267. Calf Intestinal Alkaline Phosphatase I. Improved Isolation Method and Molar Composition of the Purified Phosphatase

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Summary

A modified and improved method for isolation of calf intestinal alkaline phosphatase is described. By this method 300 to 400 mg of pure enzyme was prepared in a relatively short time. On the basis of the results of ultracentrifugation and of the free, polyacrylamide and immunoelectrophoresis the phosphatase obtained is found to be a homogenous glycoprotein, containing firmly bound zinc, magnesium and phosphoric acid. The molar composition of the enzyme and the catalytic activity were determined with different substrates and buffers.

1. Introduction. – The affinity chromatography has been already introduced as an isolation procedure of the alkaline phosphatase of calf and human intestinal mucosa [1–4]. This method certainly simplifies the rapid preparation of very small amounts of highly purified phosphatase, but the poor capacity of the ligand-charged resins does not allow an easy isolation of larger quantities of pure enzyme. Furthermore, there is no sufficient experimental proof, that the phosphatase preparations isolated by affinity chromatography are homogenous proteins. It is even doubtful, that the nature of the employed ligands, all having an ion-exchange side-effect, and their rather low inhibitor specificity permit the isolation of a homogenous phosphatase. Theoretically none of these ligands can distinguish between the always present isoenzymes and phosphatase molecular variants easily produced during the solubilization of the membrane-bound enzyme. These enzyme variants have different sugar content and can, according to our experience, only be separated by the classical isolation methods. Our method, refined and tested during many years, opens a possibility with reproducibility.

2. Results. – By the method presented in the *Scheme* about 350 mg of pure alkaline phosphatase can be isolated from 20 kg of calf duodenal mucosa in a relatively short time.

The specific-activity increase of the main fractions (indicated with capital letters in the *Scheme*, the enzyme yield and the purification factors of each stage are listed in *Table 1*.

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Scheme. *Purification procedure*

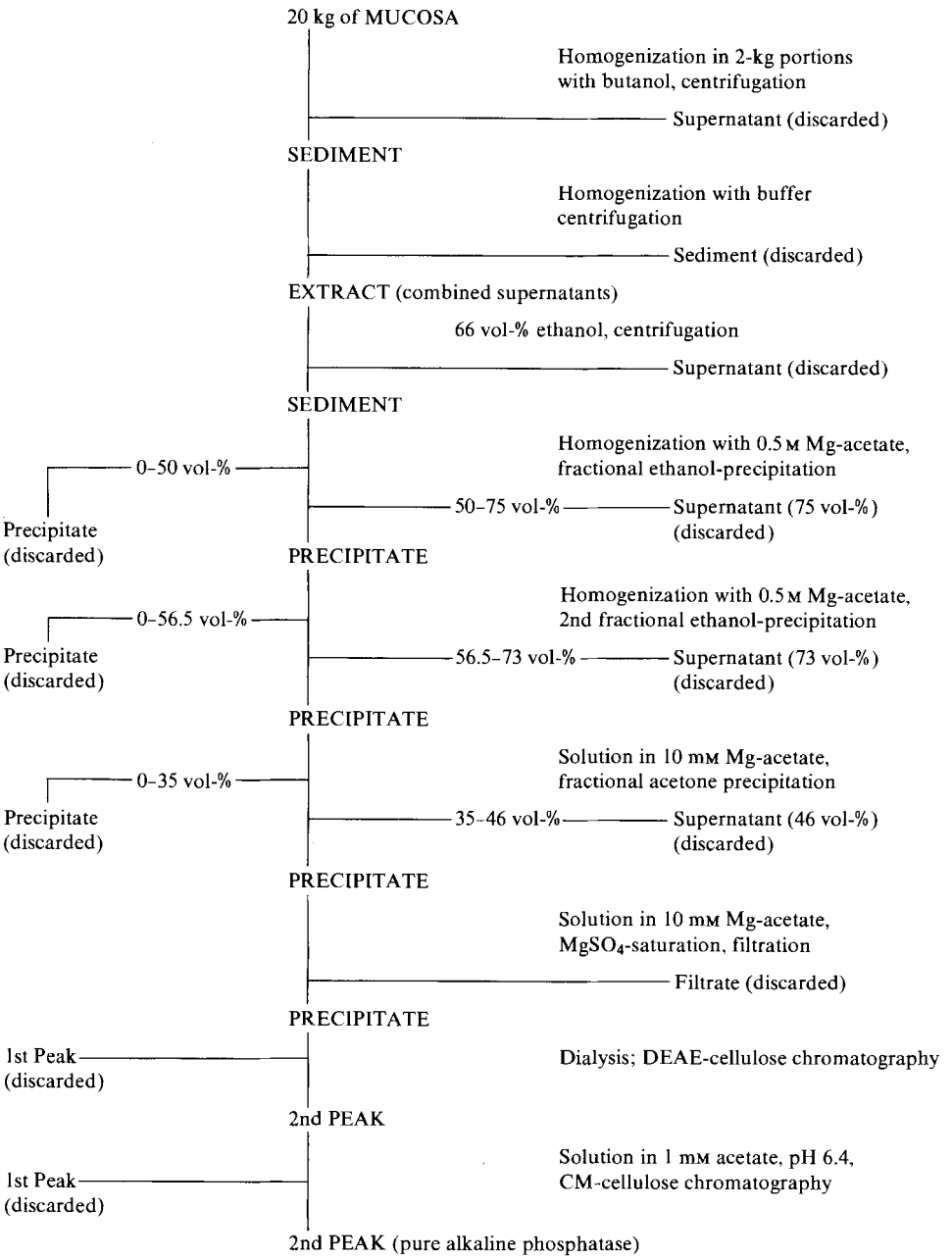

Table 1. *Purification of the calf intestinal alkaline phosphatase: mean values of 10 isolations each starting from 20 kg of mucosa*

