Purification of Kidney Alkaline Phosphatase

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In order to study the chemical properties and the nature of phosphatase it is of prime importance to obtain pure preparations, especially in work concerned with tests for a coenzyme or prosthetic group. In this connexion, numerous hypotheses have been advanced based on results with preparations of low purity, and their confirmation with the pure or a highly purified enzyme is called for.

It has been known for some time that kidney alkaline phosphatase is associated with insoluble cellular particles (Kabat, 1941; Morton, 1950, 1954, 1955; Mathies, 1951). Its purification demands a previous solubilization to liberate it from lipids or phospholipids, and a subsequent separation of non-enzymic protein material. This last process is difficult because the inert proteins which accompany the enzyme usually display similar properties.

The solubilization of the enzyme has been achieved by several procedures. The original method for the extraction of phosphatases from animal tissues by controlled autolysis (Albers & Albers, 1935) has been employed by several authors. This procedure has been frequently accompanied by a simultaneous or separate treatment with proteolytic agents, such as trypsin or pancreatin (Bodansky, 1937; Abul-Fadl & King, 1949; Abul-Fadl, King, Roche & Thoai, 1949; Mathies, 1951; Roche & Bouchilloux, 1953; Arai, 1956). Morton (1950, 1954) considers that these treatments are unsuitable, for during their application alterations of the native structure of the enzyme can take place, even without any loss of activity. It is difficult to confirm or discard this hypothesis, and controlled autolysis is undoubtedly a good method for enzyme extraction. Morton (1950, 1955) has introduced the use of butanol in the solubilization of enzymes in general, and has employed it with success in the purification of alkaline phosphatases from the intestine (Morton, 1954) and from milk (Morton, 1953*b*). The application to kidney phosphatase of the method employed for the purification of intestinal phosphatase did not yield good results, but this does not exclude the effectiveness of butanol in the solubilization and purification of kidney enzyme. In the procedure to be described controlled autolysis and treatment with butanol have both been adopted.

Once the enzyme is in solution, the methods employed by several authors for subsequent purification include fractionation with organic solvents (Kabat, 1941; Abul-Fadl *et al.* 1949; Mathies, 1951; Arai, 1956), precipitation with ammonium sulphate (Abul-Fadl *et al.* 1949; Mathies, 1951) and adsorption of impurities with several materials (Roche & Bouchilloux, 1953). Electrophoresis, in different forms, has also been employed in the purification of alkaline phosphatases. Roche & Bouchilloux (1953) and Lora-Tamayo & Elorriaga (1956) purified intestinal alkaline phosphatase by paper electrophoresis. Carlson (1954) has studied column electrophoresis of kidney alkaline phosphatase, employing starch as support. Harris & Mehl (1955) describe electrophoresis in agar gel and Mathies (1952) has obtained a good purification of kidney alkaline phosphatase by convection electrophoresis in a Kirkwood apparatus. Nevertheless, several of these authors (Carlson, 1954; Harris & Mehl, 1955) point out a progressive inactivation of the enzyme in the course of electrophoresis, attributed to the loss of a metal or a coenzyme. Experiments carried out by the authors (E. F. Alvarez & M. Lora-Tamayo, unpublished work) on column electrophoresis of kidney alkaline phosphatase, with a cellulose-powder support, also reveal an inactivation of the enzyme, due not to the loss of magnesium but to some group with absorption in the ultraviolet-light band and of a non-peptide nature. Lately, Arai (1956) has shown that alkaline phosphatase, inactivated by electrophoresis, can be reactivated by incubation in sodium carbonate–bicarbonate buffer.

These various methods have yielded kidney phosphatase preparations of different purities but none has been pure, and knowledge of the biological functions, action mechanisms and nature of the enzyme has been gathered from work with these impure preparations.

The procedure we describe for obtaining the enzyme has been studied in detail. In drawing it up we have examined the different purification and fractionation techniques for proteins and enzymes such as ion-exchange chromatography (Amberlite IRC-50, IR-120, IRA-410; Dowex-2), column electrophoresis with cellulose-powder support and tryptic digestion. Each of the purification steps has been closely examined before inclusion in the method, and in Table 1 we summarize the procedure finally adopted, together with specific activity and yields at each stage.

Table 1. *Summary of the purification process of kidney alkaline phosphatase*

The results of each operation are described in Tables 2–7. Specific activity is defined in the Materials and Methods section. The yield is expressed as a percentage of the amount of enzyme used in the operation.

Step no.	Operation	Specific activity	Yield (%)
1	Controlled autolysis at pH 8, filtration or centrifuging	97–320	—
2	Precipitation with 60% (v/v) acetone and treatment with butanol	570–860	60–92
3	Fractionation with acetone (0–45%, v/v)	2 500	93
4	Fractionation with ammonium sulphate at pH 8, between 1·95 and 2·92 M	8 300	98
5	Adsorption of impurities on magnesium carbonate	15 000–16 000	102
6	Chromatography on calcium phosphate and dialysis	30 000	40–50
7	Adsorption of impurities on activated carbon	42 000	98
8	First fractionation with ethanol at pH 8, between 50 and 65% (v/v)	85 000	100
9	Second fractionation with ethanol, at pH 8, between 54 and 62% (v/v)	166 000	73

We have considered it convenient to express the yields in terms of each purification step, and not as total yields related to the starting material, owing to the fact that the material prepared in each purification step has not always been employed fully in the next step.

