Tissue Sources of Human Serum Alkaline Phosphatase, as Determined by Immunochemical Procedures*

MAX SCHLAMOWITZ AND OSCAR BODANSKY

From the Division of Research Biochemistry, Roswell Park Memorial Institute for Cancer Research, Buffalo, New York, and the Division of Metabolism and Enzyme Studies, Sloan-Kettering Institute for Cancer Research, New York, New York

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The immunochemical differentiation of the alkaline phosphatases from tissues of the dog has been reported (2, 3). Antisera against dog intestinal phosphatase quantitatively precipitated this enzyme under conditions which were without effect on the alkaline phosphatases from liver and kidney of the same species. The potential value of this immunochemical approach for studies of tissue specificity of functionally similar enzymes and for establishing the origin of serum enzymes in normal and diseased states stems from the ability of antibodies to react specifically with and precipitate homologous antigens from mixtures of antigenically unrelated substances. This has prompted the present immunochromic investigation of preparations of alkaline phosphatases from human tissues.

Studies on the specificity of the antibodies against several human tissues will be reported, as well as the use of these antibodies to establish the contribution by the tissues to the total alkaline phosphatase activity in serum.

EXPERIMENTAL

Materials and Methods

Human Intestinal Phosphatase—The enzyme(s) from the mucosa of the normal entire small intestine was prepared in a partially purified form by a procedure involving autolysis and fractionation with ethanol similar to that described for the dog intestinal enzyme (3). The tissue was homogenized in a Waring Blender with an equal volume of 50 per cent by volume aqueous ethanol and 0.1 volume of a 1:1 mixture of ethyl acetate and toluene. The mixture was adjusted to pH 6.9 to 7.0 and permitted to autolyze for 72 hours at room temperature with periodic adjustments of the pH to neutrality with sodium hydroxide. The mixture was then centrifuged at 18,000 × g for 20 minutes at about 5°C; the supernatant solution was cooled to about 10°C, and the pH was adjusted with glacial acetic acid to pH 4.6 at 10°C. After standing for 1 hour at −18°C, the solution was clarified by centrifugation and the phosphatase was precipitated at about 5°C by addition of 1.14 volumes of absolute ethanol. The precipitate was collected by centrifugation, washed as before and dried, was a white powder, soluble at pH 7. It contained 28 per cent protein and had a turnover number per 100,000 gm. of protein of about 2075 when assayed by the procedure already described (2).

Human Bone Phosphatase (A)—This phosphatase preparation was isolated essentially according to the procedure of Martland and Robison (4) by extraction with water and chloroform of the tumor mass and distal head of the femur from a patient with osteogenic sarcoma. The use of this material was dictated by the fact that normal bones contain relatively low levels of phosphatase, so that enormous quantities would have been required to obtain adequate amounts of enzyme for use in the immunization studies. The use of osteogenic sarcomatous bone as a source of bone phosphatase is based on the assumption that there is no significant immunochemical difference between the enzymes from this tumorous bone and that from normal bone.

Tumor material, 50 gm., and bone, 39 gm., from the epiphysis were homogenized in a blender with H2O (5 and 3 volumes, respectively) and CHCl3 (1 ml.) and set for 48 hours at room temperature. At the end of this time, the supernatant fluids from these mixtures, obtained by filtration or centrifugation, were dialyzed in the cold for 27 hours against distilled water, frozen and lyophilized. The residues were then extracted with 50 ml. of H2O and 0.5 ml. of CHCl3 for 2 days at room temperature followed by 5 days at about 5°C. The extracts were frozen and lyophilized. Because the available amount of this enzyme was limited, it was not subjected to further fractionation with ethanol as was done with the intestinal enzyme.