Purification step	Volume [l]	Protein[1]) [g]	Total activity[2]) [U × 10^6]	Specific activity [U/mg]	Yield [%]	Purification factor
Homogenate	40	3975	1.51	0.38	100	1
Extract	19	703	1.35	1.85	90	5
Fractional precipitations:						
Ethanol: 66 vol-%	2.4	95	1.23	13	81	34
50–75 vol-%	0.5	21	1.17	56	77	147
56.5–73 vol-%	0.15	9	0.90	100	60	263
Acetone: 35-46 vol-%	0.05	3.9	0.80	205	53	540
$MgSO_4$-precipitation	0.1	2.1	0.76	362	50	952
DEAE-cellulose: 2nd peak	0.02	0.7	0.68	971	45	2555
CM-cellulose: 2nd peak	0.02	0.353	0.55	1555	36	4092

[1]) (Protein nitrogen) × 7.25. [2]) 1 U = 1 unit of enzyme activity = 1 µmol of *p*-nitrophenylphosphate (*p*-NPP) hydrolyzed/min at 25°. (1 M diethanolamine pH 9.8; 10 mM *p*-nitrophenylphosphate (*p*-NPP); 0.5 mM Mg-acetate).

The elution diagrams of the protein fractions and the phosphatase activity during the final chromatographic purification on DEAE- and CM-cellulose are given in *Figure 1* and *2*. The enzyme is firmly retained on the first resin, but only weakly on the second. Nevertheless the CM-cellulose chromatography could not be omitted because it turned out to be the unique method to separate completely

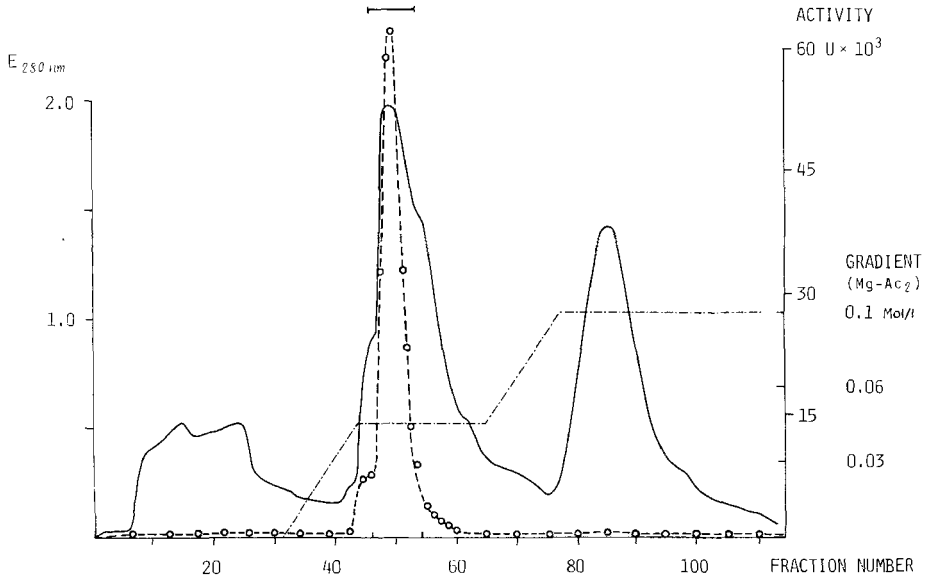

Fig. 1. *Elution profile of the DEAE-cellulose chromatogram* (——— protein; -o-o- phosphatase activity; -·-·- magnesium acetate gradient)

Table 2. *Results of the CM-cellulose chromatography*

Fraction	Hexoses	Hexosamines	Specific activity
1st Peak (CM_1)	38.8%	27.5%	208 U/mg
2nd Peak (CM_2)	6.5%	4.1%	1550 ± 150 U/mg

the last part of inactive or poorly active glycoproteins from the phosphatase. The comparison of the proteins present in the first (CM_1) and in the second (CM_2) peak of the CM-cellulose chromatogram (*Fig. 3* and *Table 2*) shows, that the first fraction is a very heterogenous mixture of glycoproteins with some phosphatase activity and it contains about seven times more protein-bound carbohydrates than in the second homogenous fraction CM_2.

