Purification and Partial Characterization of the Alkaline Phosphatase of Swine Kidney*

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In 1957, we reported (1) the purification of alkaline phosphatase of swine kidney to a specific activity of about 150,000 units on a basis of total nitrogen content; the units were those as defined by Roche and Bouchilloux (2). At that time, this was the highest activity reported for any alkaline phosphatase, but one or two other workers have since appeared to achieve a similar order of activity. However, certain of the claims of higher activity must be questioned. Mathies (3) reported a specific activity of 10,500 units according to the assay of King et al. (4), and Schales and Arai (5) reported an activity of 11,600 King units; in both cases, the nitrogen content was measured by indirect methods, and the specific activities are to be considered as reflecting only the order of magnitude of activity. Schramm and Armbruster (6) have proposed a factor of about 7 to convert King units to Roche units; we have confirmed this factor, and, since Mathies performed his assays at 25°, have determined the coefficient for conversion of results at 25° to those at 37° to be about 1.4. Thus, the preparation of Schales and Arai would have a specific activity of 10,500 x 7 x 1.4, or 102,900 Roche units, and the preparation of Mathies would have a specific activity of 11,600 x 7, or 81,200 Roche units. Preparations somewhat more comparable to ours have been reported by Portmann (7) and by Alvarez and Lora Tamayo (8); in their procedures, however, the yields were quite low.

Since 1957, we have investigated a number of methods for further purification and have settled upon gradient elution from Ecteola cellulose (9) as the most useful method; with this method and with a variety of less suitable methods, it has been possible to achieve a purification to about 300,000 units per mg of total nitrogen. The particular value of the methods as developed is the excellent yield. Another problem, common to all studies of alkaline phosphatase, that of testing of homogeneity, has not been entirely overcome. Practically all workers with preparations of 50,000 units or greater have claimed homogeneity and have presented electrophoretic and other data to document their claims. As we have pointed out (1), electrophoretic methods are incapable of separating materials as unrelated as the peptidases and the alkaline phosphatases of swine kidney; claims of homogeneity on the basis of electrophoresis would seem to be worthless. The material prepared by us is homogeneous in electrophoresis, in paper chromatography, by rechromatography on substituted cellulose and by countercurrent distribution, but, perhaps more to the point, we have not been able to exceed 300,000 units of activity by a great variety of techniques of chromatography or electrophoresis. It is to be admitted that the problem remains; the amounts of material necessary for conclusive studies are not available. The total purification in our procedure is in excess of 30,000-fold and one must process many kilograms of tissue with excellent yield to obtain a few milligrams of purified material.

The characterization of an alkaline phosphatase has not been previously achieved; several investigators (3, 10) have published absorption spectra results and have concluded that the material was a simple protein. Others have indicated that carbohydrate is present (5, 7), and it has been claimed that nucleotides are present (11). Few if any of these reports have been convincing, but our work would appear to confirm these claims in part. As others had observed with less purified materials, the absorbancy in the ultraviolet range would appear to be that of a simple protein; there is no indication of any other component. Such an absorbancy with our materials was somewhat surprising because, first, the material is completely resistant to all proteolytic enzymes that were tried and, second, the color yield in the procedure of Lowry et al. (12) was well below that of any protein tested even though the absorbancy per milligram of nitrogen was greater than for any protein tested. In order to explain the high absorbancy and relatively low color yield with the phosphomolybdate reagents, it was postulated that the tyrosine groupings presumed to be present were covered by some groupings that might be removed by denaturation or hydrolysis. Accordingly, the material was treated with dilute acid with the finding that not only did the reaction with phosphomolybdate reagent (13) increase markedly but that there was a marked increase in the absorbancy of the material in the ultraviolet range; no protein was found to duplicate this behavior.

Inasmuch as it appeared that a chromophore in the ultraviolet range reacted with phosphomolybdate after hydrolysis with dilute acid, it was suspected that the phenolic groupings of tyrosine were covered with some grouping that inhibited the absorbancy in the ultraviolet range. After hydrolysis with strong acid, however, no tyrosine could be demonstrated and, indeed, as the hydrolysis proceeded, the absorbancy shifted to shorter wave lengths, indicating the relative absence of aromatic amino acids. Other reactions of the material were investigated with the finding that, after but not before hydrolysis, the material reacted with ferricyanide and ferric chloride or with ferricyanide alone to give an immediate deep purple reaction. In a study of model compounds no phenol or catechol was found to give a similar reaction, although some photographic developers were found to give a pink

