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# ALKALINE PHOSPHATASES OF THE CHICKEN DUODENUM

## I. ISOLATION AND PARTIAL CHARACTERIZATION OF THE MULTIPLE FORMS OF DUODENAL PHOSPHATASE IN PRE- AND POST-HATCHING STAGES

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SUMMARY

1. Butanol-solubilized alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) from chicken duodenum has been resolved into several isozymic forms: (1) Two forms (F) that migrate fast in starch gel and have molecular weights of about 200 000; $F_1$ migrates slightly further than $F_2$. (2) A polydisperse series of molecules (S) that move slowly in starch gel; they vary in molecular size from slightly larger than the F-forms to a size completely excluded from Sepharose 6B. The proportion of F- and S-phosphatase obtained from butanol extracts is age-dependent, the F-enzyme constituting about 20% of the extracted activity in the 20-day embryo, but more than 55% in post-hatching stages.

2. The isolated S-complex does not yield additional F-enzyme on a second extraction with butanol. $F_1$ can be transformed into $F_2$ by desialation with neuraminidase.

3. Purified $F_1$, $F_2$ and S are virtually indistinguishable in several catalytic properties. They do however differ in $Na_2EDTA$ sensitivity and thermal stability, and to some extent in susceptibility to inactivation by acid or urea.

## INTRODUCTION

The alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) of the vertebrate intestine is an integral part of the outer membrane of the microvilli that covers the lumenal surface of the absorptive epithelium[1-3]. Purification of the enzyme requires the disruption of the membrane in order to release the enzyme molecules into solution. Solubilization of the enzyme is generally accomplished by the use of *n*-butanol[4], which is thought to rupture the lipoprotein linkages that bind the phosphatase molecules to the membranous structure. Butanol-extracted phosphatase is almost always heterogeneous.

The significance of this heterogeneity is not understood. In the duodenum of the mouse, a series of alkaline phosphatase isozymes is distributed in linear order from the tips to the bases of the villi[5]. This indication that the isozymes differ in their functional roles is supported by the fact that new isozymes appear around the time of weaning, when there is a strong upsurge of duodenal phosphatase activity[6,7]. In the duodenum of the chicken a similar increase in alkaline phosphatase activity just before and after hatching[8] has been reported to involve an increased preference for phenylphosphatase over $\beta$-glycerophosphate as substrate[9], a fact which might reflect the appearance of a new form of the enzyme. This paper reports the results of a further investigation of this situation, in which we have found that the chicken duodenum contains two related phosphatases of relatively small molecular size, plus a polydisperse series of larger molecules; the latter can be converted into the former by appropriate enzymic treatment[10,11].

## MATERIALS AND METHODS

### *Animals*

A hybrid line of White Leghorn chickens (Kimber 137) was used throughout this study. Eggs were kept in a forced draft incubator at 38°, humidity 70%. Embryos were used after 20 days of incubation. Hatched chicks were given access to water but were not fed; they were sacrificed at 3 or 4 days. 20-week pullets were maintained in the laboratory for 1 day after arrival, with water but no food.

### *Preparation of enzyme extract*

Embryos and chickens were decapitated. The whole duodenal loop was excised, and the attached pancreas and mesentery were quickly stripped off. Each loop was blotted and stored at 0° until all were collected. From 20-day embryos, 265 loops weighing 50 g were collected; from 3–4 day chicks 96 loops weighing 38 g were collected, and from 20-week pullets 10 loops weighing 65 g. After freezing and thawing 6–10 times, each duodenal pool was homogenized in a Waring blendor, then diluted with double distilled water to a final tissue concentration of 150 mg/ml. The homogenate was stirred with an equal volume of *n*-butanol at room temperature for 1 h, followed by heating to 40° for 10 min. The solubilized enzyme in the water phase was recovered after centrifugation at 4080 $\times g$ for 15 min; it was then dialyzed against distilled water at 2–4° for 60–70 h. After concentration by ultrafiltration in cellulose acetate tubing (Union Carbide Co.), the solution was centrifuged at 109 000 $\times$ *g* for 1 h to remove any contaminating tissue particles. The supernatant was stored at −15 to −20°.