The homogeneity of the CM_2-fraction was assessed by five different methods: the ultracentrifugation (sedimentation equilibrium[1]), *Fig. 4*) the free and polyacrylamide electrophoresis (*Fig. 5* and *6*) and the one- and two-dimensional immuno-

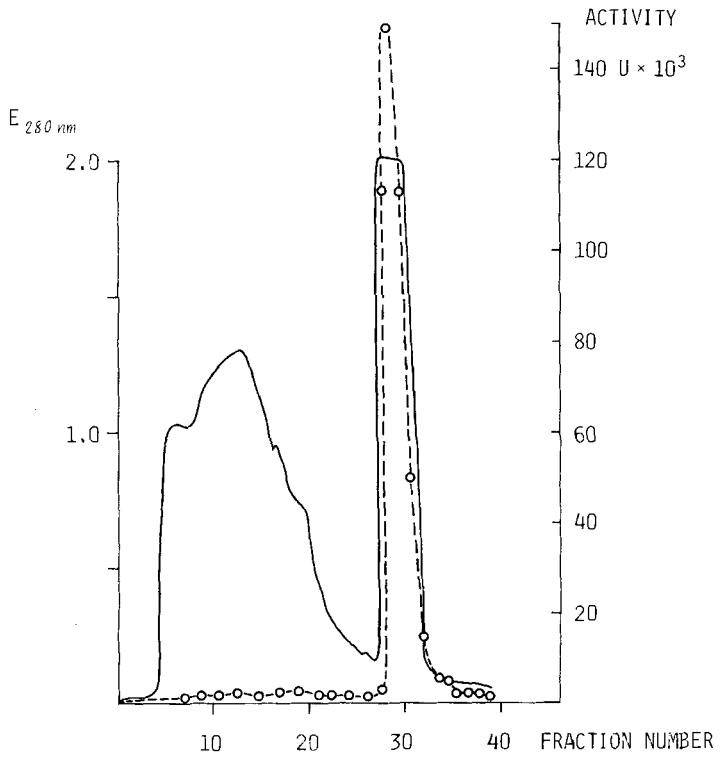

Fig. 2. *Elution profile of the CM-cellulose chromatogram* (——— protein; -o-o- phosphatase activity)

[1]) We thank Prof. *R. Schwyzer* and PD Dr. *J.-L. Fauchère*, Institut für Molekularbiologie und Biophysik, ETH Zürich, for this determination.

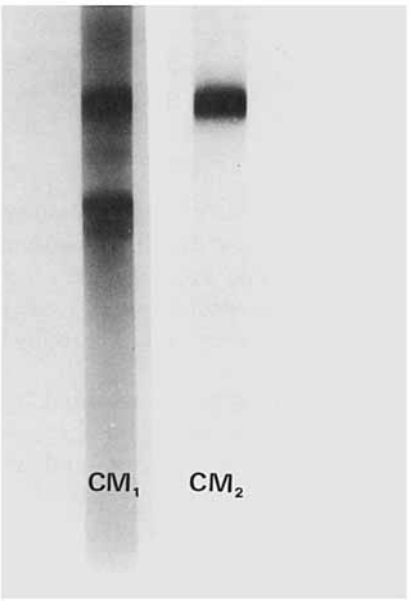

Fig. 3. *Polyacrylamide gel electrophoresis of the first and second elution peak of the CM-cellulose chromatogram* (100 µg of protein separated on a 7.5% polyacrylamide gel at pH 8.6 and stained with *Amidoschwarz 10B*)

electrophoresis (*Fig. 7* and *8*). All these methods indicated the presence of only one protein in the CM_2-fraction. The same degree of homogeneity was found in about 20 different enzyme preparations isolated previously by this method, indicating the good reproducibility of the method proposed. The molecular weight of CM_2 was determined by four different methods *(Table 3)*.

The mean molecular weight found by these four methods was 116500. In the presence of sodium dodecylsulfate (SDS) the polyacrylamide electrophoresis gave half the molecular weight, indicating the presence of at least two subunits. On the base of the analytical data and assuming a molecular weight of about 117000, we found the molar composition listed in the *Table 4*.

On the basis of different, separately isolated enzyme preparations, the uncertainity of the values obtained for amino-acid and sugar contents varied between

Table 3. *Molecular weight of the intestinal alkaline phosphatase (CM_2)*

Method	Molecular weight found (D)
Ultracentrifugation	110000 ± 2000
Polyacrylamide electrophoresis	125000
Polyacrylamide-SDS-electrophoresis (monomer)	58–62000
Sephadex G-200	110000
Osmometry	121000 ± 5000
Mean value:	116500 D

Table 4. *Molar composition of calf intestinal alkaline phosphatase*

A.	Compound	nmol found	Molar relation[a])	Residues/mol[b])
	Asp	177.3	114.2	114
	Thr	110.5	71.2	71
	Ser	81.9	52.7	53
	Glx	145.4	93.6	94
	Pro	86.3	55.5	55
	Gly	127.3	81.9	82
	Ala	166.3	107.1	107
	Val	131.7	84.8	85
	Cys/2	17.0	10.9	10[c])
	Met	39.4	25.3	25
	Ile	42.5	27.3	27
	Leu	111.5	71.8	72
	Tyr	59.0	38.0	38
	Phe	43.8	28.2	28
	His	40.7	26.2	26
	Lys	65.5	42.2	42
	Arg	61.9	39.9	40
	Trp[c])	12.8	8.2	8

B.	Compound	g-%	Molar relation[a])	Residues/mol[b])
	Hexoses	6.0%	39.0	39
	Hexosamine	4.1%	26.8	27
	Sialic acid	1.07%	4.1	4
	Zinc	0.23%	4.1	4
	Magnesium	0.085%	4.1	4
	Protein-bound phosphate	0.165%	1.98	2
	Amides (Asn + Gln)			70
	Nitrogen	13.8%[d])		8
	Cystin (S-S)[c])			4
	Cystein (SH)[c])			2

[a]) Tyrosin = 38 mol; [b]) Nearest entire giving a molecular weight of about 117000; [c]) Based on spectroscopic results; [d]) N-content calculated from the values indicated in the last column: 13.7%.