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or red reaction. With these reactions as tracers, the hydrolysates were subjected to chromatography. It was soon evident that the chromophoric grouping was associated with carbohydrate; the reaction with aniline phthalate indicated a mixture of carbohydrates and the reaction of Dische (14) with cysteine and sulfuric acid indicated that a mixture of glucose and pentose was associated with the chromophore. In the hydrolysis mixtures, a second compound was found that absorbed maximally near 270 mp but did not react with potassium ferricyanide; more of this compound was formed with prolonged hydrolysis or with hydrolysis with more concentrated acid. At the same time, several fractions that did not absorb in the ultraviolet range but gave reactions characteristic of pentose or glucose were formed. The pentose was associated with a ninhydrin-reactive material and appeared to survive prolonged hydrolysis, but the glucose disappeared more or less coincident with the formation of a chromogenic group with an absorbancy near 330 mp. The hexose was identified as glucose by the reaction with cysteine and sulfuric acid and, in hydrolysates, by the reaction with glucose oxidase. Although several ninhydrin-reactive materials were found in the hydrolysates, none of these materials could be identified with the known amino acids; red, pink, or yellow colors resulted from the reactions with ninhydrin.

In further attempts to identify the chromophoric grouping, a variety of compounds were investigated with the finding that many tri- and tetra-substituted pyrimidines would react with the phosphomolybdate reagent either as originally used or as in the method of Lowry et al. (12) for the determination of protein, with the International model PR-2 centrifuge and discarded. Cold ethanol, 1000 ml, was added to the supernatant solution (per liter of digest) and the precipitate was removed by centrifugation in the International model PR-2 centrifuge and discarded. Cold ethanol, 1000 ml, was added to the supernatant solution (per liter of digest), and the precipitate was removed by centrifugation in the International model PR-2 centrifuge at 0° and was dissolved in about 500 ml of the pH 8 Tris buffer for each 1000 ml of the digest. This solution, "second ethanol," was treated exhaustively by the procedure of Sevag et al. (15) (shaking with a mixture of chloroform and octanol at room temperature as vigorously as possible for 30 minutes followed by centrifugation); the procedure was continued until no more emulsion was formed. For each 1000 ml of the solution of enzyme at about 4-5°, sufficient barium acetate was added to make the solution 0.1 m in respect to barium acetate, and ammonium hydroxide was added dropwise until the pH reached 9.6 (pH meter at 4-5°); the mixture was centrifuged in the International model PR-2 centrifuge at 0° to remove the precipitate which was discarded. For each 1000 ml of solution, 300 ml of ethanol at 0° were added rapidly, and after 30 minutes, the precipitate was collected and discarded. An additional 1200 ml of ethanol at 0° was added, and after 30 minutes, the precipitate was collected and dissolved in about 300 ml of water with the correction of the pH to about 8 by the addition of dilute acetic acid. This "third ethanol" fraction was dialyzed with stirring at room temperature against several changes of about 10 liters of 0.01 m barium acetate, pH 9.0, over a period of 24 hours. The dialyzed material was then treated batchwise with Dowex 2-X8 in the acetate form; about 690 ml of dialyzed material were added 100 g of wet resin at room temperature. After thorough mixing, the mixture was filtered on a sintered glass filter with suction, the solution was assayed for activity, and successive additions of 100 g batches of Dowex 2-X8 were made until the resin began to absorb the activity. The material, "Dowex 2-treated," was then fractionated with barium acetate and ethanol; the solution of enzyme was cooled to 0° and mixed with 0.1 volume of 1 m barium acetate, pH 9, 0.3 volume of ethanol at 0° was added rapidly, and after 30 minutes, the precipitate was collected and dissolved in 100 ml of water. An additional 0.3 volume of ethanol at 0° was added rapidly, and after 30 minutes, the precipitate was collected; this procedure was repeated with addition of 0.3 volume of ethanol until a total of 1.5 volumes of ethanol had been added. The activity was usually found in the 0.6 to 0.9 fraction, although in a few cases the bulk of activity was found in the 0.9 to 1.2 fraction. Nevertheless, the procedure is quite reproducible and, as noted in Table 1, the recovery is excellent. It is to be remembered that the average crude digest was found to contain about 100 units per ml and to have a specific activity of about 10 on the basis of total N (1).

**Assay of Activity**—The assay was as previously described (1) and was according to the procedure outlined by Morton (16).