The material thus prepared from 14 normal intestines (about 2.4 gm.) was combined and extracted with 150 ml. of water. The aqueous extract containing about 900 mg. of protein was refractionated in the cold, as above, with 25 per cent ethanol at pH 4.6, followed by precipitation of the enzyme in presence of 70 per cent ethanol at pH 5.0 (glass electrode). The final product, washed as before and dried, was a white powder, soluble at pH 7. It contained 28 per cent protein and had a turnover number per 100,000 gm. of protein of about 2075 when assayed by the procedure already described (2).

Human Kidney and Human Liver Phosphatase—The enzyme preparations from kidney cortex and whole liver were obtained by autolysis and ethanol fractionation, essentially as described for the intestinal enzyme. They were prepared by Dr. Allan Grossberg of the laboratory of one of the authors (M. S.). Approximately 88 and 77 per cent of these kidney and liver enzyme...
samples, respectively, were soluble protein and their turnover numbers were about 23 and 80, respectively.

Antihuman Intestinal Phosphatase Serum—A total of 133 mg. of the soluble antigen protein in an alum suspension was administered intravenously to a rabbit in a series of 18 graded-dose injections. The injections were given on alternate days. The animals were bled on the 7th, 9th, and 14th days following the last injection. The pooled serum from these bleedings was preserved with phenol (0.2 per cent), adjusted to pH 7.8 at 25° and stored at about 5°. The smallest volume of antiserum which precipitated 90 per cent or more of the maximal amount of activity precipitable from 30 μg. of intestinal phosphatase was 0.04 ml. (5).

It was shown earlier (2) that the system, partially pure dog intestinal phosphatase-antidog intestinal phosphatase, contained several precipitating antigens and antibodies of which the enzyme and its antibody constituted one pair. It must be presumed that the same is true in the case of the human phosphatases which are even less pure than the one from the dog. It should therefore be pointed out that the loss of enzyme activity due to precipitation rather than analysis of total protein precipitated was the measurement used to follow the reaction of the enzyme with its antibodies.

Antihuman Bone Phosphatase Serum—The serum was prepared in the manner described for the anti-intestinal phosphatase serum except that 485 mg. of the bone phosphatase was used as antigen. The corresponding titer for this antitase phosphatase serum is 0.3 ml. for 136 pg. of the enzyme.

Horse Antirabbit γ-Globulin Antibodies—The preparation of horse antibodies against rabbit γ-globulin has already been described (6).

Human Serum—Serum from hospitalized patients and from normal fasted individuals were obtained. The pH of each serum was adjusted to pH 7.8 at about 25° and clarified by centrifugation for 10 minutes at 18,000 × g at 3°. Aliquots of the sera were analyzed by the King-Armstrong procedure to establish the level of phosphatase activity. The remaining serum was preserved with phenol (0.14 per cent) and used for the immunochromatical studies.

Determination of Protein Concentration and Phosphatase Activity—Analysis of protein was carried out by the biuret method as previously described (6). For the assay of phosphatase activity in supernatant fluids from the precipitation reactions, samples were incubated at 37° in a medium containing sodium γ-glycero phosphate (2.4 × 10⁻⁴ M), MgCl₂ (1.2 × 10⁻⁴ M), and Veronal buffer (0.12 to 0.26 M), pH 9.7 at 37°. The mixtures were incubated for periods ranging from 0.5 to 3.0 hours, depending on the level of activity. Aliquots, 1.0 ml., of the mixtures were deproteinized with 0.5 ml. of a 5 per cent solution of trichloroacetic acid, and 2.0- or 4.0-ml. aliquots of the protein-free filtrates were assayed for inorganic phosphate (7).

Results

Precipitation of Human Intestinal Phosphatase by Its Antiserum—In these experiments different amounts of the enzyme were added to antiserum (0.20 ml.) and the mixtures were made up to 2.10 ml. with 0.15 M saline solution. The amounts of enzyme chosen cover the range of activity that would be found in 1.40 ml. of sera of 20 to 100 King-Armstrong units (cf. experiments on human serum). The mixtures were incubated for 5 minutes at 37°, at 25° (room temperature) for 1 hour, and then with occasional mixing at 4° for 148 hours. They were centrifuged at 18,000 × g for 0.5 hour at 4° to remove precipitates. Portions of the clear supernatant fluids were assayed for phosphatase activity at pH 9.7 as described.