± 3 and 5%. This can be taken as a further indication of the enzyme homogeneity if the error limits of the analytical methods and the high molecular weight of the phosphatase are considered. Only the carbohydrate content of different preparations has an uncertainity of about $\pm 8\%$. Beside the technical difficulties of an exact determination of protein-bound carbohydrates, this variation is probably also due to the dependence of the phosphatase-sugar content upon the animal age. It was not always possible to obtain intestines from calves of the same age. However, the electrophoretic homogeneity and the specific activity were not influenced by this (relatively small) variation of the carbohydrate content.

The determination of the individual carbohydrates by GC., by HPLC. and by the amino-acid analyzer gave the results in *Table 5*.

The specific catalytic activity of the enzyme preparations (U/mg protein) was determined as indicated in *Exper. Part* and verified using commercial kits *(Merck, Boehringer)*. On the other hand the V_{max}/mg protein and the K_M-values of two

Table 5. *Molar carbohydrate content of the calf intestinal alkaline phosphatase*

Carbohydrate	Residues/mol		
	Amino-acid analyzer	GC.	HPLC.
Fucose	–	1	3
Mannose	–	12	12
Galactose	–	25	21
Glucose	–	1	+
Glucosamine	27	–	–
Galactosamine	1	–	–

substrates were determined on the base of the activity found with at least six different substrate concentrations. These parameters were calculated according to *Lineweaver & Burk* [5]. The results *(Table 6)* show the high specific activity of the enzyme preparations isolated by this purification method. The specific activity exceeded those so far described in the literature, except [4], where an activity of 2000 U/mg is reported after an enzyme activation by diethanolamine. *Table 6* also shows that the phosphatase activity is strongly influenced by the nature and the concentration of the buffer: 1 M diethanolamine activates, glycine inhibits the

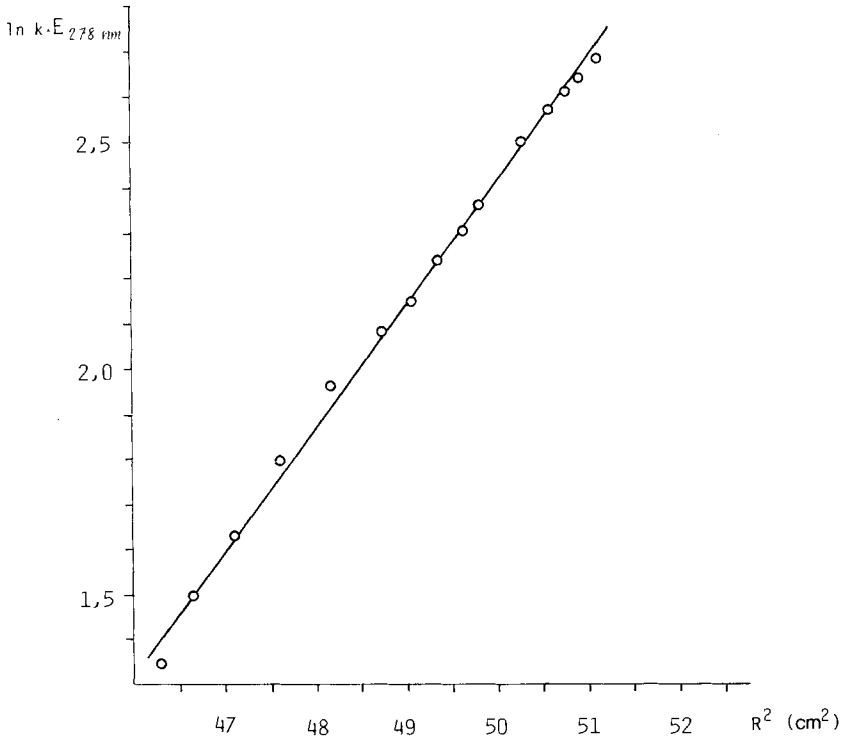

Fig. 4. *Ultracentrifugation* (sedimentation equilibrium) *of the phosphatase* (CM_1-fraction) ($\ln(kE_{278\,nm})$ *vs.* R^2 (cm^2); sedimentation: 6640 rev./min during 105360 sec at 21.5° in 0.05 M Tris-buffer, pH 7.5)

Table 6. *Specific activity of one calf intestinal alkaline phosphatase preparation in different buffer solutions at 25°*

Substrate	Buffer	pH	Mg (mM)	U/mg[a])	V_{max}/mg	K_M (mM)
p-NPP	1 M Diethanolamine/HCl	9.8	0.5	1554	1710[b])	0.7
p-NPP (diluted enzyme solution stabilized with albumine 1 mg/ml)	1 M Diethanolamine/HCl	9.8	0.5	1930	–	–
p-NPP	1 M Diethanolamine/HCl (Kit *Merck)*	9.8	0.5	1503	–	–
p-NPP	0.2 M Diethanolamine/HCl	9.8	0.5	1200	–	0.26
p-NPP	0.05 M Diethanolamine/HCl	9.8	0.5	1121	–	0.19
p-NPP	0.9 M 1-Amino-2-methyl-2-propanol/HCl (Kit *Boehringer*)	10.5	1	1204	–	12
p-NPP	0.1 M Tris/HCl	9.95	0.5	1430	–	0.36
p-NPP	0.1 M Glycine/NaOH	10.5	0.5	612	–	4.8
p-NPP	0.05 M Sodium barbital/HCl	9.95	0.5	1147	–	0.55
β-Glycerophosphate	1 M Diethanolamine/HCl	9.8	0.5	1531[c])	2000[d])	8
Bis(nitrophenyl)/phosphate	0.1 M Tris/HCl	9.95	–	0	–	–
Adenosine	0.1 M Tris/HCl	8	–	0	–	–
Adenosine	0.1 M Phosphate	6.5	–	0	–	–