### *Gel filtration and DEAE-Sephadex chromatography*

Sepharose 6B (Pharmacia) was equilibrated with 0.01 M Tris–HCl, pH 8.0, containing 0.005 M $MgCl_2$. Downward-flow columns of various sizes were used. The columns were packed in the cold room, and the flow rate was adjusted to about 2 ml/cm$^2$ per h. Void volumes ($V_0$) of the columns were determined with blue dextran 2000 (Pharmacia) by reading at 625 nm in a Beckman DU spectrophotometer.

DEAE-Sephadex A25 (Pharmacia) was equilibrated with 0.01 M Tris–HCl, pH 8.0, containing 0.005 M $MgCl_2$. The columns were packed in the cold room, and washed with buffer overnight before use.

*Electrophoresis in starch gel and Geon-421*

Horizontal starch-gel electrophoresis was run at 2–4° with a discontinuous buffer system[12]. The enzyme samples were applied to 4 mm × 5 mm pieces of Whatman 3MM paper, which were then inserted into slots in the gel. Electrophoresis was continued for 8–10 h with a voltage drop of 18 V/cm at 15–20 mA. The gels were sliced horizontally and stained for phosphatase activity at room temperature in a solution containing 0.05% sodium β-naphthyl phosphate, 0.05% Fast Blue BB, 0.125 M sodium barbital, and 0.001 M $MgCl_2$.

For preparative purposes, the enzyme sample was mixed with Geon-421 (a high molecular weight copolymer of polyvinyl acetate and polyvinyl chloride; B. F. Goodrich Chemical Co., Cleveland, Ohio) that had been suspended in 2 M HCl overnight, washed with distilled water until neutral, and equilibrated with the buffer. The enzyme–Geon mixture was applied in a 7 cm × 2 cm × 0.3 cm slot cut 3 cm from the cathode end in a starch gel measuring 26 cm × 9 cm × 3 cm. Electrophoresis was carried out for 23 h at 400 V and 20 mA in the cold room. The gel was then sliced horizontally into halves, and enzyme bands were located by applying staining medium to the cut surface of the gel. The enzyme-containing strips were cut out and then enclosed between a pair of buffer-equilibrated beds of geon built up to the same height and width as the starch. Current of 400 V and 10 mA was again applied, so that the enzyme was driven into one of the geon beds. The enzyme was then extracted from the geon with distilled water, and concentrated by ultrafiltration. This procedure takes advantage of the fact that the enzyme does not bind to Geon-421.

*Determination of enzyme activity and protein content*

Phosphatase activity was determined with phenyl phosphate as substrate according to the technique of KING AND ARMSTRONG[13], in which released phenol is measured with the Folin–Ciocalteu reagent. Optimal conditions, established with butanol extracts, proved similar at all stages used. The reaction mixture therefore consisted in all cases of 1.25 ml of 0.2 M $Na_2CO_3$–$NaHCO_3$ buffer, pH 10.5, 0.25 ml of 0.1 M $MgCl_2$, 1.0 ml of 210 mM disodium phenylphosphate (Sigma Chemical Company), 0.4 ml of double distilled water, and 0.1 ml of enzyme preparation. The reaction was run at 37.5°, at which it is linear with time for at least 1 h; it was stopped after 10–30 min by addition of 1 ml of diluted Folin–Ciocalteu reagent, followed by 1.6 ml of 20% $Na_2CO_3$. After color was allowed to develop for 15 min at 37.5°, the tubes were read in a Klett–Summerson colorimeter with a 540 nm filter. Protein content was determined by the method of LOWRY *et al.*[14].

*Neuraminidase digestion of phosphatase samples*

The phosphatase preparation (50 μl containing 45–60 μg protein) was incubated with 100 μl of neuraminidase (*Vibro cholerae*, 50 units activity, Calbiochem Company), 10 μl of 0.01 M $MgCl_2$, 10 μl of 0.01 M $CaCl_2$ and 50 μl of 0.4 M sodium acetate buffer at pH 5.3. In controls, the neuraminidase was replaced with an equal volume of buffer. After incubation at 37.5° for 12–24 h, the digests were subjected to electrophoresis without further treatment.