Under optimum conditions, the purest material obtained liberated 166 mg. of phosphorus/mg. of nitrogen/min., with sodium β-glycerophosphate as substrate. Its purity, as judged by paper electrophoresis, is from 85 to 90%, and it shows only one active component with a trace of another protein of a slightly higher mobility. Examination of the solubility of this preparation in aqueous ethanol showed that it was possible to achieve a slightly greater specific activity by making a third fractionation between narrower limits than those set by 54–62% (v/v) of ethanol. However, this is not advisable, as the yield decreases considerably. Ten kilograms of pig kidneys were used in this work and 12·4 mg. of protein material with the above mentioned specific activity was obtained.

EXPERIMENTAL AND RESULTS

General methods

Determination of proteins and protein nitrogen. Unless otherwise indicated, the procedure adopted is an adaptation of the microbiuret method described by Goa (1953). A preliminary calibration curve was constructed with crystalline bovine-serum albumin (Armour and Co., Chicago, Ill., U.S.A.) and checked against Ma & Zuazaga's (1942) micro-Kjeldahl method. The differences were never greater than ±3%. The method allows determination, with sufficient precision, of 50–100 μg. of protein, when the reaction is carried out in a total vol. of 2·7 ml. and 1 cm. cells are used for the extinction readings in a Beckman DU spectrophotometer at 330 mμ. The determinations were made in duplicate with 0·5–1·5 ml. of protein solution, 0·2 ml. of Benedict's qualitative reagent, and the volume was adjusted to 2·7 ml. with M-sodium hydroxide. After 15 min. the extinction is read against a blank with water instead of protein solution.

With purified preparations, both the relation mg. of protein/ml. $= E_{280\ m\mu/1\cdot40}$, and Kalckar's (1947) formula mg. of protein/ml. $= 1\cdot45\ E_{280\ m\mu} - 0\cdot70\ E_{260\ m\mu}$, yield good results. To express protein in terms of protein N, the factor 1/6·25 has been used.

Determination of enzymic activity. This has been determined under optimum conditions (Alvarez, 1956) against 20 mM-sodium β-glycerophosphate (British Drug Houses, Ltd.), 10 mM-magnesium acetate, in a medium buffered with 50 mM-sodium veronal, pH 9·9. For each determination 4·5 ml. of substrate–Mg^{2+} ions–buffer solution is taken, and kept in a thermostat for 5–15 min. at 38°. Next 0·5 ml. of enzyme solution, suitably diluted with water, is added, and the reaction is allowed to proceed for 5–15 min. The enzymic activity is stopped by addition of 2 ml. of 25% (w/v) trichloroacetic acid. After filtration (if necessary) the inorganic phosphorus is determined. Filtration is needed only with very impure preparations. Determinations must be made immediately after diluting the enzyme, for very dilute aqueous solutions are unstable. Not more than 5% of the substrate should be allowed to hydrolyse; otherwise the determinations must be repeated with a more dilute enzyme solution. Under these conditions the substrate hydrolysis is strictly proportional to the concentration of the enzyme and to time (Alvarez, 1956).

In agreement with Roche & Bouchilloux (1950, 1953) and Morton (1953*a*) the enzyme unit adopted is expressed as the amount of enzyme which, under optimum conditions, hydrolyses 1 μg. of P/min. The specific activities are expressed in μg. of P hydrolysed/min./mg. of protein N (units/mg. of N).

Inorganic phosphorus. This was determined colorimetrically (Briggs, 1924) with a Leitz photocolorimeter.

Solubility curves. These were obtained by using constant amounts of solution (0·5–1 ml. with 1–4 mg. of protein/ml.) with variable concentrations of the precipitating agent. After 3–6 hr., at 0°, the precipitates were centrifuged and redissolved at fixed volumes (2–4 ml.) in water or 10 mM-

sodium bicarbonate. Protein and enzymic activity in each tube were then determined, and the curves for each of these variables drawn as a function of the concentration of the precipitating agent. The determination of the solubility curve has always preceded any precipitation step, and it has been taken as a base on which to establish the optimum concentrations of the precipitating agent in fractionation.

Paper electrophoresis. These tests have been carried out in a Grassman–Hannig apparatus (Grassman & Hannig, 1952) with Franz Bergmann paper (Franz Bergmann K.-G., Berlin-Zehlendorf; 7 cm. × 40 cm.), with sodium veronal–HCl buffer (pH 8·6; *I*, 0·05) during 14–16 hr. at 175–200 v (2–4 mA). Development, determination of activity and construction of the fractionation curve were undertaken as described by Lora-Tamayo & Elorriaga (1956). In every test a control containing crystalline bovine-serum albumin was employed and relative mobilities were determined in respect to it, by analogy with *R* in paper chromatography.

Preparation of adsorbents and chromatographic techniques. The magnesium carbonate employed was obtained by precipitating 0·5 M-magnesium chloride with 0·5 M-sodium carbonate. The precipitate was filtered off on a Büchner funnel and washed with 25 mM-sodium carbonate until all chloride was eliminated. The preparation was then suspended in a 25 mM-sodium carbonate–bicarbonate buffer (pH 9) and stored for use.

Specially activated carbon for the adsorption of nucleotides was prepared from active carbon (E. Merck, Darmstadt, Germany) according to Morton (1953*b*).