[a]) Substrate concentration 10 mM; [b]) substrate concentrations used: 0.2, 1, 2, 3, 5 and 10 mM; [c]) substrate concentration 40 mM; [d]) substrate concentrations used: 1, 5, 10, 20, 30 and 40 mM.

enzyme. In 1 M diethanolamine buffer, pH 9.8, seven different enzyme preparations showed with *p*-nitrophenylphosphate (*p*-NPP) a mean specific activity of 1500 ± 150 U/mg with a V_{max} of approximately 1800 ± 100 U/mg.

The enzymatic purity of the phosphatase preparations was also established by the absence of contaminant enzyme activities, like that of phosphodiesterase and of adenosine deaminase, enzymes present in the intestinal mucosa.

The determination of zinc, magnesium and phosphoric acid was achieved using dialyzed phosphatase preparations. Therefore the values obtained prove that these three constituents are really bound to the enzyme protein.

Finally, the amount and the purity of these phosphatase preparations allowed us to start with the study of the amino-acid sequence. By the *Edman* degradation of the native enzyme we determined the following amino-terminal sequence: Leu-Gly-Pro-Thr-Asp-Val-Asn-Phe-×-Ala-...

3. Discussion. – Three main difficulties are encountered during the isolation of the calf intestinal alkaline phosphatase: the solubilization of the membrane-bound enzyme, the separation of contaminant glycoproteins from the phosphatase, itself a glycoprotein, and finally the removal of the always present isoenzymes and artificial enzyme variants, easily produced during the solubilization of the enzyme. *Morton* [6] solved the first problem by the treatment of the mucosa with butanol, the second and the third problems could be solved by the $MgSO_4$-precipitation and the CM-cellulose chromatography. We introduced this salt precipitation [7] eliminating a great part of the inactive glycoproteins, but lacking the inhibitory effect of the $(NH_4)_2SO_4$ frequently used [1] [2]. By the CM-cellulose chromatog-

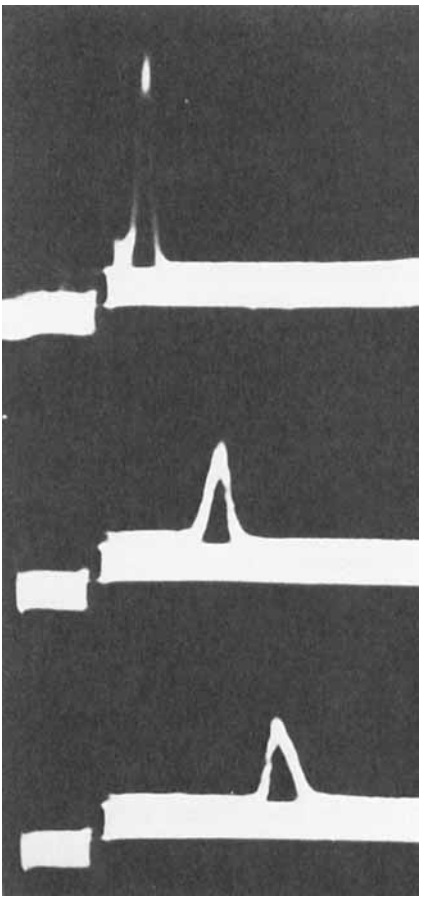

Fig. 5. *Migration of the calf intestinal alkaline phosphatase in the free electrophoresis* (*Boskamp* apparatus for free microelectrophoresis according to *Antweiler;* 6.5 mg of enzyme in 0.4 ml Na-barbital/HCl buffer (0.12 M; pH 9.5); 70 V and 1.8 mA; photographs taken after 3, 10 and 15 min)

raphy, not employed so far for the phosphatase purification, the last part of accompanying poorly active or inactive glycoproteins are separated if the following experimental conditions are very strictly maintained: the use of a short column, allowing a rapid chromatography (2–3 h) and exhaustive equilibration of the CM-cellulose by 1 mM Mg-acetate brought to pH 6.4 by a small amount of acetic acid (conductibility 3–4 μS). Under these conditions the poorly active glycoproteins are separated with the equilibration buffer; the phosphatase is retained and it can be eluted by 0.1 M Mg-acetate of pH 6.4.

An important criterion of purity is the highest attainable specific activity of the enzyme preparation. In the case of the purified calf intestinal alkaline phosphatase very different values (*p*-NPP; U/mg; 25°) have been reported: 1200 [1], 150–200 [3], 2000 [4] and 500 [8]. Besides various degrees of purity attained, these

differences are also due to the different enzyme-assay techniques employed and the strong dependence of the phosphatase activity from the pH-value and the type of buffer used. On the other hand, the exact determination of the protein concentration is difficult since the enzyme solutions are always very diluted. We preferred therefore to determine the protein nitrogen and to multiplicate this value by the factor 7.25. Separate determinations showed, that the N-content of pure alkaline phosphatase is 13.8%.