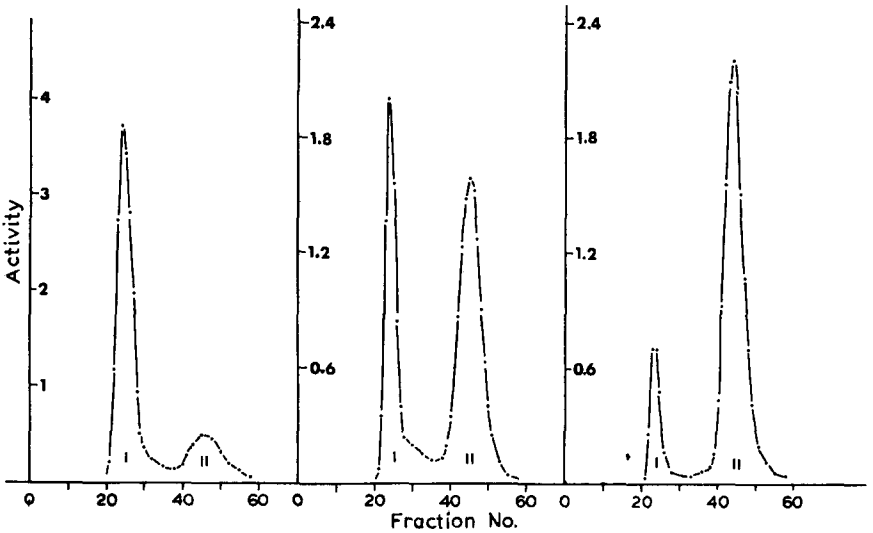


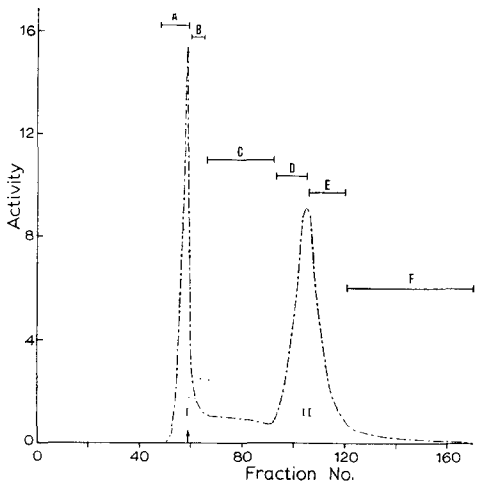

Fig. 1. Sepharose 6B chromatograms of butanol extracts of duodenum from 20-day chick embryo (left), 3–4 day chick (middle) and 20-week pullet (right). 50 $\mu$l of extract containing 30–50 $\mu$g protein was applied to a 0.9 cm × 28.5 cm column and eluted at 1.8–2.0 ml/cm$^2$ per h. 0.26-ml fractions were collected. Activity is expressed as Klett units per 0.01 ml per 30 min. About 90% of activity applied to the column was recovered in all cases. In each test the apex of Peak I was at Fraction 24, which overlapped the blue dextran peak; apex of Peak II was at 45. Sum of activity of Fractions 20–35 was taken as Peak I activity, that of Fractions 36–58 as Peak II activity; these were expressed as percent of total activity recovered. For 20-day embryo: Peak I = 77.1%, Peak II = 22.9% with $V_e/V_o$ = 1.87. For 3–4-day chick: I = 41.0%, II = 59.0% with $V_e/V_o$ = 1.86. For pullet: I = 17.0%, II = 83.0% with $V_e/V_o$ = 1.84.

## RESULTS

### *Phosphatase variants in the duodenum in embryonic and post-hatching stages*

When butanol extracts of duodena of 20-day embryos, 3–4-day-old chicks or 20-week pullets were subjected to filtration on a Sepharose 6B column (0.9 cm × 28.5 cm) under similar conditions, two peaks of phosphatase activity appeared (Fig. 1). In each case Peak I is excluded, but Peak II has a $V_e/V_o$ value of 1.84 to 1.87. Approx. 90% of the activity applied to the column was recovered. The distribution of total activity between the two peaks varied according to the age of the animals. In the extracts reported in Fig. 1, the activity in Peak II constituted only 23% of the activity recovered from the column with 20-day embryo material, but 59% and 83%, respectively, with 3–4-day chick and 20-week pullet material. With extracts of other batches of duodenal tissue, these percentages varied somewhat, but there was always a substantial increase in proportion of activity in Peak II with increasing age.