Calcium phosphate (hydroxyapatite) for column chromatography of proteins was prepared according to Tiselius, Hjertén & Levin (1956). The only difference in behaviour between the freshly prepared material and that kept during several months in mM-sodium phosphate buffer (pH 6·8) is that, with old preparations, the columns are more compact and the rate of flow is lower. The chromatographic technique with this material has been described by Tiselius (1954*a*, *b*, 1955*a*, *b*) and Tiselius *et al.* (1956) and it has been used without making any fundamental modifications. Column elution was effected by increasing the ionic strength of the eluting phosphate buffer in a continuous form or with suitable linear or curved gradients, obtained according to Drake (1955). It has been determined experimentally that these check with the equations given by this author. Each one of the elution techniques described has both advantages and disadvantages, which have been already pointed out by Tiselius *et al.* (1956). For preparative-scale work, we adopted the gradient-elution technique.

Preparation and purification of the enzyme

(1) *Autolysis.* We used the procedure recommended by Abul-Fadl *et al.* (1949) but the operation was carried out at pH 8. Fresh pig kidneys, deprived of their capsules, are sliced longitudinally and fat is removed. The material is washed two or three times with water and put through a mincer. The slurry thus obtained is suspended in 1 l. of 25% (v/v) aqueous acetone and 100 ml. of a mixture of equal parts (v/v) of ethyl acetate and toluene/kg. The mixture is adjusted to pH 8 with conc. sodium carbonate solution and kept at room temperature for 2–3 days with occasional stirring. Autolysis sets in after 3–4 hr. with liberation of acid substances, and it is therefore necessary to add sodium carbonate frequently to maintain the pH between 7·5 and 8·5. If this precaution is not observed, the pH falls to around 5–6 and activity decreases. After 48 hr. the pH is stable and autolysis is considered to be ended. The mixture is passed through a sieve or cloth and the solid residue extracted for 24 hr. with 0·2–0·3 of the volume employed before. The two filtrates are combined and centrifuged in a Sharples Supercentrifuge (Expt. I) or filtered off, under vacuum, through a layer of paper pulp 2–4 cm. thick (Expt. II). The filtrate is greenish red or reddish, depending on the efficiency of the preliminary washing of the material. Lots of 5 kg. of kidneys were used to start at once. More than this weight is difficult to handle at this step. Table 2 summarizes two different extractions.

(2) *Acetone precipitation and treatment with butanol.* The solutions obtained according to step 1 are cooled to −5° in a bath of ice and salt, and for each litre of solution 1·5 l. of rectified acetone, precooled to between −10 and −15°, is added with continuous stirring. The temperature increases to about 0°. The precipitate is left to sediment for about 12 hr., the supernatant liquid is poured off and the remainder filtered through paper in a funnel (25–30 cm. diam.). The precipitate is then washed with 60% (v/v) acetone, drained and suspended in 50 mM-sodium carbonate–bicarbonate buffer (pH 9).

Rectified butanol, to 25% (v/v) concentration, is added slowly, with stirring, to the suspension and the mixture is heated to 35–37° in a water bath and held at this temperature for 5–10 min. It is then cooled in tap water and in an ice bath. Then it is filtered under vacuum through paper pulp and the residue is washed with the carbonate buffer. The dark-yellow or reddish filtrate is extracted two or three times with freshly distilled, peroxide-free ether to remove butanol. Table 3 summarizes two different experiments.

Table 2. *Extraction of kidney phosphatase by autolysis*

Expt. no.	Material		Units ml.	Volume (ml.)	Total units ($\times 10^{-3}$)	Protein N (mg./ml.)	Specific activity	Colour
I	Autolysis of 5 kg. of kidneys and washing of residues	First extraction	374	4700	1758	1·17	320	Greenish yellow
		Second extraction	116	1350	157	1·62	71	Yellow
				6050	1915		206	
II	Autolysis of 5 kg. of kidneys (4·45 kg. of slurry) and washing of residues	First extraction	348	4850	1688	3·57	97	Ruby-red
		Second extraction	132	2220	293	0·77	172	Yellow
				7070	1981		103	

(3) *Fractionation with acetone.* Fractionation with acetone of the preparations obtained after step 2 showed that after precipitation between 0 and 45% (v/v), the enzyme was recovered quantitatively with a threefold purification. Only a small amount of material precipitated before 35% (v/v).

The enzyme solution obtained in the previous step is cooled to −5° in a salt-and-ice bath. Rectified acetone, precooled to between −10° and −15°, is slowly added with continuous stirring (820 ml./l.). The suspension is left at 0° during 12 hr. Supernatant liquid is then suctioned off, and the precipitate centrifuged or filtered off through paper.

The precipitate is suspended in 50 mM-sodium carbonate–bicarbonate buffer (pH 9) and after stirring mechanically during 30–60 min. it is then vacuum-filtered through paper pulp 1–2 cm. thick. The solution thus obtained is reddish yellow. The diagram of precipitation with acetone after step 3 (Fig. 1) shows that further fractionation is not useful. Table 4 summarizes one of these operations.

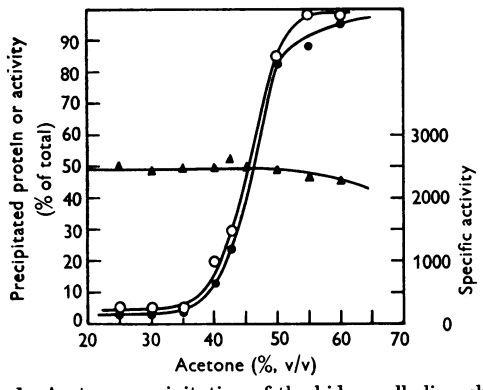

Fig. 1. Acetone precipitation of the kidney alkaline phosphatase preparation obtained after step 2 (see text). Conditions were as described in the text. ●, Protein; ○, activity; ▲, specific activity.

