by the substrate or a cofactor (2, 4, 9, 10). In this investigation, it was found that, when increased concentrations of G-1-P were used in the test system, no effect was observed on the antiphosphorylase activity. Moreover, preincubation of phos-

### Table II

**Antiphosphorylase Activity of Rooster Serum after Injection of Dog Liver and Dog Heart Phosphorylase**

Highly purified LP (8.8 mg. of protein per series) was injected intravenously in solution or adsorbed on gel. Partially purified HP (5.5 mg. of protein per series) was adsorbed on gel before injection. At the times indicated blood samples were taken and the sera were assayed for antiphosphorylase activity in the standard test system, except that the antisera to heart phosphorylase were tested against heart phosphorylase. The results are expressed as the per cent inhibition of phosphorylase by 0.01 or 0.05 ml. of antisera.

<table>
<thead>
<tr>
<th>Time of assay</th>
<th>Enzyme preparation injected</th>
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<tbody>
<tr>
<td></td>
<td>LP without gel</td>
</tr>
<tr>
<td>After 4th injection</td>
<td></td>
</tr>
<tr>
<td>&quot; 6th &quot;</td>
<td>4</td>
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<tr>
<td>&quot; 8th &quot;</td>
<td>4</td>
</tr>
<tr>
<td>&quot; 10th &quot;</td>
<td>8</td>
</tr>
<tr>
<td>&quot; 11th &quot;</td>
<td>6</td>
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</tbody>
</table>

For this reason, all sera used in subsequent experiments were first fractionated with (NH₄)₂SO₄ to remove most of the albumin component.
phorylase with 5-AMP did not protect the enzyme from the inhibitory effect of the serum. On the other hand, when increased amounts of glycogen were preincubated with the enzyme, a significant amount of protection of the enzyme resulted. An amount of serum which inhibited the enzyme 30 per cent in the absence of glycogen inhibited only 6 per cent of the enzyme activity when the enzyme and glycogen (5 to 25 mg. per ml.) were preincubated for 10 minutes at 37°. High concentrations of NaF (to 0.3 M), one of the chief constituents of the reaction mixtures, had no effect on the inhibitory properties of the serum. Ammonium sulfate, present in many of the enzyme preparations used later, similarly had no significant effect on the antigen-antibody reaction when tested at a final concentration of 0.2 M.

Precipitates of Antigen-Antibody—Sera which had been precipitated with ammonium sulfate and treated with NaCl as described were used to study the possible precipitation of the antigen-antibody complex. In one series of experiments, control serum and liver phosphorylase antiserum were added to radioactive liver phosphorylase (labeled with P32). The radioactive phosphorylase (0.2 ml.) was mixed with 0.5 ml. of the sera and 0.05 ml. of 0.75 M NaF, which was included in order to minimize the possibility of enzymatic inactivation of the phosphorylase. The mixtures were allowed to stand for 3 hours, at which time heavy precipitation had occurred in the tubes containing antiserum, while no precipitation had occurred in the tubes containing the control serum. After centrifugation, the antiserum precipitate contained most of the radioactivity; little radioactivity remained in the supernatant fluid. No visible precipitate was obtained from the tubes containing the control sera, and the radioactivity remained in the supernatant fluid after centrifugation.

In other experiments, enzymatic activity of precipitates and supernatant fluids was determined after incubation of control serum and antiserum with phosphorylases. Antisera to liver phosphorylase were incubated with liver phosphorylase, and antisera to heart phosphorylase with heart phosphorylase. No precipitate was formed when the enzymes were mixed with control sera, but they were formed when the phosphorylases were combined with their respective antisera. After centrifugation and removal of the precipitate, phosphorylase activity in the supernatant fluid was decreased or absent, depending on the relative amounts of antigen and antibody. The washed and resuspended precipitates were not enzymatically active in the standard assay system for phosphorylase. It seems clear that the antibodies were able to form precipitates with the antigen as well as to obliterate the enzymatic activity of the antigen.