In several experiments, 1.0-ml. portions were withdrawn after standing 120 hours, mixed with equal volumes of horse antirabbit γ-globulin antibody solution and set at 37° for 5 minutes, at 25° for 1 hour, and then for 23 hours in the cold before centrifugation and analysis as described above. The horse antibodies, present in amounts capable of precipitating all the rabbit γ-globulin, serve to bring down enzyme-antienzyme complexes not otherwise precipitated under the conditions used.

Normal rabbit serum causes no precipitation or loss of enzyme activity under any of the conditions described above.

The results of these experiments are summarized in Table I, where it may be seen that the precipitation of low concentrations of intestinal phosphatase(s) is incomplete but rises with increasing enzyme concentration to a maximal figure of approximately 90 per cent. Other experiments have shown that the use of larger amounts of antiserum does not alter this maximal value. In the presence of the horse antibodies, this maximal value is attained with all the concentrations of enzyme tested.

Precipitation of Human Bone Phosphatase by Its Antiserum—The authors are indebted to Drs. Avery Sandberg and William Staubitz of Roswell Park Memorial Institute for making available this clinical material and to Miss Jean Schubarg for preparing the sera.

1 These analyses were performed by the Clinical Biochemical Laboratory of Roswell Park Memorial Institute.

2 This value for maximal precipitation was obtained with aged (approximately 3 years old) samples of the antiserum. Fresh (less than 2 months old) samples yielded the same value.
Studies analogous to the ones described for the intestinal enzyme were carried out with the phosphatase(s) from bone and its antisera. The levels of activity contained in the amounts of enzyme used roughly correspond to those used in the intestinal phosphatase experiment. Because of its lower antibody titer, 0.50 ml. of the antible phosphatase serum was used. All other conditions were as described for the experiment with the intestinal enzyme.

As with the intestinal enzyme, precipitation of bone phosphatase by its antisera increased with increasing amounts of the antigen (Table I) to a maximal figure of about 85 per cent. This value was not increased by the use of greater amounts of antisera. Upon addition of horse antibodies it will be noted that precipitation of the enzyme becomes almost complete (91 to 96 per cent) for all concentrations of bone phosphatase used.

Precipitation of Mixtures of Intestinal and Bone Phosphatases by Their Antisera—In these experiments, intestinal and bone phosphatase in amounts that were approximately equal in their enzyme activity and at concentrations which provided for maximal precipitation by the homologous antisera, were tested with anti-intestinal phosphatase and antibone phosphatase serum. Mixtures of the enzymes were similarly tested. The amounts of antisera used were sufficient to precipitate all of the enzyme present, if it was at all precipitable. In the case of the systems containing anti-intestinal phosphatase serum, the precipitation time was 4 hours; for the systems containing antibone phosphatase serum, it was 48 hours. Centrifugation of all mixtures after precipitation was carried out at about 1,800 x g. Analysis of supernatant fluids for residual activity was carried out as usual.

From Table II, it is seen that at the concentrations used, the intestinal enzyme(s) was precipitated by its antisera (86 per cent in 4 hours) but scarcely by the antibone phosphatase serum (4 per cent in 48 hours). Conversely, an equivalent amount of activity in the form of bone phosphatase(s) was not precipitated (0.7 per cent in 4 hours) by anti-intestinal phosphatase serum, but was maximally precipitated (87 per cent in 48 hours) by the antibone phosphatase serum. Further, from mixtures of the two enzymes, the anti-intestinal phosphatase serum selectively precipitated the intestinal phosphatase, and the antibone phosphatase serum selectively precipitated the enzyme from bone. These results show that the enzymes and their respective antisera are immunochemically different. The experiments do not delimit the extent of these differences (cf. experiment on cross-reactions, and Table III).