If butanol extract of 3–4-day chick duodenum was chromatographed on Sepharose 6B in a larger (2.5 cm × 89 cm) column, the amount of activity eluted in Peak II was not much changed (54%), but the two peaks were further separated (Fig. 2). When several series of consecutive fractions from this column were pooled, concentrated and subjected to electrophoresis on starch gel, a relationship between the molecular sizes of the enzyme variants and their electrophoretic mobilities appeared



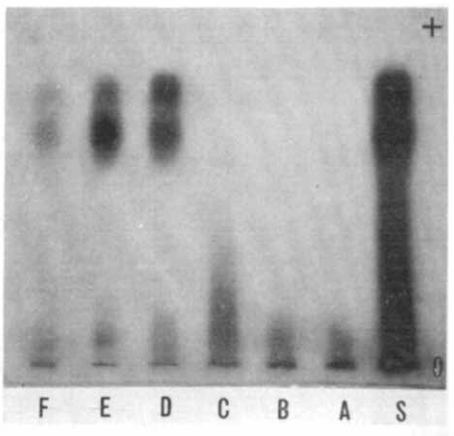




Fig. 2. Sepharose 6B chromatogram of extract of 3–4-day chick duodenum. 100 $\mu$l of concentrated extract containing 900 $\mu$g protein was applied to a 2.5 cm × 89 cm column and eluted at 15 ml/h. 2.5-ml fractions were collected. Activity is expressed as Klett units 0.1 ml per 10 min. 90% of applied activity was recovered. Peak I (Fraction 59, arrow) overlapped the blue dextran peak. Peak I (Fractions 50–65) contained 30% of the activity recovered and Peak II (Fractions 92–121) contained 54%. About 11% of the total activity was between the peaks (Fractions 66–91), and 5% in the trailing edge beyond Fraction 121. A–F represent fractions pooled for electrophoresis (Fig. 3).

Fig. 3. Zymogram showing electrophoretic mobilities of pooled fractions of duodenal phosphatase as indicated on Fig. 2. Pooled samples were concentrated before being placed on starch. S, crude extract before chromatography. O, origin. All active components migrate toward anode.

(Fig. 3). The excluded peak was found to contain only phosphatase of rather low mobility, which remained at or near the origin, and the trough region between the two peaks contained additional components of somewhat higher mobility than those in the excluded peak. The second peak however yielded two prominent fast-moving forms, although a small amount of slow-migrating material was also present. The fast migrating forms are apparently of similar molecular size, as both are eluted in one symmetrical peak (Fig. 2).

The two fast-moving phosphatases found in butanol extracts of chick duodenum we designate $F_1$ and $F_2$, $F_1$ being the variant that migrates furthest. The slow-migrating polydisperse series of enzymatically active molecules we designate S. Both S- and F-forms are present before and after hatching, but not in the same proportions. In a molecular weight determination in which bovine hemoglobin, rabbit $\gamma$-globulin, bovine catalase, and bovine thyroglobulin were used as markers in a Sepharose 6B column, $F_2$ gave a $V_e/V_o$ value slightly lower than that of catalase, indicating a mol. wt. of about 200 000. S-phosphatase evidently comprises a series of molecules ranging from slightly larger than F to a size that is excluded from Sepharose 6B.

*Isolation of $F_1$-, $F_2$-, and S-forms on a preparative scale*

The starting material was a butanol extract of 3–4-day chick duodenum; this extract contained 54% F-enzyme, and 30% excluded S-enzyme (Fig. 2). The sample

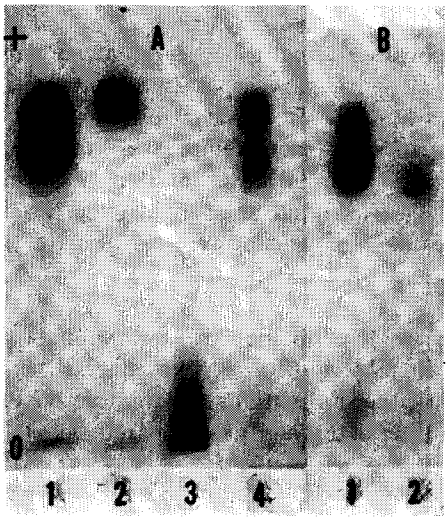



Fig. 4. Zymograms of purified preparations of duodenal phosphatases. (A) Slot 4, F-enzyme and Slot 3, S-enzyme, both after rechromatography on Sepharose 6B; Slot 2, $F_1$-enzyme and Slot 1, $F_2$-rich fraction, both after preparative electrophoresis on starch gel. (B) Slot 1, partially purified F-enzyme; Slot 2, $F_2$-enzyme purified by chromatography on DEAE-Sephadex. O, origin.