LP Antiserum; Species Variation—Antiserum prepared from roosters injected with dog liver phosphorylases was incubated with liver phosphory-
lase from several species. The phosphorylases tested in the following experiments were prepared by homogenization in fluoride, centrifugation, gel treatment, and ammonium sulfate precipitation. The results of these experiments are summarized in Fig. 1. It can be seen that the antiserum had less of an inhibitory effect on the other enzymes tested than it did on dog liver phosphorylase. No effect was observed when rooster liver phosphorylase was used. These quantitative differences in effect were observed with all volumes of antiserum used. The antiserum was also incubated with potato phosphorylase, and no antiphosphorylase activity was detected.

**HP Antiserum; Species Variations**—Antiserum to dog heart phosphorylase was prepared by injecting roosters with the enzyme. This antiserum was incubated with phosphorylase from hearts and livers of several species.

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**Fig. 1.** Effect of different amounts of dog LP antiserum on liver phosphorylases from four animals. Liver phosphorylases were incubated for 10 minutes at 37° in the standard assay with 0.003, 0.01, or 0.05 ml. of antiserum or control serum from chickens. Phosphorylase activity was then measured, and the per cent inhibition caused by the antiserum is represented by the height of the bars.
of animals. The latter enzymes were prepared by homogenization in fluoride, centrifugation, gel treatment, and ammonium sulfate precipitation (see above). It was found that this antiserum inhibited cat heart phosphorylase 30 and rooster heart phosphorylase 7 per cent, whereas it inhibited dog heart phosphorylase 75 per cent. Phosphorylases from rooster, rabbit, and cat livers were not inhibited at all by this antibody.

**Fig. 2.** Effect of dog LP antiserum on dog tissue phosphorylases. The antiserum from chickens was added in amounts (0.05 ml.) sufficient to abolish the activity of approximately 4 times the amount of LP added. After 10 minutes at 37°, phosphorylase activity was measured in the presence or absence of $1.5 \times 10^{-3}$ M 5-AMP. The results are those obtained in the presence of 5-AMP.

**LP Antiserum; Tissue Variation**—Phosphorylase was prepared from tissues of dogs, namely brain, diaphragm, liver, heart, smooth muscle from small intestine, and gastrocnemius muscle, by the same method as already described for hearts and livers of various species. The results, after these enzymes were incubated with antiserum to dog liver phosphorylase, are summarized in Fig. 2. Liver phosphorylase was inhibited much more by LP antiserum than were the other phosphorylases. In these studies, the amount of antiserum necessary to produce significant effects on other phosphorylases was sufficient to abolish the activity of about 4 times the amount of liver phosphorylase added. Since the enzymes from tissue other than liver were composed of approximately equal
amounts of "a" and "b" forms, they were tested without and with $1.5 \times 10^{-2}$ M 5-AMP. (The "b" forms are enzymatically inactive in the absence of 5 AMP and are active when tested in the presence of 5-AMP.) It was found that phosphorylase from brain, skeletal muscle, and diaphragm were inhibited only slightly more (5 per cent) in the absence of 5-AMP. Smooth muscle phosphorylase (intestinal) was inhibited 17 per cent in the presence of 5-AMP and 45 per cent without 5-AMP, and was the only phosphorylase tested which showed this considerable difference. The per cent inhibition of dog heart and liver was unaffected by the addition of 5-AMP.

**HP Antiserum; Tissue Variation**—Antiserum to dog heart phosphorylase was tested against the tissue preparations described previously. These results are summarized in Fig. 3. It can be seen than this antiserum was less inhibitory toward the other phosphorylases than toward the enzyme to which it was prepared. Phosphorylase from intestinal smooth muscle was inhibited slightly, and liver phosphorylase activity was not affected by this antiserum. It may be noted that the antiserum to HP is relatively more effective against phosphorylases from striated muscle than the antiserum to LP.

![Diagram](http://www.jbc.org/)

Fig. 3. Effect of dog HP antiserum on dog tissue phosphorylases. The antiserum from chickens (0.05 ml.) was incubated with the phosphorylases for 10 minutes at 37°; then phosphorylase activity was measured in the presence of $1.5 \times 10^{-2}$ M 5-AMP.
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Specificity of LP Antiserum—That the difference in the inhibitory effect of the LP antiserum on different enzymes was not owing to the presence of an interfering substance in the crude extracts was shown in two ways. First, dog liver phosphorylase used in the studies of species and tissue variation was prepared in the same manner as the phosphorylases from other sources. Moreover, it was shown in the standard assay that this antiphosphorylase activity of the antiserum was the same when tested with the cruder liver phosphorylase as when tested with a highly purified phosphorylase. Second, a purified preparation of dog liver phosphorylase was mixed with the crude extract of a tissue which was not inhibited by the serum (Table III). The amount of activity remaining after incubation with an excess of antibody was equal to that of the added crude phosphorylase preparation.