(4) *Fractionation with ammonium sulphate.* The solubility diagrams against ammonium sulphate of the preparation obtained in step 3 revealed the presence of at least three easily separable components. Enzymic activity was associated with the one precipitating between 1·95 and 2·92 M-ammonium sulphate. Recovery is quantitative and a threefold purification is possible.

Fractionation is carried out in a graduated beaker at 0° through the addition, in small portions, of ammonium sulphate until the suspension attains 1·95 M in SO_4^{2-} ions. After each addition of the reagent the pH is checked, for it must be kept around 8 by the addition, drop by drop, of dilute aq. NH_3 soln. The precipitate is separated by filtration or centrifuging and, observing identical precautions, the solution is brought to 2·92 M in SO_4^{2-} ions. After separation of the precipitate the last solution is discarded.

Both precipitates are dissolved separately in 25 mM-carbonate–bicarbonate buffer (about pH 9) and the solutions are filtered or centrifuged. Table 5 summarizes the process.

The enzyme solution obtained preserves all of its activity for months if it is kept at 0° with a few drops of toluene. In this state, an inert protein precipitate separates and specific activity attains 10 000 units/mg. of protein N. The solubility diagram against ammonium sulphate (Fig. 2) shows that further purification with this reagent is impossible.

(5) *Adsorption of inert material on magnesium carbonate–* The enzyme solution obtained according to step 4 is dialysed for 12 hr. at 0–5° against 25 mM-carbonate–bicarbonate buffer (pH 9). It is next cooled in ice and, during stirring, an excess of magnesium carbonate is added. The magnesium carbonate suspension, prepared as described above, was filtered on a Büchner funnel and about 50 g. of the cake was used in the experiment in Table 5. The enzyme is not adsorbed on this material. After contact for 30–60 min. the suspension is filtered

Table 3. *Precipitation of phosphatase with acetone and treatment with butanol*

Expt. no.	Material	Units (ml.)	Volume (ml.)	Total units ($\times 10^{-3}$)	Protein N (mg./ml.)	Specific activity	Yield (%)	Colour
I	Initial	316	6050	1915	1·27	250	—	—
	Final	986	1790	1764	1·74	569	92	Dark yellow
II	Initial	280	7070	1981	2·73	103	—	—
	Final	701	1680	1178	0·82	855	60	Dark yellow

Table 4. *Fractionation of phosphatase with acetone*

Material	Units/ml.	Volume (ml.)	Total units ($\times 10^{-3}$)	Protein N (mg./ml.)	Specific activity	Yield (%)	Colour
Initial	848	3470	2930	1·30	653	—	—
Final	3900	700	2730	1·58	2470	93	Reddish yellow

Table 5. *Fractionation of phosphatase with ammonium sulphate*

Material		Units/ml.	Volume (ml.)	Total units ($\times 10^{-3}$)	Protein N (mg./ml.)	Specific activity	Yield (%)	Colour
Initial		3 900	700	2730	1·58	2470	—	—
Final	0–1·95 M	438	251	109	2·00	219	4	Reddish yellow
	1·95–2·92 M	13 440	200	2688	1·62	8300	98	Salmon
				2797			102	

under vacuum through paper pulp and the residue washed with the same buffer. Table 6 summarizes the results.

This purification step can be omitted, the procedure being as indicated under step 6, for its main object is the elimination of that inert material which has an electrophoretic mobility similar to that of serum albumin and which separates well from the enzyme by chromatography on calcium phosphate.

(6) *Chromatography on calcium phosphate (hydroxyapatite).* The enzyme solution, treated or not according to step 5, is dialysed for 24 hr. at 0° against a solution 5 mM in Na_2HPO_4 and 0·2 mM in Na_2CO_3. It is then applied to a hydroxyapatite column, prepared according to Tiselius *et al.* (1956), cooled to about 0° and equilibrated with the phosphate–carbonate solution. The column employed was 2·5 cm. × 16 cm. (retention volume about 60 ml.). These columns have a great adsorptive capacity, and the one employed is sufficient to chromatograph several grams of material without achieving saturation.

Elution is carried out with a curved gradient (Drake, 1955), obtained with a closed mixture chamber of 120–250 ml. capacity according to the amount of material to be chromatographed. It is charged with a solution 5 mM in Na_2HPO_4 and 0·2 mM in Na_2CO_3 and fed with a solution of 25 times this strength. Finally, elution with 0·2 M-Na_2HPO_4 is effected at room temperature in order to regenerate the column and eliminate inactive material.

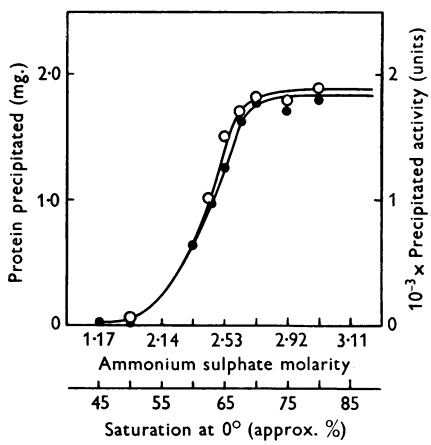

Fig. 2. Ammonium sulphate precipitation of the kidney alkaline phosphatase preparation obtained after step 4 (see text). Conditions were as described in the text. ●, Protein; ○, activity.