### Table I

**Purification of alkaline phosphatase**

See the text for the description of the procedures.

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume</th>
<th>Total units</th>
<th>Total N</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude digest</td>
<td>30,000</td>
<td>3,300,000</td>
<td>240.00</td>
<td>14</td>
</tr>
<tr>
<td>First ethanol</td>
<td>4,000</td>
<td>3,500,000</td>
<td>56,000</td>
<td>166</td>
</tr>
<tr>
<td>Second ethanol</td>
<td>1,200</td>
<td>3,100,000</td>
<td>1,920</td>
<td>1,030</td>
</tr>
<tr>
<td>Third ethanol</td>
<td>300</td>
<td>2,800,000</td>
<td>200</td>
<td>14,000</td>
</tr>
<tr>
<td>Dowex 2*</td>
<td>650</td>
<td>1,900,000</td>
<td>26.3</td>
<td>72,000</td>
</tr>
<tr>
<td>Barium fraction, 0.6 to 0.9*</td>
<td>100</td>
<td>1,370,000</td>
<td>10.9</td>
<td>125,000</td>
</tr>
<tr>
<td>Peak 1*</td>
<td>1,300</td>
<td>900,000</td>
<td>3.4</td>
<td>265,000</td>
</tr>
<tr>
<td>Barium fraction, 1.0 to 1.5*</td>
<td>50</td>
<td>860,000</td>
<td>2.8</td>
<td>308,000</td>
</tr>
</tbody>
</table>

* Data in these steps are from a different preparation; at this stage the preparation described had a total activity of 2,200,000 units.
The units were those described by Roche (2). The unit was defined as the amount of enzyme which liberated 1 µg of phosphorus (as inorganic phosphate) per minute at 38°C when the concentration of β-glycerophosphate was 0.02 M, the concentration of magnesium ion was 0.005 M, and the pH was 9.5 (ethanolamine-HCl buffer, 0.02 M). Specific activity was defined as units per milligram of total N. In the expression of the specific activity, some concern over the method of determination of the N content should be expressed; as will become evident, the method of Lowry et al. (12) is not valid with highly purified alkaline phosphatase, and the only N value that can be justified is that from the determination of total N by micro-Kjeldahl procedures. Equally invalid are results from precipitation procedures such as with trichloroacetic acid or sulfosalicylic acid; purified alkaline phosphatase in the concentrations available does not precipitate with these reagents.

**Activators and Inhibitors**—The purified material required magnesium ion for optimal activity, but, in the presence of the optimal concentration of magnesium ion (0.005 M), activation by amino acids or other divalent metal ions could not be demonstrated. In fact, many other divalent metal ions, otherwise with little effect, inhibited in the presence of the optimal concentration of magnesium ion. These results will be discussed in full in subsequent publications.

**Other Activities**—Our digests and many of the crude fractions are known to be excellent sources of certain peptidases (1) and of diesterase activity. Peptidases have been followed by the methods described with leucinamide and cysteinylglycine as substrates (1), and diesterase was assayed by the method of Heppe1 and Hilmo (17). Purified materials with a specific activity of over 100,000 were found to be inactive in the hydrolysis of peptides and as a diesterase when tested undiluted, whereas the assay for phosphatase required a dilution of 1:10,000 or 1:100,000 to permit assay. Inorganic pyrophosphatases and ATPases were absent after the very first few steps of the procedure. 5' Adenylic acid, p-nitrophenylphosphate, glucose 6-phosphate, and phenylphosphate were found to be hydrolyzed by the preparations.

**RESULTS**

**Purification on Ecteola Cellulose**—A column of Ecteola cellulose (9), 5 x 100 cm, was washed thoroughly with 1 M NaOH and then with water until the excess alkali had been removed. Then the solution of alkaline phosphatase containing from 1,000,000 to 2,000,000 units in about 500 ml was placed on the column and was followed by several hold-back volumes of water. A discontinuous two-chamber gradient was applied by gravity and at room temperature as follows: the bottom chamber contained 2 liters of water, and to the top chamber were added 4 liters of 0.02 M barium acetate. The rate of flow was about 1 ml per minute. Absorbancy in the ultraviolet range at 260 and 280 nm and the phosphatase activity were followed; the results are summarized in Table I and Fig. 1. The material in the active peak was concentrated by fractionation with barium acetate and ethanol as described above; fractions representing 0.0 to 0.5, 0.5 to 1.0, and 1.0 to 1.5 volumes of ethanol were collected and dissolved in 50 ml of water. The fraction "1.0 to 1.5" contained the bulk of the activity, and, on the basis of total nitrogen, had a specific activity of 398,000. Similar preparations have varied from 255,000 to 315,000 units, identical values, considering the error of assay and determination of nitrogen.