Effect of LP Antiserum on Liver Slices—There is some evidence indicating that antibodies are unable to penetrate or cross cell membranes readily and the following experiment supports this evidence. Thin slices of cat liver weighing between 52 and 63 mg. were incubated, with shaking, for 45 minutes at 37° in a medium consisting of 0.7 ml. of control or LP antiserum, 0.5 ml. of 0.05 N HPO₄ (pH 7.2) in 0.308 N NaCl, and 0.2 ml. of 1:5000 epinephrine. It was found that the antiserum had no effect on glucose output or glycogen content of the slices.

DISCUSSION

The primary purpose of this investigation was to determine whether the enzyme phosphorylase from one tissue was identical to or different from phosphorylase from another in the same animal, with the belief that this
information might be helpful in understanding some variations in the response of different tissues to chemical or pharmacological agents. For example, a given chemical agent A may be more effective than a closely related chemical B when one tissue is tested for some response, while, on another tissue, chemical agent B may be more effective than A. It is probable that a number of factors play a role in these variations, and such factors as cell permeability, different rates of modification of the chemical, etc., may be most important in any given case. The possibility also existed that a specific component of the cells might differ from tissue to tissue. Since phosphorylases from dog liver and heart were available for study, they were used as an example of a specific component of cells. Antibodies to these phosphorylases were produced, and the effect of these antibodies on phosphorylases from different tissues of a dog was studied. The data presented in this paper, based on experiments with two different antibodies, indicate that the phosphorylases from different organs in the same animal are not identical. In some cases the immunological differences were very marked; e.g., the antiserum to heart phosphorylase did not inhibit liver phosphorylase at all in the amounts tested.

The genetic control of enzyme formation is being actively investigated by numerous investigators, and there is considerable evidence that even a single gene may control the formation of a single enzyme. If one or more genes in an animal control enzyme formation, one might expect the enzyme structure in different tissues of the same animal to be identical. It was concluded from the experiments reported here that the enzyme phosphorylase differed in various tissues of an animal. It seems probable that a given tissue modifies the specific enzyme (phosphorylase) if there is indeed a close relationship of genes to enzymes.

Several investigators have reported antibodies to enzymes which are specific for one species of animals (1–4). In the present investigation, similar findings were noted; i.e., the phosphorylase of liver and heart from other species differed in their response to the dog antisera. The species differences were sometimes marked, but in some cases there was less difference noted between species than between different tissues of the same animal.

The current study does not yield information regarding the nature or the magnitude of differences in the structure of the phosphorylases. It is possible that variations in chemical composition or spatial arrangements, or both, may account for the immunological differences. Certain biochemical or kinetic differences of phosphorylases have been observed; e.g., dog heart phosphorylase can be converted to a “b” form enzymatically while liver phosphorylase is converted to a different “inactive” form by the same enzymes (7). These findings are compatible with the conclusion
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drawn from immunological studies that the phosphorylases from different tissues of the dog are different.

SUMMARY

1. Liver phosphorylase and heart phosphorylase from dog were injected into roosters and rabbits, and antibodies to these enzymes were obtained.
2. The antibodies reacted with their respective antigens (phosphorylases) so that enzymatic activity was lessened or abolished. Antigen-antibody precipitates were also formed, and these complexes were enzymatically inactive.
3. When the antisera were tested against phosphorylases from different organs of the dog, it was found that the phosphorylases from different organs did not react to the same extent with the antisera. In some cases, the differences were great; e.g., the antiserum to heart phosphorylase did not inhibit liver phosphorylase at all in the amounts tested.
4. Phosphorylases from the same organ but from different species also varied in the extent of reaction with the antiserum.
5. The results of these experiments demonstrate that the phosphorylases from different organs of the same animal are immunologically different. They also confirm the reports that an enzyme from the same organ of different species may differ immunologically.

BIBLIOGRAPHY

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