used contained 45 mg protein, with specific activity sufficient to liberate 303 mg phenol from phenyl phosphate per mg protein per 30 min. This material was first placed on a 2.5 cm × 90 cm Sepharose 6B column under conditions described previously. The fractions of the excluded S-peak and those of the included F-peak were pooled separately, the trough region between the two peaks being discarded. The recovered S- and F-enzymes were rechromatographed under the same conditions. Fig. 4A (Slot 3) shows that the S-phosphatase thus isolated is completely free of F-forms; its activity was 2558 mg phenol per mg protein per 30 min, a 150-fold increase over the activity of the original homogenate.

The F-enzyme fraction (about 15 mg protein in 2 ml) recovered from the second Sepharose 6B column was subjected to electrophoresis in a large starch gel, and subsequently recovered from Geon-421, as described under MATERIALS AND METHODS. When the 2 fractions thus obtained were examined electrophoretically, the $F_1$-preparation proved to be devoid of $F_2$ activity (Fig. 4A, Slot 2); the specific activity of this $F_1$-fraction was 7423 mg phenol per mg protein per 30 min, 436 times that of the original homogenate. The other preparation was rich in $F_2$, but also contained a substantial amount of $F_1$ (Fig. 4A, Slot 1).

Preliminary experiments with DEAE-Sephadex had shown that chromatography on this material would not resolve the S-phosphatase into discrete forms; it did however prove useful in separating the F-forms. The $F_2$-rich fraction obtained with the use of Geon-421 was therefore applied to a DEAE-Sephadex column (0.9 cm × 24 cm). After washing with 600 ml of 0.02 M NaCl to remove contaminating non-phosphatase protein, the $F_2$-enzyme was recovered by elution with 600 ml of 0.04 M NaCl. Fig. 4B (Slot 2) shows that the purified $F_2$ thus obtained was apparently devoid of $F_1$ and S; its activity was 9571 mg phenol per mg protein per 30 min, a 563-fold increase in specific activity above that of the original homogenate.

*Butanol resistance of S-phosphatase*

To examine the possibility that the S-variant might be phosphatase still associated with membrane fragments, 0.1 ml of the purified S-preparation (containing 25.7 μg protein) was mixed with 0.1 ml of *n*-butanol. In the control, water was substituted for butanol. The mixtures were shaken at room temperature for 1 h, heated at 40° for 10 min, and then dialyzed against distilled water for 48 h at 4°. On electrophoresis the butanol-treated S-phosphatase was found to be slightly increased in mobility compared to the control, but no F-like components were released.

*Neuraminidase sensitivity of F-phosphatase*

When purified $F_1$- and $F_2$-preparations were incubated with neuraminidase individually under identical conditions and then subjected to electrophoresis, it was observed that the $F_2$-variant did not change in either mobility or apparent activity. The mobility of $F_1$ was however reduced to that of $F_2$, with no apparent loss of activity (Fig. 5).

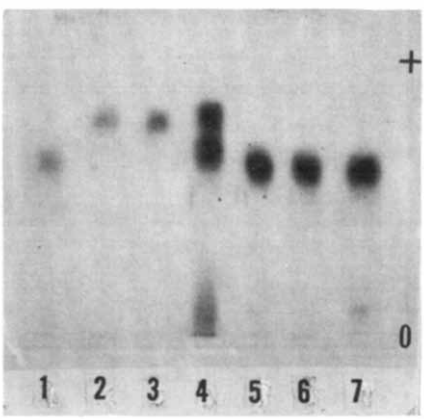



Fig. 5. Effect of neuraminidase on purified F-phosphatases. Slot 1, $F_1$-phosphatase after incubation with neuraminidase; Slot 2, $F_1$ control after incubation with buffer; Slot 3, untreated native $F_1$; Slot 4, crude unfractionated extract; Slot 5, untreated purified $F_2$; Slot 6, $F_2$ buffer control; Slot 7, $F_2$ after incubation with neuraminidase.