**Further Studies of Homogeneity**—A preparation obtained in the manner described above was rechromatographed on a small column of Ecteola, 2 x 25 cm. After application of the 50 ml of concentrated enzyme solution, the column was washed with about 4 hold-back volumes of water, and then gradient elution was undertaken with 250-ml lower and upper chambers at room temperature. Barium acetate, 250 ml, 0.01 M, pH 9, was placed in the upper chamber with 250 ml of water in the lower chamber, and, when this was exhausted, 250 ml of 0.05 M barium acetate, pH 9, were placed in the upper chamber; the upper chamber was replenished with the 0.05 M solution as needed. As may be seen in Fig. 2, there was only one peak of ultraviolet absorbing material, and the activity coincided exactly with the absorbancy in the ultraviolet range. It was found that the material would not
stain properly with bromthymol blue or other protein stains on paper chromatography or paper electrophoresis but would react immediately with ninhydrin; thus, the ninhydrin test was used to detect alkaline phosphatase. In a system of 1 X ammonium acetate-ethanol (30:70), with descending chromatography, the active material was found to have an Rf of about 0.6; no other component could be detected with ninhydrin, by absorbancy in the ultraviolet range, or by phosphatase assay (sprayed with 1% p-nitrophenylphosphate, pH 9, kept in air at 37° for 15 minutes, dried at 100°, and sprayed with dilute alkali).

Countercurrent Distribution—A solution of alkaline phosphatase with a specific activity of about 250,000 and a total of 150,000 units was precipitated with barium acetate and ethanol and was dissolved in 10 ml of the lower phase of the system devised by Warner and Vainberg (18) for the distribution of nucleic acids. The material originally had a specific activity of 300,000 but had lost some activity upon standing. This solution was centrifuged to remove barium phosphate and placed in a 100-cell distribution apparatus maintained at 4°; the results of the distribution are shown in Fig. 3. There were two major complications to the study; first, the material could not be assayed in the conventional manner because phosphate was present and, second, in the presence of the phosphate buffer and formamide, the alkaline phosphatase gradually lost activity. The assay was with p-nitrophenylphosphate as the substrate, and the units are those of Bossuyt, Lowry, and Brook (19); we have not attempted a conversion of these units to the Roche units used in the rest of the study. Even with the limitations, it is apparent that the material absorbing at 280 m¿ was distributed in exactly the same manner as the alkaline phosphatase; thus, we may be assured that the material we are studying represents the alkaline phosphatase.

These studies are being continued to develop a more suitable system to permit recovery of the fully active material.

Preliminary Observations with Alkaline Phosphatase—Throughout the purification, the absorbancy of the material in the ultraviolet range was followed. For the average solution of final material as described above, the absorbancy per ml at 280 m¿ was about 0.5; such solutions contained about 0.05 mg of N per ml and 15,000 units of alkaline phosphatase. Thus, the absorbancy per 1 mg of total N would be about 10; for crystalline ovalbumin (used in this laboratory as the standard for protein determinations), we find the absorbancy for 1 mg of total N to be 4.5. The absorbancy value for chymotrypsin was somewhat higher, and that for ribonuclease was less, but there is little in this absorbancy to indicate the presence of materials other than protein and indeed, the absorbancy versus wave length (Fig. 4) would appear to be typical for proteins (a maximum at 278 to 280 m¿ with a minimum at 250 m¿ and a shift of maximum in alkaline solution to about 285 to 287 m¿); under similar conditions, trypsin gave almost identical shifts of absorbancy. There were, however, other observations that were not entirely consistent with a content of protein alone; first, the material was completely resistant to proteolysis by chymotrypsin, trypsin, carboxypeptidase, aminopeptidase, ficin, and papain; second, the content of protein as determined by the method of Lowry et al. (12) accounted for less than half the total N. It was found, however, that proteins differed considerably in the reaction with the Lowry reagents; it was found, for example, that the yield of color from ribonuclease per milligram was about twice that from ovalbumin, reflecting the relatively high content of tyrosine in ribonuclease.