Cross-reaction of Anti-intestinal Phosphatase Serum and Anti-bone Phosphatase Serum with Alkaline Phosphatases from Intestine, Bone, Kidney and Liver—It has already been observed (Table I) that maximal precipitation of low concentrations of the intestinal and bone enzymes can be achieved by the addition of horse antirabbit γ-globulin antibodies to the systems. In the use of the antibone and anti-intestine rabbit sera for studies on the tissues of origin of the phosphatases in human serum (cf. next section), concentrations of serum phosphatase (5 to 50 King-Armstrong units) which are not maximally precipitated except in the presence of horse antibodies were often involved. It thus became necessary to investigate the possibilities for cross-reactions in these systems containing horse antibodies.

Toward this end, precipitations were carried out with the anti-intestinal and antibone phosphatase sera and amounts of enzyme corresponding roughly to the phosphatase activity contained in 1.40 ml. of a human serum of about 50 King-Armstrong units.

The enzymes tested were the alkaline phosphatase preparations from intestine, bone, kidney, and liver. The precipitation reaction mixtures, made up to 2.10 ml. with saline solution were incubated for 5 minutes at 37°, 1 hour at 25°, (room temperature), and 119 hours at 4°. Then, 1.0-ml. portions were withdrawn, mixed with equal volumes of the horse antibody solution, and then incubated at 37° and 25° as described above, followed by 23 hours in the cold. At the end of this time (144 hours total),
all of the mixtures, those treated with horse antibodies and those not so treated, were centrifuged at about 18,000 \( \times g \) for 0.5 hour. Aliquots of the clear supernatant fluids were then analyzed in the customary manner for phosphatase activity.

From the results of these experiments, Table III, it is seen that the anti-intestinal phosphatase serum causes no more than a small amount of precipitation of any of the phosphatases from the other tissues, in the presence or absence of horse antibodies. On the other hand, the antibone phosphatase serum precipitates the enzymes from other tissues to varying degrees (4 to 74 per cent) in the absence of horse antibody, and precipitates them extensively (61 to 92 per cent) in its presence.

The use of horse antibodies has revealed cross-reactions of human intestinal phosphatase(s) with antibone phosphatase serum that were not evident from the precipitation studies carried out in their absence (cf. Table II).

Because of lack of antikidney and antiliver phosphatase sera, it is not known whether these would cross-react with the enzyme(s) from bone.

Precipitation of Human Serum Phosphatase by Anti-intestinal Phosphatase Serum and by Antibone Phosphatase Serum—For the precipitation of human serum phosphatases by the two antisera, 1.40 ml. of serum were mixed with anti-intestinal phosphatase serum (0.20 ml.) or with antibone phosphatase serum (0.50 ml.) or with both. Normal rabbit serum was added to bring the volume of these mixtures to 2.10 ml. The conditions for their incubation, subsequent treatment with horse antibodies and analysis for phosphatase activity are exactly as described in the previous experiment.

From Table IV, it may be seen that the anti-intestinal phosphatase serum precipitates 13 to 29 per cent (presumably phosphatase(s) of intestinal origin) of the total serum enzyme from normal individuals, and 0.5 to 11 per cent from sera of the cancer patients. In three of the five sera from cancer patients, the actual number of units of enzyme precipitated by the anti-intestinal phosphatase serum was within the normal range. The percentage of total serum enzyme precipitated by the antibone phosphatase serum was 60 to 79 per cent for the normals and 80 to 94 per cent for the cancer patients. A mixture of anti-intestinal phosphatase serum and antibone phosphatase serum brought down no more enzyme than did the antibone phosphatase serum alone (cf. Table IV, columns 5–8). This finding is consistent with the results of the previous experiment (Table III) where it was shown that, in the presence of horse antibody, intestinal phosphatase(s) is precipitated by antibone phosphatase serum.