To identify the material liberated from $F_1$-enzyme after neuraminidase digestion, the absorption spectra of the chromogens produced by pure synthetic sialic acid and by an aliquot of $F_1$-digest were examined with a Beckman DU spectrophotometer at 510–585 nm by AMINOFF's[15] thiobarbiturate procedure scaled down to 1/10 volume. The substance released from $F_1$ proved to have the same absorption maximum (549 nm) as the pure sialic acid. The amount of sialic acid removed by neuraminidase was 3.65 μg/mg $F_1$-protein.

*Catalytic properties of the purified phosphatase variants*

The purified $F_1$-, $F_2$- and S-enzymes were dialyzed against 0.01 M Tris–HCl buffer at pH 7.8 before being used in kinetic analyses. Except where otherwise specified, tests reported below were run at 37.5°. The results of these studies have been reported in detail[16].

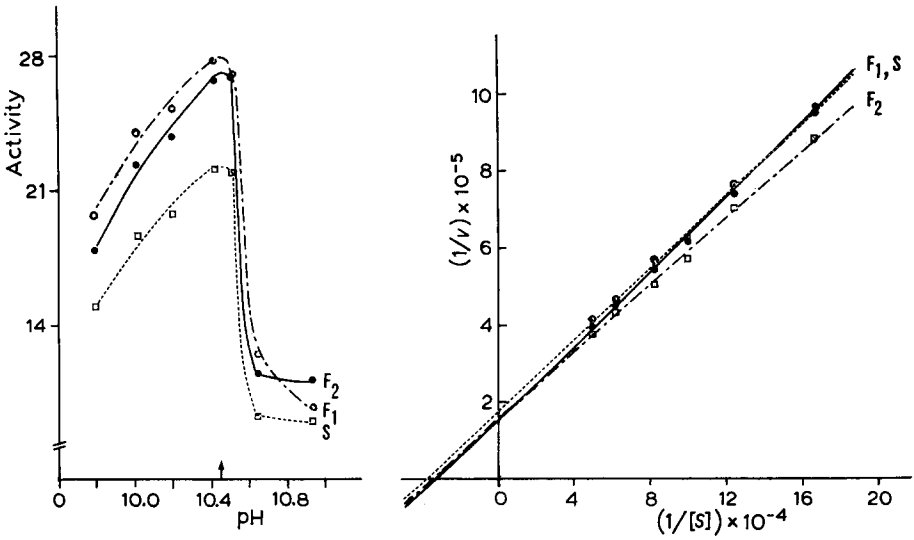



Fig. 6. pH optima of phosphatase isozymes acting on 70 mM phenyl phosphate with 8.33 mM $MgCl_2$. 0.2 M carbonate–bicarbonate buffer was used. Reactions were run for 30 min at 37.5°. Arrow indicates pH optimum of 10.45 for all 3 forms of the enzyme.

Fig. 7. Lineweaver–Burk plots for determining $K_m$ values of phosphatase isozymes. Assay mixtures contained 1.25 ml of 0.2 M carbonate–bicarbonate buffer, pH 10.45; 1.0 ml phenylphosphate, 18–60 mM; 0.25 ml of 0.1 M $MgCl_2$, 0.4 ml water and 0.1 ml enzyme solution. Reactions were run for 30 min at 37.5°. Intercept of plotted line with abscissa gives $-1/K_m$.

(1) pH optimum was found to be 10.45 for all three isozymes (Fig. 6). Activity was sharply reduced at pH 10.65.

(2) Substrate optimum was determined with phenyl phosphate concentrations from 10 to 140 mM at pH 10.45 and in the presence of 8.33 mM $MgCl_2$. All three isozymes showed maximal activity at about 70 mM; $F_1$ and S were inhibited slightly at concentrations above 90 mM.

(3) Optimal $Mg^{2+}$ concentration was determined at pH 10.45 in the presence of 70 mM phenyl phosphate. All three isozymes showed maximal activity with 8.33 mM $MgCl_2$.

(4) Michaelis constant ($K_m$) was determined at phenyl phosphate concentrations between 5 and 20 mM at pH 10.45, with 8.33 mM $MgCl_2$. The Lineweaver–Burk plot was employed to estimate $K_m$ from a line determined by the least squares method (Fig. 7). Averaging the results of 6 tests for each enzyme gave the following $K_m$'s: $F_1$, $2.6 \cdot 10^{-2}$ M; $F_2$, $2.4 \cdot 10^{-2}$ M; S, $2.8 \cdot 10^{-2}$ M.