The alkaline phosphatase had a higher than usual absorbancy on a nitrogen basis but gave a lower than usual yield of color in the Lowry method, possibly indicating a low content of tyrosine and a high content of tryptophan; yet the shift upon the addition of alkali was characteristic of a protein with a fairly high content of tyrosine. The material was treated with dilute acid and the absorbancy in the ultraviolet range increased markedly (Fig. 4); the absorbancy in dilute acid was identical with that in neutral solution. However, as may be seen in Table II, the reaction with the reagents of Lowry (Column 1) did not increase as might be expected. When the copper reagent was removed (thus converting the method essentially back to the method of Polin and Ciocalteu for phenols), a marked increase was observed. Ribonuclease, as a control, was not influenced by heat in its response in the presence or absence of the copper reagent. Next, the hydrolysis was investigated under conditions of treatment with 6 N HCl; as may be seen in Fig. 5, there was a marked change in

Fig. 3. Countercurrent distribution of purified alkaline phosphatase. Shown is a 106-tube transfer with a 10-ml upper and a 10-ml lower phase.

Fig. 4. Absorbancy of purified alkaline phosphatase. ▼—▼, original in 0.3 N HCl; ○—○, original in 0.1 N NaOH; and ●●, original after heating for 2 hours at 100° in 0.3 N HCl.
absorbancy in the course of a hydrolysis of 12 hours. A new peak appeared at 330 mμ, and absorbancy increased throughout the ultraviolet range. This behavior was characteristic of nothing we have encountered and the problem became one of identification of the unknown material or materials.

Identification of Components of Alkaline Phosphatase—A hydrolysate prepared by reflux with 6 N HCl for 24 hours was concentrated in a vacuum, dissolved in water, and taken to dryness several times to remove the HCl, and, finally, was dissolved in about 0.1 volume of water (about 0.5 mg of N per ml). This concentrate was used for spot tests on filter paper preparatory to paper chromatography. Among the tests performed for the detection of phenols and catechols were the tests with potassium ferricyanide plus ferric chloride, ferric chloride alone, and potassium ferricyanide alone. The hydrolysate responded weakly to the test with ferric chloride but responded immediately to both the other tests to give an intense blue color. Phenol, tyrosine, and dihydroxyphenylalanine were among the many control compounds tested; none of these compounds responded to the test with potassium ferricyanide alone. Phenolic compounds found to react were the photographic developers, p-methylaminophenol and 2,4-diaminophenol; however, these compounds gave red colors and reacted strongly with ferric chloride (in contrast to the hydrolysate). Thus, it would appear that the unknown compound is not a simple phenol and certainly is not tyrosine.

Separation of Components of Hydrolysates—The hydrolysates were subjected to chromatography with a variety of solvent systems, and the various components were detected by observation with ultraviolet light, by spraying with 1% aqueous solution of potassium ferricyanide, by spraying with the ferricyanide plus 1% ferric chloride, by spraying with a solution of ninhydrin (0.1% in acetone or butanol) followed by a short period of heating, and by spraying with a solution of aniline phthalate (20). It had been determined previously that the hydrolysates were positive with all these reactions. It should be noted, however, that the hydrolysates did not respond to tests for orthophosphate or for phosphate esters. Various solvent systems were found useful for different purposes but propanol-water (80:20) was found to resolve the hydrolysates as effectively as more complicated systems. In Table III, the results with two hydrolysates, 1 N, 2 hours, 100°, and 6 N, 24-hour reflux, are given; the final solutions after removal of HCl and neutralization contained about 1 mg of N per ml. Other strips run at the same time and superimposable on the basis of the materials detectable by ultraviolet light were eluted with 1 N HCl, and the absorbancy in the ultraviolet range and the general reaction of Dische with sulfuric acid and cysteine (14) were determined for each component. There was considerable end absorption in the eluates and the maxima as recorded are only approximate. Two components with significant absorbancy in the ultraviolet range were detected in both hydrolysates (Components 6 and 10 of Table III); only one of these, Component 6, was rich in carbohydrate. Component 0 was found to be responsible for the reaction with ferricyanide alone and with ferricyanide plus ferric chloride. Component 0 of the 2-hour hydrolysate appeared to contain more pentose than Component 6 of the 24-hour hydrolysate; inasmuch as the absorbancy was less, it may be that longer hydrolysis had made the pentose available for the test. When this possibility was tested, cysteine, which responds very poorly to the test with cysteine and sulfuric acid, was found to have a markedly increased value after prolonged hydrolysis with acid. Component 6 was found to have a considerable increase in absorbancy when treated with alkali; this component from the 2-hour hydrolysate was found to have an increase in absorbancy from 0.345 at 275 mμ to an absorbancy of 2.16 at 265 mμ upon the addition of alkali to about 0.1 N. Thus, the material reacting with ferricyanide contains carbohydrate and has a marked shift of absorbancy in alkaline solution.