A tentative approximation of the contribution to the serum phosphatase by the enzyme(s) of osseous origin can be made by subtracting the amount precipitated by anti-intestinal phosphatase serum from the amount precipitated by the mixture of both antisera. This contribution (Table IV, columns 9–10) in the case of the normals is approximately 40 to 59 per cent of the serum phosphatase. For the cancer patients, the contribution of bone phosphatase is considerably greater. It ranges from 75 to 86 per cent, being greatest in the cases with skeletal metastases. In the absence of any information regarding the presence in serum of phosphatases from liver or kidney which were shown to be capable of cross-reacting with antibone phosphatase serum, this enzyme must be referred to as "bone" phosphatase with reservations.

The ratio of "bone" to intestinal phosphatase is 1.4 to 4.6 for the normals and from 6.9 to 100 for the cancer patients (Table IV, column 11).

In the last two columns of Table IV is recorded the fact that approximately 28 to 39 per cent (1.8 to 2.6 units) of serum phosphatase from the normal individuals was not precipitated by anti-intestinal plus antibone phosphatase sera, whereas for the cancer patients, 6 to 22 per cent (3.9 to 13.6 units) could not be precipitated. The nature and origin of this phosphatase(s) remains to be established.

### Table IV

The precipitation of human serum phosphatase by anti-intestinal phosphatase and antibone phosphatase sera in the presence of horse antibody

<table>
<thead>
<tr>
<th>Clinical diagnosis</th>
<th>Serum phosphatase</th>
<th>Phosphatase precipitated by</th>
<th>Units$</th>
<th>Ratio of &quot;bone&quot;:intestinal phosphatase</th>
<th>Phosphatase not precipitated by AntiHIP + AntiHBP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AntiHIP$ ^*</td>
<td>AntiHBP</td>
<td>AntiHIP + AntiHBP</td>
<td>AntiHIP + AntiHBP</td>
<td>AntiHIP + AntiHBP</td>
</tr>
<tr>
<td></td>
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<td>$%$</td>
<td>$%$</td>
<td>$%$</td>
<td>$%$</td>
</tr>
<tr>
<td>Normal patient...</td>
<td>8.4</td>
<td>29</td>
<td>2.4</td>
<td>67</td>
<td>5.6</td>
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<tr>
<td>Normal patient...</td>
<td>4.7</td>
<td>13</td>
<td>0.6</td>
<td>67</td>
<td>2.8</td>
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<tr>
<td>Normal patient...</td>
<td>8.4</td>
<td>13</td>
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<td>6.0</td>
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<td>Cancer patients</td>
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<td></td>
<td></td>
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<td>50</td>
<td>11</td>
<td>5.5</td>
<td>87</td>
<td>44</td>
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<td>liver metast......</td>
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<td>Breast; liver and</td>
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<td>8</td>
<td>9.4</td>
<td>92</td>
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<tr>
<td>Cystic duct; liver</td>
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<td>1.9</td>
<td>80</td>
<td>50</td>
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<tr>
<td>metast...</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Prostate; skeletal</td>
<td>48</td>
<td>3</td>
<td>1.4</td>
<td>93</td>
<td>45</td>
</tr>
<tr>
<td>metast...</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Prostate; skeletal</td>
<td>122</td>
<td>0.5</td>
<td>0.6</td>
<td>94</td>
<td>115</td>
</tr>
</tbody>
</table>

* See footnote, Table I.

† "Bone" phosphatase values were obtained by subtracting the values in column 3-4 from those in column 7-8. See text for explanation.