(5) Relative activity toward phenyl phosphate and β-glycerophosphate was determined for each enzyme variant because relative increase in activity with phenyl phosphate is a significant aspect of the differentiation of the phosphatase isozymes of the mouse duodenum[7]; in addition, the ratio of P cleaved from phenyl phosphate to that cleaved from β-glycerophosphate has been reported to increase in the chicken duodenum in both pre-hatching[9,17] and post-hatching[18] stages. In the present study, β-glycerophosphate activity was assayed under optimal conditions for this substrate (70 mM β-glycerophosphate, pH 9.2, 8.33 mM $MgCl_2$), with P released being determined by the method of FISKE AND SUBBAROW[19]. $F_1$, $F_2$ and S were all found to have

phenyl phosphate to β-glycerophosphate ratios of about 2.2. In addition, assay of partially purified fractions from 20-day embryo and 20-week pullet tissue yielded ratios ranging only from 2.0 to 2.5.

(6) L-Phenylalanine inhibition, which is an important characteristic of intestinal phosphatases[20], was examined by adding 2–24 mM L-phenylalanine to the assay mixtures under optimal conditions. All three isozymes exhibited the same sensitivity to the inhibitor, the initial activity being lowered in all cases about 70% by 20 mM L-phenylalanine.

(7) Energy of activation was determined by assaying under optimal conditions at 23–42°. All three isozymes proved to have energies of activation of about 5000 cal/mole.

*Stability tests of the purified phosphatase variants*

(1) $Na_2EDTA$ inactivation was examined with 1.2 and 2.5 mM $Na_2EDTA$ at 24°, and with 1.2 mM at 37.5°; pH was 7.8 in all cases. $F_1$ (sialoenzyme) had higher resistance to EDTA than $F_2$ under all conditions and S was of intermediate sensitivity. The three forms could be best distinguished by incubation in 1.2 mM EDTA at 37.5°, in which case the retained activities of $F_1$, $F_2$ and S after 15 min of incubation were 35%, 14% and 22%, respectively (Fig. 8).

(2) Thermal denaturation studies showed that all three forms are quite heat

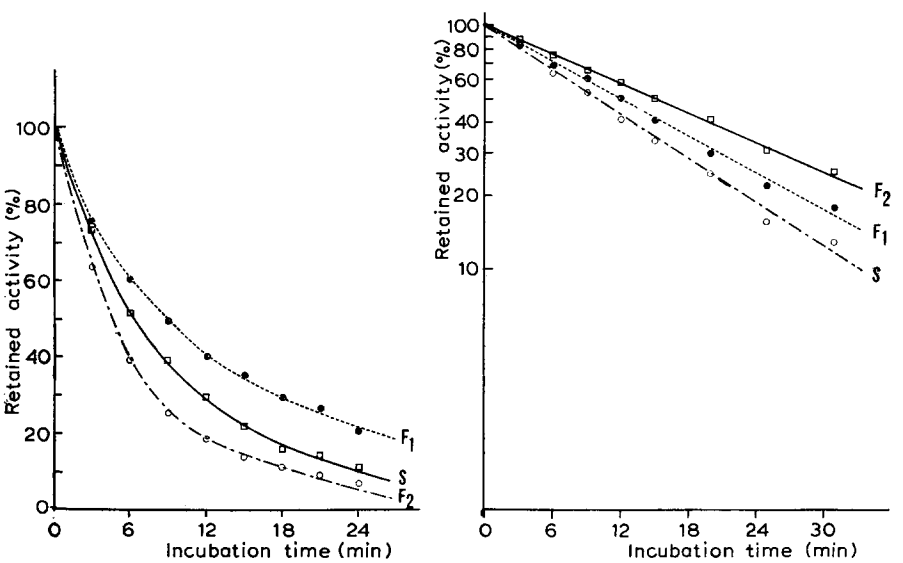



Fig. 8. Inactivation of phosphatase isozymes by $Na_2EDTA$. 0.9 ml of enzyme was incubated at 37.5° after determination of initial activity, then 0.1 ml of warmed 0.012 M $Na_2EDTA$ in 0.01 Tris–HCl, pH 7.8, was added (time zero). 0.1 ml aliquots of this mixture were removed at 3-min intervals and added to 2.9 ml of assay mixture. Activity is expressed as percent of activity in to absence of the inhibitor.