Identification of Carbohydrates—The general reaction with cysteine and sulfuric acid of the carbohydrate containing components indicated that pentoses and hexoses were present (A_{max} much greater than A_{min} but, with time, becoming essentially equal). In various systems, we have observed pink reactions with aniline phthalate (indicating pentoses) but no further identification has been made. The reaction with orcinol is not satisfactory in the presence of glucose and, especially, the orcinol reaction after bromination has proven meaningless in the presence of glucose.
TABLE III

Paper chromatography of acid hydrolysates

Descending chromatography with propanol-water (80:20) was used; the solvent flow was 38 cm. Aniline phthalate was used to detect the sugar components. By the techniques used, five components were found in the 1 N HCl, 2-hour hydrolysate, and eight components were found in the 6 N HCl, 24-hour hydrolysate.

<table>
<thead>
<tr>
<th>Component</th>
<th>Cm.</th>
<th>2 hour, 1 N HCl</th>
<th>24 hours, 6 N HCl</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Ninhydrin</td>
<td>KFe(CN)</td>
</tr>
<tr>
<td>1.</td>
<td>5.0</td>
<td>++, p*</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>7.8</td>
<td>++, p</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>9.0</td>
<td>++, y</td>
<td>++, b</td>
</tr>
<tr>
<td>4.</td>
<td>10.6</td>
<td>+, y</td>
<td>+, p</td>
</tr>
<tr>
<td>5.</td>
<td>13.0</td>
<td>+, p</td>
<td>+, p</td>
</tr>
<tr>
<td>6.</td>
<td>18.0</td>
<td>+, p</td>
<td>+, p</td>
</tr>
<tr>
<td>7.</td>
<td>18.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.</td>
<td>20.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.</td>
<td>31.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.</td>
<td>35.0</td>
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2 hours, 1 N HCl hydrolysate

<table>
<thead>
<tr>
<th>Component</th>
<th>cm max</th>
<th>A max</th>
<th>A 260</th>
<th>A 410</th>
<th>Dische test</th>
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<tr>
<td>6.</td>
<td>275</td>
<td>0.345</td>
<td>0.077</td>
<td>0.192</td>
<td>0.217, 0.107</td>
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<tr>
<td>10.</td>
<td>270</td>
<td>0.215</td>
<td>0.092</td>
<td>0.192</td>
<td>0.064</td>
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24 hours, 6 N HCl hydrolysate

<table>
<thead>
<tr>
<th>Component</th>
<th>cm max</th>
<th>A max</th>
<th>A 260</th>
<th>A 410</th>
<th>Dische test</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.</td>
<td>275</td>
<td>0.162</td>
<td>0.088</td>
<td>0.283</td>
<td>0.212, 0.088</td>
</tr>
<tr>
<td>10.</td>
<td>270</td>
<td>0.412</td>
<td>0.130</td>
<td>0.283</td>
<td>0.042</td>
</tr>
</tbody>
</table>

*p = pink; y = yellow; b = blue; g = grey; r = red.

Fig. 6. Release of reducing sugar in hydrolysis with dilute acid. Micrograms of glucose are plotted versus minutes of hydrolysis. The determination by the method of Dische was with untreated material; the determination with glucose oxidase was with the neutralized hydrolysate at the completion of the experiment.

The identification of the hexose as glucose has been reported briefly (21). In Fig. 6, the release of reducing sugar (22) by hydrolysis with dilute acid is illustrated, alkaline phosphatase was heated in a boiling water bath in 1 N HCl, aliquots were removed, neutralized by the addition of sodium carbonate, centrifuged, and the supernatant solution was analyzed for reducing sugar with the results as indicated. An aliquot, at the end of the hydrolysis, after suitable neutralization, was treated with glucose oxidase (Glucostat, Worthington Biochemical Laboratories) with the result as indicated. A sample of the same preparation of alkaline phosphatase was treated with cysteine and sulfuric acid in the primary reaction of hexoses of Dische (14); after 48 hours, the absorbancy of the test solution was determined along with that of standard amounts of glucose, galactose, and mannose. The results given in Fig. 7 demonstrate that the material is glucose; the amount of glucose indicated by the method for an equivalent aliquot is shown in Fig. 6 as somewhat lower.