On this base, the pure intestinal alkaline phosphatase isolated by the method described in this paper showed at 25° a specific activity of 1500 ± 150 U/mg if

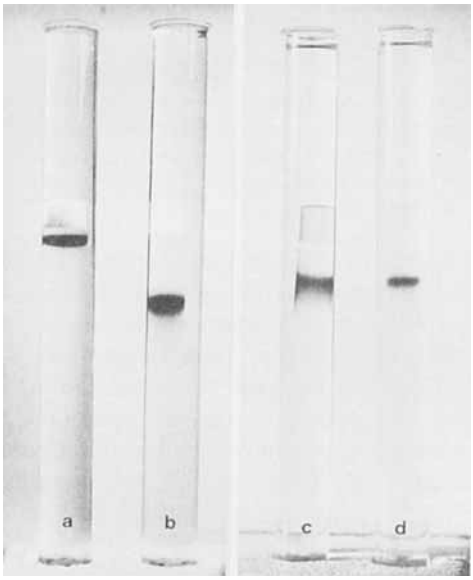

Fig. 6. *Polyacrylamide gel electrophoresis of the pure phosphatase preparation at two pH-values* (Gel: 7.5% polyacrylamide in Tris-buffer, pH 7.5 (*a*); pH 8.5 (*b*, *c* and *d*); migration 4 h at 140 V; gels stained with *Amidoschwarz 10B* (*a*, *b* and *d*). Specific stain of gel *c*: incubation during 3 min at 0° in a solution containing 25 mg of naphthylphosphate and 50 mg of *Fast Blue B* salt in 100 ml of 0.02 M Tris-buffer pH 8.5. Sample 70 μg in gels *a* and *b*, 20 μg in *c* and *d*)

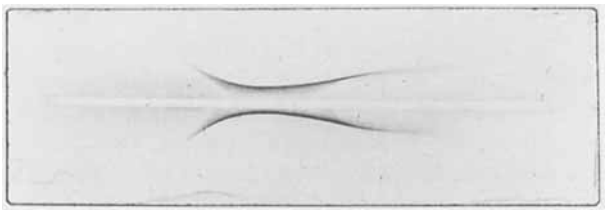

Fig. 7. *Immunoelectrophoresis of the pure phosphatase preparation on agarose gel* (0.8%) *at pH 8.6* (After the electrophoretic migration, the gel was incubated with a phosphatase antiserum (rabbit) obtained with a partially purified phosphatase preparation (450 U/mg). With the preparation used for the antibody production the immunoelectrophoresis gave two precipitation lines (not represented))

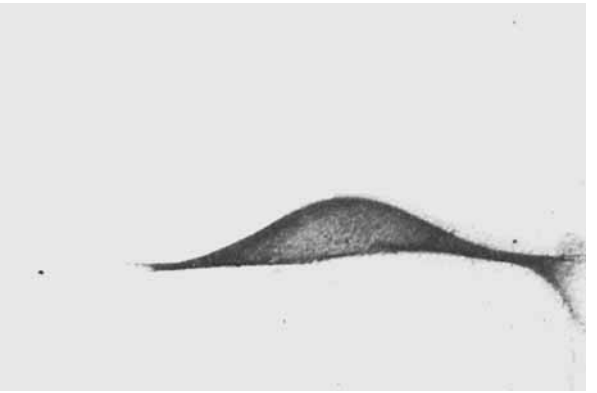

Fig. 8. *Two dimensional immunoelectrophoresis of a pure alkaline phosphatase preparation* (Sample 110 µg; electrophoresis: first direction (anode left), 40 min at 250 V in 0.8% agarose, pH 8.6; second direction (anode above) 15 h at 100 V in antiserum containing agarose (2 ml of rabbit antiserum + 38 ml of 0.8% agarose) in Na-barbital buffer, 0.1 M, pH 8.6; Stain: *Amidoschwarz 10B*)

p-NPP was used as substrate and 1 M diethanolamine (pH 9.8) containing 0.5 mM Mg-acetate as buffer. This value corresponds to the mean obtained with seven separately isolated, electrophoretically homogenous enzyme preparations. It is very difficult to evaluate the specific activity by a single standard determination with an accuracy $> \pm 10\%$. Therefore we have measured the activity with six different substrate concentrations and calculated the V_{max}/mg of enzyme by the method described in [5]. These values characterize the enzyme better than those obtained by a single determination with only one substrate concentration. Under these conditions the phosphatase preparations showed in 1 M diethanolamine-buffer values *ca.* 1800 U/mg with *p*-NPP and *ca.* 2000 U/mg with *β*-glycerophosphate. In nonactivating buffer systems the V_{max}/mg-values were 1350 ± 150 U/mg.

A careful determination of the Zn- and Mg-content in dialyzed preparations showed that the pure alkaline phosphatase contains not only Zn but also Mg firmly bound. Until now this was admitted only for the Zn. The isolation of the phosphatase from solutions containing Mg-salts increases the Mg-content to more than 10 g-atom per mol of the purified enzyme, but 4 g-atom of Mg remain tightly bound on the phosphatase, even if it is dialyzed during seven days against quartz distilled water at 4°.

We think that the determination of the carbohydrate content is an important criterion for the evaluation of the homogeneity of the intestinal phosphatase preparations, because the major contaminant proteins co-purified with the enzyme until the latest stages are the mucosal glycoproteins. Our pure preparations contained not more than about 6% of hexoses and 4% of hexosamines.