The eluate is taken in 3·5–4 ml. fractions and the determination of its extinction at 280 mμ is carried out in a Beckman DU spectrophotometer. These chromatographic fractions are gathered conveniently in accordance with their content of protein. They are then dialysed against distilled water at 0–5° until elimination of phosphate ions is achieved (2–3 days), and finally, against 10 mM-sodium bicarbonate (12 hr.). During dialysis the separation of needle crystals takes place. They are magnesium phosphate crystals with a small amount of occluded protein, but they have no enzymic activity. Similar crystals are also formed when the solutions obtained in the first purification steps are kept a few days at 0°. Albers & Albers (1935), Abul-Fadl *et al.* (1949), Abul-Fadl & King (1949), Arai (1956) and Mathies (1951) have reported similar results.

Excepting that one which elutes with 0·2 M-Na_2HPO_4 (coloured fraction), all of these fractions are active. The most important fraction, with about 30 000 units/mg. of protein N, represents about 40–50 % of the activity applied to the column. It elutes in a large volume, giving very dilute fractions, starting from a 0·09 M-phosphate ion concentration under the conditions shown. The other fractions display activities lying between that value and 3000 units/mg. of protein N and have not been further studied.

The total recovery of activity is around 85–95 % and that of protein material of the same order. Table 7 summarizes one of the operations in which the material employed was not previously treated according to step 5.

The most active fraction (about 30 000 units/mg. of protein N) can be concentrated by adsorbing it on a hydroxyapatite column and eluting with 0·15 M–0·2 M-Na_2HPO_4. It behaves as if it were homogeneous and does not tolerate a subsequent chromatographic fractionation. Nevertheless it is preferable to proceed to its concentration, precipitating it with an excess of acetone at −5°. The mixture is left to stand for 24–48 hr. at 0° and the precipitate is separated by centrifuging. Next it is washed with cold acetone and dried under vacuum over phosphoric oxide at 0°. The preparation is stable and can be kept in this way to mix with material obtained in further preparations.

(7) *Adsorption of impurities on activated carbon.* The preparation obtained according to step 6 is dissolved in 0·01 M-

Table 6. *Adsorption of inert material on magnesium carbonate*

Material	Units/ml.	Volume (ml.)	Total units (× 10^{-3})	Protein N (mg./ml.)	Specific activity	Yield (%)	Colour
Initial	11 200	175	1960	1·10	10 200	—	Salmon
Final	7 700	260	2000	0·48	16 000	102	Yellow

Table 7. *Chromatography of phosphatase on calcium phosphate*

Material		Units/ml.	Volume (ml.)	Total units (× 10^{-3})	Protein N (mg./ml.)	Specific activity	Yield (%)	Colour
Initial		5720	22	125·8	0·620	9 380	—	—
Final fractions	I	271	92	24·9	0·072	3 760	19·8	Colourless
	II	102	45	4·6	0·010	10 200	3·7	
	III	234	130	30·4	—	16 200	24	
	IV	270	203	54·8	0·009	30 000	43·5	
	V	—	170	—	—	—	—	Reddish yellow
				114·7			91·0	

sodium bicarbonate. The precipitate does not dissolve completely as it is made up partly of scarcely soluble mineral salts. The solution is cooled to 0°, and 5 mg. of active carbon/mg. of protein in solution is added with stirring. A higher proportion of adsorbent irreversibly eliminates activity. Stirring is continued for 20–30 min. and the solution is then centrifuged. Table 8 summarizes the process.

(8) *First fractionation with ethanol.* The solubility curve against ethanol of the solution obtained in step 7 indicates that a twofold purification is achieved by fractionation between 50 and 65% (v/v) of ethanol with quantitative recovery of activity.

The enzyme solution is cooled to 0° in an ice-bath and 1 ml. of absolute ethanol/ml. of solution is added, with stirring, drop by drop. The suspension is left to stand for

Table 8. *Adsorption of impurities on activated carbon and fractionation with ethanol*

Operation	Material		Units/ml.	Volume (ml.)	Total units ($\times 10^{-3}$)	Protein N (mg./ml.)	Specific activity	Yield (%)	Colour
Adsorption on activated carbon	Initial		14 900	32·5	484	0·450	33 100	—	Pale yellow
	Final		11 300	42·0	475	0·270	41 800	98	
First fractionation with ethanol	Final		43 000	11·0	473	0·502	86 000	100	Colourless
Second fractionation with ethanol	Initial		32 800	13·8	452	0·380	86 300	—	
	Final	0–54%	3 300	6·0	19·8	0·020	165 000	4	
		54–62%	25 000	12·5	313	0·150	166 000	69	
		>62%	5 800	13·0	75·4	0·270	21 500	17	—
					408·2			90	