Fig. 7. Reaction of cysteine and sulfuric acid with alkaline phosphatase and glucose (the secondary reaction of hexoses). Absorbancy is plotted versus wave length in millimicrons.
higher than the value indicated by the oxidase or by the reducing sugar method. After prolonged hydrolysis, such as in the 6 N hydrolysate, 24 hours, very little glucose can be detected. Presumably, the glucose is destroyed in reactions with other products of hydrolysis; at the same time, the absorbancy of the solution at 330 mμ increases (Fig. 5). The percentage content of glucose as determined by the method of Dische was about three times the content of nitrogen or, as one may calculate on a molar basis, there were approximately 4 nitrogen atoms per molecule of glucose.

Ninhydrin-reactive materials containing carbohydrates were formed during prolonged hydrolysis; in an experiment to prevent the destruction of such materials, hydrolysis was carried out in an atmosphere of nitrogen with 6 N HCl for 24 hours, the solution was concentrated in a vacuum and the final solution was subjected to chromatography with propanol-water as in Table III. In this case a component with a Rf somewhat greater than the material reacting with ferricyanide was detected by a pink reaction with ninhydrin. The material did not absorb in the ultraviolet range but responded to the test of Dische as did ribose to give a maximal absorbancy at 330 mμ (λmax = 0.520 compared with 0.660 for the ferricyanide-positive component). Apparently, under these conditions, it was possible to find a breakdown product of the material with a ninhydrin-reactive material attached to the pentose. With the exclusion of oxygen during the hydrolysis, materials similar to Component 10 of Table III were not detected.

Identification of Other Compounds—Hydrolysates of alkaline phosphatase were prepared with 6 N and 9 N HCl with refluxing or at 105° in sealed tubes; the hydrolysates were concentrated in a vacuum over drying agents or by exhaustive vacuum distillation; these hydrolysates, containing 1 to 3 mg of total N per ml, were examined for amino acids by paper chromatography with phenol-water and with butanol-acetic acid-water systems in one- and two-dimensional runs. Six ninhydrin-reactive materials were detected but, in no case, could any of the components be identified as known amino acids. Furthermore, in all the systems, amino acids, other than the prolines, responded to the ninhydrin stain with blue colors; the unknown ninhydrin-reactive materials gave pink or red colors in the various systems.

In attempts to characterize the component responsible for the reaction with potassium ferricyanide, a variety of compounds were investigated. Mono- and disaccharides, commercially available amino acids, the various vitamins, the known coenzymes, and a variety of purines and pyrimidines were investigated. In these studies, it became clear that the only materials of those tests giving suitable reactions (ninhydrin plus the reaction with ferricyanide) were certain substituted pyrimidines. Thus, it was considered desirable to extend the studies of the pyrimidines. Although it is recognized that the material in the hydrolysates was combined with carbohydrates, a comparison with uncombined materials seemed a reasonable place to start.

The pyrimidines were obtained from the California Corporation for Biochemical Research as described in their listing of July, 1959; the listing is stated to be according to the system approved by Chemical Abstracts. It is apparent from Table IV that a variety of substituted pyrimidines react as the ferricyanide-positive material in the hydrolysates of alkaline phosphatase. The most reactive compounds were the tetra-substituted pyrimidines containing amino groupings; none of these compounds are known to be naturally occurring. A tetra-substituted pyrimidine of known biological occurrence is divicine, a compound thought to have the 2,4-diamino, 5,6-dioxy structure. No authentic compound of this structure has been available for testing but dilute acid hydrolysates of vicine, isolated from vetch seeds, have as has been reported (23) and much as do the hydrolysates of alkaline phosphatase. A search is underway to locate vicine-like compounds containing pentose as well as glucose. In agreement with Morton (10) and others, we have found no phosphate in the preparations; the possible content of phosphate permits less than 1 phosphate per 10 molecules of glucose.