Finally we could also purify by this method the intestinal alkaline phosphatase of other species (rat, dog) and the renal alkaline phosphatase of the horse.

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Experimental Part

1. *Extraction.* Mucosa (20 kg) was scrapted from cautiously rinsed, 1–1.5 m long pieces of calf duodenum. It was treated in 2-kg portions, to which 1.8 l of butanol were added. After homogenization (20 min, *Polytron PT 45/50, Kinematica,* CH-6010 Kriens), the homogenate was centrifuged (2000 × g, 30 min), the butanol was decanted and the sediment was homogenized *(Polytron PT 45/50)* during 10 min with 2 l of buffer (100 mmol of Na-acetate and 10 mmol of Mg-acetate/l; pH 9 by addition of NaOH). The homogenate was centrifuged (3 to 4 h, at not less than 3000 × g, 4°), the turbid supernatant was decanted, and the sediment was discarded. The ten supernatants resulting from 20 kg of mucosa (about 19 l extract) were combined. Two volumes of abs. ethanol denatured with 4% CH_3OH were added with good stirring. After spontaneous sedimentation of the precipitate during the night, the clear supernatant was siphoned off and the residue was centrifuged (1500 × g; 20 min). The sediment was homogenized with 2.5 l of 0.5 M Mg-acetate *(Polytron PT 45/50)* and the homogenate was centrifuged (9500 × g, 60 min at 4°; *Sorvall,* rotor *GS-3*). The supernatant was decanted, and the sediment was discarded.

2. *Fractional precipitations.* The protein concentration of the supernatant was brought to 13 mg/ml (2 mg nitrogen/ml) by addition of 0.5 M Mg-acetate. The solution was thoroughly and mechanically stirred, and an equal volume of C_2H_5OH was added very slowly; 30 min later, the sediment (0–50% ethanol) was centrifuged (1500 × g, 20 min) and discarded. The supernatant was vigorously stirred, and C_2H_5OH was dropped in until a concentration of 75 vol-% was attained. The centrifuged precipitate (50–75 vol-%) was homogenized with 500 ml of 0.5 M Mg-acetate *(Polytron PT 45/50)*, and the turbid solution was centrifuged (48000 × g, 2 h, 4°; *Sorvall,* rotor *SS-34*). The supernatant was diluted with 0.5 M Mg-acetate to adjust the protein concentration to 10 mg/ml (1.4 mg nitrogen/ml). To this solution C_2H_5OH was added slowly by vigorous mechanical stirring until the alcohol concentration was brought to 56.5 vol-% (the last portion of C_2H_5OH must be added drop by drop). The sediment was centrifuged (1500 × g, 30 min) and discarded. With vigorous stirring, the C_2H_5OH-concentration of the decanted supernatant was brought to 73 vol-%. The enzyme-rich sediment (56.5–73 vol-%) was centrifuged and dissolved in 150 ml 10 mM Mg-acetate. A small turbidity was centrifuged off (48000 × g, 30 min), and the protein concentration of the clear supernatant was brought to 20 mg/ml (2.75 mg nitrogen/ml) by addition of 10 mM Mg-acetate. Then, acetone was slowly added with vigorous stirring (final concentration = 35 vol-%). The poorly active sediment was centrifuged and the acetone concentration of the decanted supernatant was increased to 46 vol-%. The precipitate, containing most of the enzyme, was centrifuged and dissolved in 50 ml of 10 mM Mg-acetate.

The protein concentration was brought to 29 mg/ml (4 mg nitrogen/ml) by addition of 10 mM Mg-acetate, then, $MgSO_4$ (1.3 g of $MgSO_4 \times 7\,H_2O$/ml) was added, and the resulting mixture was stirred (3–4 h) at r.t. to reach complete saturation and a fine floculation of the protein precipitate. The precipitate was filtered off without suction through a sintered glass filter *(Schott 25 D-3)*. If the first parts of the filtrate were turbid they were poured on the filter again until the filtrate remained clear. Salt cristallization was avoided by covering the filter with a watch glass and by filtering at 37° (temperature-controlled oven). The precipitate was washed on the filter with 10 ml of saturated $MgSO_4$-solution, the filtrate was discarded, and most of the precipitate was transferred to a beaker. The precipitate remaining on the filter was dissolved by eight to ten successive filtrations of 10 ml of dist. water. Each of these filtrates was transferred to the beaker, so that the main precipitate was finally dissolved in 80–100 ml. After homogenization *(Polytron PT 10/35)* the turbid solution was centrifuged (13500 × g; 4°, 2 h), the clear supernatant was decanted and dialyzed at 4° (*Visking* dialysis tubing) first against water, then against 10 mM Mg-acetate containing 1 μmol of Zn-acetate/l.