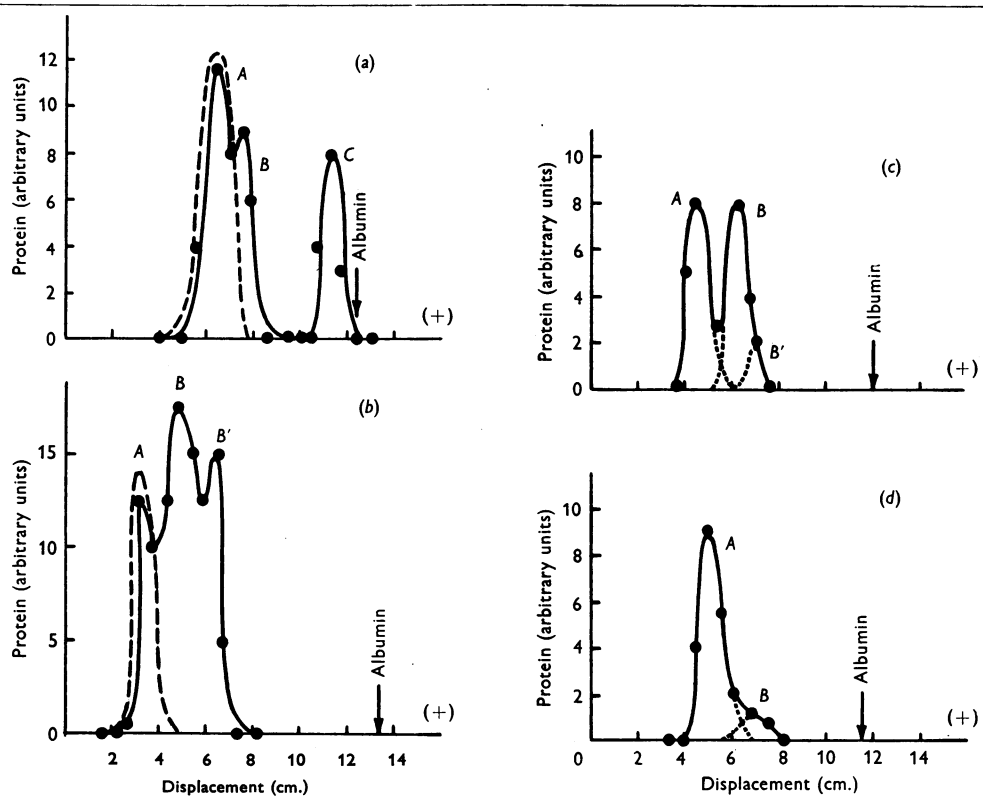

Fig. 3. Paper-electrophoresis diagrams of kidney alkaline phosphatase preparations at different stages of purification. Conditions were as described in the text. μ_r = Relative mobility against crystalline bovine-serum albumin. In the following, figures in parentheses represent percentage of total protein. Protein, —; activity, - - -. (*a*) Preparation after step 4: specific activity 8300 units/mg. of protein N; μ_{rA} = 0·52 (45%); μ_{rB} = 0·62 (30%); μ_{rC} = 0·92 (25%). (*b*) Preparation after step 6: specific activity 30 000 units/mg. of protein N; μ_{rA} = 0·25; μ_{rB} = 0·38; $\mu_{rB'}$ = 0·46. (*c*) Preparation after step 8: specific activity 85 000 units/mg. of protein N; μ_{rA} = 0·36 (active component) (50%); μ_{rB} = 0·52 (45%); $\mu_{rB'}$ = 0·59 (5%). (*d*) Preparation after step 9: specific activity 166 000 units/mg. of protein N; μ_{rA} = 0·43 (active component) (85–90%); μ_{rB} = 0·56 (10–15%).

24 hr. at 0° and then centrifuged. A further 0·86 ml. of absolute ethanol/ml. of the original solution is then added to the solution and, after leaving it to stand for 24–48 hr. at 0°, the precipitate is centrifuged. The solution is discarded and the precipitates are redissolved in 0·01 M-sodium bicarbonate. Table 8 summarizes the operation.

(9) *Second fractionation with ethanol.* The enzyme solution is cooled to 0° and fractionated between 54 and 62 % (v/v) in ethanol. First, 1·17 ml. of absolute ethanol/ml. of solution is added, and the precipitate separated by centrifuging, after leaving the suspension to stand for 24 hr. at 0°. Then, 0·46 ml. of ethanol/ml. of the original enzyme solution is added and the precipitate separated as before. Finally, the solution is treated with an excess of absolute ethanol and the precipitate recovered.

The three precipitates are redissolved in 0·01 M-sodium bicarbonate. Table 8 summarizes the operation described.

Analysis and absorption spectrum of the purified enzyme

Electrophoretic analysis. It is known that electrophoresis inactivates alkaline phosphatase (Carlson, 1954; Harris & Mehl, 1955; E. F. Alvarez & M. Lora-Tamayo, unpublished work). Nevertheless, in the course of kidney phosphatase purification, we have considered as a criterion of purity, not only the increment of specific activity but also the protein composition of the preparation obtained, which was established by paper electrophoresis. Fig. 3 shows the diagrams obtained for preparations of varying degrees of purity. As can be deduced from these diagrams, inert material which goes along with the enzyme is made up mainly of proteins of very similar electrophoretic mobilities. They are very close to those of β and γ serum globulins. Because of this, preparations corresponding to that of diagram (*a*) (8300 units/mg. of protein N) could be deemed 45 % pure, when the fact is that they are much less pure. The purest preparation obtained (diagram *d*) is made up of 85–90 % enzymic component and traces of an inert protein of somewhat greater mobility. It is impossible to affirm that the preparation is equally pure in absolute terms, for the purity of a protein or enzyme can be judged only in a relative way, and cases are known in which a product found to be homogeneous by electrophoresis is not so when examined by another analytical technique. Nevertheless, high specific activity (166 000 units/mg. of protein N), the characteristics of the solubility curves obtained and the results of electrophoretic analysis lead us to suppose that the preparation is practically pure.