**DISCUSSION**

The characterization of the alkaline phosphatases of swine kidney (or of other tissues) presents several unique problems. The high order of purification necessary has limited the material available and truly satisfactory data as to homogeneity are not available. Also, the extent and nature of the modification of the alkaline phosphatase by the proteolytic digestion utilized in the release of the activity from the ribonucleoprotein particles and in the purification cannot be assessed at present. We have adopted the attitude that a reasonable goal is the characterization of the smallest particle or molecule bearing the catalytic activity; therefore, we have attempted to remove as much protein as possible and to characterize the active material surviving this removal. The data presented in this report would indicate that the material obtained is essentially devoid of amino acids; the ninhydrin-reactive materials in the hydrolysates could not be identified as amino acids, and, indeed, most of the ninhydrin-reactive materials were released by mild hydrolysis unlikely to release amino acids from peptide linkage and were found to be associated with carbohydrate. Thus, it would appear that the ninhydrin-reactive materials are breakdown products of some grouping associated with carbohydrate in the alkaline phosphatase. The complete resistance of the activity to all proteolytic enzymes tested would tend to substantiate the conclusion that peptides are either not present or are not essential for activity. It is to
be admitted that it will be difficult to rule out protein, peptide, or amino acids as components of the active material. The method of Lowry et al. (12) for the detection of protein is positive, as is the ninhydrin reaction but, as has been pointed out above, the method of Lowry and the ninhydrin reaction are not specific for protein, peptide, or amino acids. Should a protein be composed of those amino acids giving the less intense reactions with ninhydrin, it may be possible that as much as 5% contamination with such a protein might not be detected by our methods of chromatography; should this possible contaminant represent the activity, we must anticipate a specific activity of 20-fold greater than achieved, about 6,000,000 Roche units, and a total purification of about 1,000,000-fold.

The tentative identification of the nitrogenous constituent of alkaline phosphatase as a tri- or tetra-substituted pyrimidine would seem the closest approximation at this time. Presumably, many more complex compounds with a similar ring system would have the proper absorbancy in the ultraviolet range and would respond to the tests with ferricyanide and with ninhydrin; of the rather long list of relatively simple materials tested, the pyrimidines were the only materials with the proper reactions.

A point of pertinence to the question of whether or not the material we have isolated is a fragment of the material originally present in the ribonucleoprotein particles is the determination of the molecular weight at various stages of purification. Although such studies are still far from complete, it may be reported that gradient centrifugation has indicated a molecular weight of the purified material to be less than 10,000 and that studies of the same material with Sephadex (Pharmacia Laboratories, Inc.) have indicated a molecular weight that is less than that of ribonuclease or lysozyme. It should be remembered, however, that our preparations are isolated as the barium salt; we have made no observations as to the possible effects of barium on the observed molecular weights.

Should one accept the conclusion that protein is not present, or if present, is not essential for activity, the problem becomes one of accounting for the catalytic activity without invoking the secondary structures of protein. Two factors seem worthy of consideration; first, activity is dependent upon the addition of a divalent metal ion (magnesium) and, second, the optimal pH is well above 9 and, depending upon the substrate and buffer employed, may be as high as 10.5. Thus, should the optimal pH be determined even partially by the pK of an essential grouping in the catalyst, the imidazole grouping would not appear to be a particularly likely candidate. On the other hand, substituted pyrimidines have appropriate pK values to satisfy these criteria. In our work on this problem we have postulated that the phosphates are of at least two types; one type, the neutral phosphates, as illustrated by the inorganic pyrophosphates, has a pH optimum near neutrality and appears to utilize the sulfhydryl grouping as an essential grouping in its catalytic activity (24), whereas the second type illustrated by the alkaline phosphatase has a pH optimum near 10 and utilizes a nonprotein nitrogenous constituent in the catalytic process. Iodoacetamide and p-hydroxymercurobenzoate inhibit the inorganic pyrophosphatase of brain (24) but are without effect on the alkaline phosphatase of swine kidney; diisopropylfluorophosphate is without effect on either activity. Unfortunately, it has not been possible to find a potent inhibitor other than materials combining with metal ions, for the alkaline phosphatase.

**SUMMARY**

Alkaline phosphatase of swine kidney has been purified about 30,000-fold to a specific activity of 300,000 Roche units per mg of total nitrogen; this material appears to be homogeneous by paper electrophoresis, paper chromatography, by chromatography on Ecteola cellulose, and by countercurrent distribution. Although the material has an absorbancy in the ultraviolet range similar to proteins, no amino acids may be detected in hydrolysates. Components of the alkaline phosphatase have been identified as glucose, a pentose, and a nitrogenous component that appears to be a tri- or a tetra-substituted pyrimidine.

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