3. *Chromatographic separations.* The dialyzed, sulfate-free enzyme solution was precipitated with 2 volumes of C_2H_5OH, the centrifuged precipitate was dissolved in 150 ml of a 10 mM Tris-buffer (pH 8.6) containing 5 mmol of Mg-acetate/l (conductibility 21 to 24 μS). Two parallel chromatograms were performed, each with 75 ml. They were applied to a DEAE-cellulose column (13 × 2.5 cm; *Whatman DE 52* microgranular) equilibrated completely with a 10 mM Tris/5 mM Mg-acetate buffer, pH 8.6. The same buffer was passed through the column until the first protein peak was completely eluted (UV.-monitoring). Then a linear Mg-acetate gradient (5 to 70 mM Tris buffer, pH 8.6 was applied *(Gilson Mixograd)*. As soon as the second protein peak started to appear, the increase of the gradient was stopped, and the peak was eluted under isocratic conditions (about 38 mM Mg-acetate). The fractions (4 ml) corresponding to the second peak were combined, the enzyme was precipitated

by addition of two volumes of C_2H_5OH (−20°) and centrifuged. The sediment was dissolved in 20 ml of 1 mM Mg-acetate. This solution was applied to a CM-cellulose column (8×2.2 cm; *Whatman CM-32* microgranular) thoroughly equilibrated with a 1 mM Mg-acetate/acetic acid buffer, pH 6.4 (conductibility 3–4 μS). With the same buffer a first, poorly active protein peak was eluted, then 0.1M Mg-acetate, brought to pH 6.4 by addition of acetic acid (120–130 μS), was applied. The pure phosphatase was eluted in a second symmetrical peak. The 4-ml fractions corresponding to this peak were combined, the enzyme was precipitated at −20° by addition of 2 volumes of C_2H_5OH. The centrifuged precipitate was dissolved in 20 ml of 10 mM Mg-acetate containing 1 μmol of Zn-acetate/l. The solution was kept frozen at −20°. At each purification stage the enzyme activity and the N-content were determined.

4. *Enzyme assay.* – 4.1. *With* p-*nitrophenylphosphate (p-PNP)*×*6 H_2O* (Fluka). Kinetic measurements were made according to [9] [10]: 20 μl of the diluted enzyme solution, containing 0.1 to 0.6 μg of phosphatase per ml of 10 mM Mg-acetate, were incubated in 2 ml of substrate-buffer solution at 25°. The extinction was registered every minute during 5 min. E^{mM}_{405} of *p*-nitrophenol in 1M diethanolamine, pH 9.8, was 17.9 (*Eppendorf* photometer). The activity of the chromatographic fractions was determined in the same way, but the enzymatic reaction was stopped after 5 or 10 min by the addition of 2 ml 1M NaOH containing 1 g of EDTA/l. With the same substrate (10 mM) other enzyme assays were prepared using the following buffers: glycine/NaOH (50 mM, pH 10.5), Tris/HCl (0.1M, pH 9.95) and Na-barbital/HCl (50 mM, pH 9.95). The activity was also determined with the commercial kits of *Merck* (No. *3356*) and of *Boehringer* (No. *396494*).

4.2. *With β-glycerophosphate.* In Na-barbital/HCl or diethanolamine buffer 4 ml of 40 mM β-glycerophosphate were incubated during 5 or 10 min at 25° with 1 ml of dil. enzyme solution. The reaction was stopped by the addition of 1 ml 25% trichloroacetic-acid solution (3 ml in the case of 1M diethanolamine). The liberated phosphate was determined after neutralization with NaOH 2N (indicator: *p*-nitrophenol) by addition of 1 ml 5% ammonium-molybdate in 6N H_2SO_4 and 0.5 ml of 0.2% metol-solution in 25% $K_2S_2O_5$-solution; exactly 15 min later 2 ml of 2.5M Na-acetate were added, the optical density was measured at 578 nm and compared with that given by phosphate standard solutions. *Nitrogen* was determined in mineralized samples containing 20 to 30 μg nitrogen by the method of *Stegemann & Loeschke* [11]. The *protein* concentration was calculated from the N-content by multiplication with the factor 7.25.

The determination of the protein-bound phosphoric acid was achieved by the [(molybdate-*Triton*) × 100]-method [12] after mineralization with HNO_3/H_2SO_4 or by alkaline fusion. *Zn* and *Mg* were determined by atom absorption photometry *(Zeiss FMD 3). The determination of the SH- and SS-groups* was achieved according to *Grassetti & Murray* [13] and to *Cavallini et al.* [14], that of tryptophan according to *Spande & Witkop* [15]. *The amides* (asn and gln) were determined in 0.5 to 1 mg of phosphatase according to *Stegemann* [16]. *Amino-acid determination:* 1 to 2 mg of phosphatase were hydrolyzed at 110° with 6N HCl under N_2 during 24, 36 and 72 h. The amino acids were analyzed in an amino-acid analyzer *Biocal BC-200.* Norleucine (0.25 μmol) was used as an internal standard. *The electrophoresis* on polyacrylamide (7.5, pH 8.6) were performed according to *Maurer* [17], the one- and two-dimensional immunoelectrophoresis on agar gel (0.8%, pH 8.6) according to *Scheidegger* [18] and *Laurell* [19] with rabbit antiserum. *The free electrophoresis* was performed with a *Boskamp* apparatus (type *Antweiler*). *The molecular weight* was determined according to *Lambin et al.* [20] and *Weber et al.* [21] by molecular-exclusion chromatography *(Sephadex G-200, Pharmacia)* and by osmometry (membrane osmometer *Knauer*). *The carbohydrate content* was determined by the anthrone method [22], individual sugars according to [23] and by HPLC. [24], the hexosamines with the amino-acid analyzer *(BioCal BC-200)* and photometrically according to *Elson & Morgan* [25], *the sialic acid* by the method of *Aminoff* [26]. *The amino-acid sequence* was determined with the *Edman* procedure according to *Schroeder* [27] using the native phosphatase. The liberated PTH-aminoacids were identified by TLC. and HPLC. [28].

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