Ultraviolet-absorption spectra. Figs. 4 and 5 show the ultraviolet-light spectra obtained in a Beckman DU spectrophotometer (1 cm. cells) with preparations of kidney phosphatase in different stages of purification with 0·01 M-sodium bicarbonate solvent.

All the preparations contain a substance with an absorption maximum at about 260 mμ, which is partly masked by the absorption due to protein, the maximum of which is 278–280 mμ. The substance, which is probably a pyrimidine nucleotide, is not eliminated from the purified enzyme after dialysis for 30 hr. at 0° against 0·01 M-sodium bicarbonate.

The quotient $E_{280\ m\mu}/E_{260\ m\mu}$ for the purified enzyme is 1·15. The extinction coefficient for 280 mμ in a 1 cm. cell is 1·39 and 1·20 for 260 mμ, for a solution with 1 mg. of protein/ml.

DISCUSSION

Purification of the enzyme

The procedure described for the purification of kidney phosphatase requires a brief commentary and a comparison with other methods previously employed.

In opposition to the results obtained by Mathies (1951) in the extraction of kidney enzyme by autolysis, we must report that the modified Albers & Albers (1935) method has proved very efficient

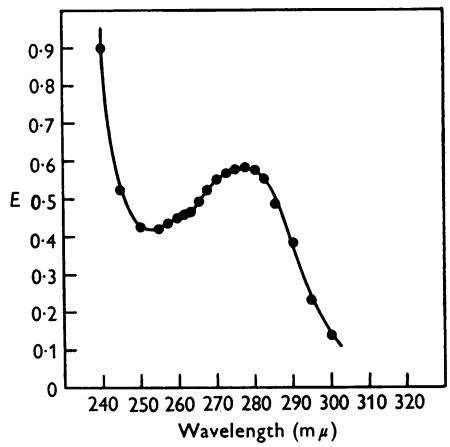

Fig. 4. Ultraviolet-absorption spectrum of kidney alkaline phosphatase preparation after step 8 of purification. Specific activity, 85 000 units/mg. of protein N. Solvent 0·01 M-sodium bicarbonate. Protein concentration, 0·45 mg./ml. Maxima at 260 and 278 mμ.

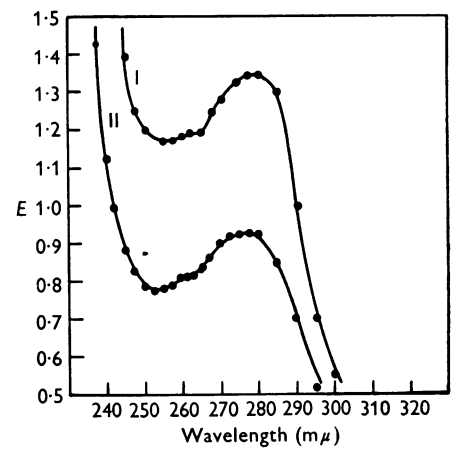

Fig. 5. Ultraviolet-absorption spectra of kidney alkaline phosphatase preparation after step 9 of purification, with 0·01 M-sodium bicarbonate as solvent. Specific activity, 166 000 units/mg. of protein N. Curve I, non-dialysed material; curve II, after dialysis for 30 hr. at 0° against 0·01 M-sodium bicarbonate. Protein concentration, 0·7 mg./ml. Maxima at 260 and 278 mμ.

without having recourse to the addition of pancreatin. Nevertheless, a careful control of pH is necessary. This must be kept at about 8. If this is not done, the yield of enzyme decreases to 3–5×10^5 units/5 kg. of kidneys, probably owing to acid inactivation of the enzyme. It is not possible to advance definite figures for the efficiency of the extraction, because the total activity in the starting material has not been determined. But if we compare liberated activity/kg. of kidney with the data reported by Mathies (1951) and by Arai (1956) a yield of 40–50 % is deduced.

The only other purification step which requires further mention is chromatography on hydroxyapatite. There is the precendent that the use of calcium phosphate is not to be recommended in the purification of alkaline phosphatase because adsorption is accompanied by partial inactivation by denaturation. However, hydroxyapatite prepared according to Tiselius *et al.* (1956) reveals conditions for protein chromatography which are not shown by other calcium phosphate preparations. Our experiments show that this material allows of a practically quantitative recovery of activity, operating at about pH 8–9. At lower values adsorption may entail denaturation, even though the enzyme is more weakly adsorbed. Addition of sodium carbonate to the disodium phosphate employed as a solvent in chromatography allows of a greater control of pH with solvent dilution. Nevertheless, the presence of this reagent modified the phosphate ion concentration necessary for the elution of the enzyme with $R = 1$ (elution concentration). Within the limits of experimental error, a linear relationship between elution concentration and the molar ratio (Na_2HPO_4): (Na_2CO_3) in the eluent (Fig. 6) is found so that the enzyme can be eluted at any desired phosphate ion concentration merely by setting the value of the molar ratio mentioned above. In the preparation described this ratio was 25, and the enzyme elutes with $R = 1$ at a 0·085–0·09 M-phosphate ion concentration. The above results, showing a deactivation effect of the CO_3^{2-} ions on the calcium phosphate, are in accordance with the suggestion of Tiselius *et al.* (1956) that the mechanism of the chromatography of proteins on this material is essentially an ion-exchange process, dependent on the affinity of proteins for calcium ions.