‡ King-Armstrong units.
The present work is a continuation of studies on the differentiation of enzymes of similar activity from different organs of a species by immunological means. It was previously shown that specific antibodies formed against alkaline phosphatase from dog intestine could be used to differentiate this tissue alkaline phosphatase(s) from those of the liver and kidney of dog (2, 3). In an analogous manner, Henion and Sutherland (8) were able to differentiate the phosphohydrolases from dog heart and liver from each other and from those of other tissues of the dog. In the present investigation, antisera against the phosphatases from human sarcomatous bone and intestine were shown capable of differentiating between these enzymes (Table II), precipitation of the one being affected under conditions in which the other remained unprecipitated, and vice versa. However, the intestinal enzyme(s) is largely precipitated by antibone serum in the presence of horse antirabbit \( \gamma \)-globulin antibodies (Table III). A reciprocal cross-reaction between the phosphatase(s) from bone with anti-intestinal phosphatase serum is not observed. These cross-reactions of the antiserum from bone with the enzymes from intestine, liver and kidney suggest that there are region(s) of antigenic or haptenic similarity in the phosphatase preparations used. The usefulness of the horse antibody system for detecting nonsedimentable antigen-antibody complexes is illustrated by these experiments (cf. also (6)).

The possibility also exists that the blood in the tumor mass of the osteogenic sarcoma contained enzymes from other tissues (intestine, liver, kidney) in amounts sufficient to elicit an antibody response in rabbits. The serum cross-reaction would then be due to the fact that the "antibone" serum contains antibodies not only to bone phosphatase but to these others as well. Absorption of the antiserum with specific enzymes could be used to test this possibility. It is also possible that most of the phosphatase isolated from liver and kidney is of bone origin.

The origin of the alkaline phosphatase in serum has been investigated by a variety of techniques (9–19), e.g. surgical removal of organs, diversion of lymph supply, alterations in diet, measurement of enzyme-substrate dissociation constants, studies on response of serum enzyme to inhibitors or activators compared with response of enzymes isolated from organs, and so on. The variety of animal species and experimental techniques used renders difficult a comparison of results of these studies with each other. For this same reason, it is not possible to compare satisfactorily the results of the present investigation with these others.

The results of the present investigation suggest that in the fasted normal human subject and in the pathological cases reported, the intestine does not contribute a very large share of the serum phosphatase. On the other hand, serum phosphatases (corrected for intestinal phosphatase) precipitable by antibone serum account for 40 to 59 per cent in normal individuals and as much as 94 per cent in individuals with elevated levels of serum phosphatases and skeletal metastases. In the light of the cross-reactions which have already been discussed, between the antibone serum and the phosphatases from liver and kidney, this phosphatase(s) cannot yet be unequivocally attributed to bone. However, the fact that the percentage of serum phosphatase precipitated by antibone serum rises markedly in the case of serum for individuals with extensive skeletal metastases strongly suggests its osseous origin.

It will be desirable to extend these studies to cover a larger number of cases, both normal and abnormal, and to investigate antisera to other tissues which may be conceived of as sources of serum phosphatase.

**SUMMARY**

The use of the tissue antisera to establish the tissues of origin of the serum alkaline phosphatases has been explored.  
1. Precipitating antisera for the alkaline phosphatases from human intestine and osteogenic sarcomatous bone have been prepared in rabbits by intravenous administration of the partially purified antigens in alum suspensions.  
2. The enzymes from intestine and bone may be differentiated by the selective precipitating action of their homologous antisera under suitable conditions.  
3. By the use of horse antirabbit \( \gamma \)-globulin antibodies to precipitate nonsedimentable complexes of enzyme with rabbit antibody, cross-reactions between the enzymes from intestine, kidney, and liver, with antibone serum have been demonstrated.  
4. For the normal fasted individual, and for individuals with a variety of tumors who show high serum phosphatase activity, the major portion of the serum enzyme appears to be of non-intestinal origin. The possibility that it is of osseous origin is discussed.

**Acknowledgment**—The continuing interest of Dr. David Pressman in these investigations is acknowledged.

**REFERENCES**

Tissue Sources of Human Serum Alkaline Phosphatase, as Determined by Immunochemical Procedures
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