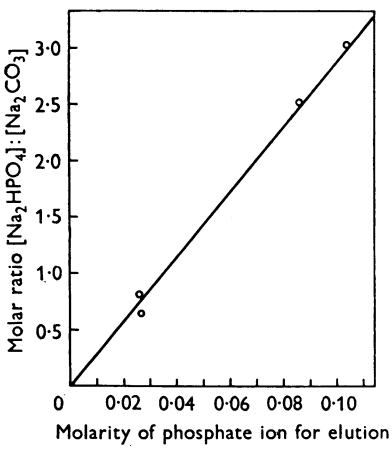

Fig. 6. Chromatography of kidney alkaline phosphatase on calcium phosphate columns. Variation of phosphate ion concentration necessary for elution of the enzyme with $R = 1$, with the molar ratio $[Na_2HPO_4]:[Na_2CO_3]$ in the eluent.

The separation of several fractions displaying enzymic activity in the course of chromatography is probably due to enzyme adsorption on inert proteins. However, we can not exclude the possibility of the existence of more than one alkaline phosphatase. Results in support of this hypothesis have been published by Gomori (1952), Gryder, Friedenwald & Carlson (1955) and Arai (1956). This question is now being studied.

The most active preparations of kidney alkaline phosphatase described up to 1956 are those of Morton (1954), with 6900 units/mg. of protein nitrogen, and Mathies (1952), with 940 King units/mg. of protein nitrogen. [Schramm & Ambrüster (1954) suggest 7·4 as the factor of conversion from the King unit to the unit employed in this paper, identical with that of Roche & Bouchilloux (1950, 1953) and Morton (1953*a*). A King unit is the amount of enzyme which liberates 1 mg. of phenol from sodium phenyl phosphate, under defined conditions, after 15 min. (Abul-Fadl *et al.* 1949).] Nevertheless Mathies (1954) has reported a specific activity of 15 200–16 800 units/mg. of protein nitrogen (about 90 % pure by electrophoresis), although neither the method of preparation nor the yield is detailed.

While this work was in progress, Arai (1956) published a thesis in which two very active preparations of kidney phosphatase are described. One of these shows 9500 King units/mg. of protein nitrogen [0–20 % (v/v) fraction in ethanol, 87 % pure by electrophoresis, 0·65 % yield], whereas the other has 5800–5900 King units/mg. of protein nitrogen [54·9–63·9 % (v/v) fraction in ethanol, 5 % yield]. The latter, much less pure than obtained by us, is precipitated, however, with the same ethanol concentration.

On the existence of a coenzyme or prosthetic group

The existence in alkaline phosphatase of a prosthetic group or coenzyme has been cited by many investigators (Roche & Thoai, 1950; Kutscher & Sieg, 1950; Zetterstrom, 1951;

Akamatsu & Kobayashi, 1951; Akamatsu & Aso, 1951; Lora-Tamayo & Municio, 1951; Lora-Tamayo & Alvarez, 1954*a*, *b*; Hofstee, 1955; Lora-Tamayo & Elorriaga, 1956). In particular, Lora-Tamayo & Alvarez (1954*a*, *b*) and Lora-Tamayo (1956) have suggested that a nucleotide, derived from uridylic acid, may be a component of alkaline phosphatase, and in point of fact the spectra in the ultraviolet-light band of the most active preparations obtained reveal the presence of a substance with an absorption maximum of about 260 mμ (Figs. 4, 5), such as the one corresponding to uridine derivatives. These results are, in part, confirmed by Arai (1956), who has detected the presence of ribose in his purest preparations.

However, the aforementioned results do not yet exclude the possibility of an impurity difficult to eliminate and the existence of a presumptive coenzyme or prosthetic group is being sought with the purified enzyme.

SUMMARY

1. Kidney alkaline phosphatase has been obtained in a high degree of purity and with an acceptable yield.

2. The method of purification includes nine steps: (1) autolysis; (2) precipitation with acetone and treatment with butanol; (3) fractionation with acetone between 0 and 45 % (v/v); (4) fractionation with ammonium sulphate; (5) adsorption of impurities on magnesium carbonate; (6) chromatography on calcium phosphate columns; (7) adsorption of impurities on activated carbon; (8) fractionation with ethanol between 50 and 65 % (v/v); (9) fractionation with ethanol between 54 and 62 % (v/v).

3. Chromatography on calcium phosphate allows of the separation of several active fractions, eluted by different concentrations of phosphate ion. The existence of more than one alkaline phosphatase in the kidney seems probable.

4. The most active product obtained liberates 166 000 μg. of phosphorus/min./mg. of protein nitrogen from sodium β-glycerophosphate under optimum conditions. Electrophoretic analysis shows that the preparation has a purity of 85–90 %.

5. The ultraviolet-light spectrum of the purified preparation reveals the presence of a substance with an absorption maximum around 260 mμ, which is not eliminated by dialysis at neutral or alkaline pH. Although the existence of an absorption maximum in the neighbourhood of this wavelength is characteristic of pyrimidine nucleotides, it is not clear if this represents a prosthetic group or an impurity extremely difficult to eliminate.

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