Fig. 9. Thermal denaturation of phosphatase isozymes. 1.2 ml of enzyme solution in 0.01 M Tris HCl, pH 7.8, was incubated at 37.5° for 15 min. 0.2 ml was removed for determination of initial activity and the remainder was transferred to a water bath at 63°. At 3–6-min intervals 0.1 ml aliquots were removed and added to 2.9 ml of assay mixture for determination of activity retained.

stable, although the F-forms are more heat resistant than S (Fig. 9). When $F_1$ and $F_2$ were combined, the heat stability of the combination was also higher than that of S-enzyme.

(3) Urea denaturation was studied by incubating S-phosphatase or combined F-phosphatases with 8 M urea in 0.01 M Tris–HCl (pH 7.8) at 24° before assay; after 25 min incubation, both preparations were only slightly reduced in activity. When the incubating temperature was 37°, however, both preparations lost more than 90% of their initial activity in 25 min.

(4) Acid inactivation was examined by incubating S-variant or combined F-variants in 0.05 M citrate buffer (pH 6.0) at 24° or 37° before assay. After incubation at the lower temperature for 30 min, the S-phosphatase retained half its starting activity, but the combined F-phosphatases retained only 30%. At the higher temperature, both preparations were almost equally affected, retaining only about 25% of the original activity after 25 min incubation.

## DISCUSSION

The alkaline phosphatase of the duodenum of the chicken has been resolved into 3 groups of molecules differing in molecular size and electrophoretic mobility. All 3 types have the same catalytic properties, however, suggesting that they may embody a common protein core with an identical active center(s). On the other hand, the differences in stability that have been observed may result from structural differences among the variant forms.

The 2 isozymes that appear as discrete, fast-moving bands on electrophoresis differ in their association with sialic acid. Because of the general impression that intestinal alkaline phosphatases are devoid of surface sialic acid[21–23], this finding was unexpected. The failure to demonstrate sialic acid in extracts of mammalian intestinal phosphatase may however be due to the action of endogenous neuraminidase during homogenization of the tissue[24]. In the young chick, the small intestine is virtually free of neuraminidase activity[25].

Butanol extracts of various tissues commonly yield, in addition to fast-moving forms, a slow-moving zone of phosphatase activity that remains at or near the origin on electrophoresis. This behavior has been demonstrated with placenta, intestine, liver and kidney, in all of which it has been shown that the poor mobility is due at least in part to large size, as reflected by the fact that the slow component is excluded from Sephadex G-200, or is eluted sooner than the more mobile forms[22,23,26,27]. A similar association of large size and poor mobility in starch gel electrophoresis also characterizes one of the phosphatase variants of unextracted whole serum[28]. Because its migration overlaps that of $\beta$-lipoprotein, MOSS AND KING[29] have suggested that the slow-moving band might represent phosphatase still associated with a lipoprotein complex. By freezing and thawing, these authors were able to release a fast-moving component from enzyme recovered from the $\beta$-lipoprotein region. By extraction with butanol, JENNINGS *et al.*[28] converted the slow-moving variant of human serum into the fast one.

The S-phosphatase of chicken duodenum seems to differ from its mammalian analogs in several ways. It is virtually unaffected by freezing and thawing[16], and is resistant to the action of butanol in a second extraction. In addition, it can to some

extent be eluted from DEAE-Sephadex with 0.1 M NaCl or higher concentrations[16], in contrast to the large components of mouse intestine[30], or of human liver or intestine[27,31], which are lost on passage of butanol extract through DEAE-cellulose.

From a developmental point of view, the increase of F-enzyme in post-hatching stages poses some interesting questions. Why does the amount of alkaline phosphatase organized into butanol-resistant complexes decrease with age? Although it is reasonable to assume that the shift in predominant form is a matter of physiological adaptation, the similarity of catalytic properties of F- and S-variants suggests that what is being adapted is not the character of the phosphatase molecules *per se*, but rather their relationship to the membranous coat of the microvilli, with which the enzyme is intimately associated[1-3]. During the first 2 days after hatching the microvillus surface expands markedly and alkaline phosphatase activity surges up at the same time[8,32]; the possibility that these events may be causally related to the developmental shift in phosphatase isozyme populations is supported by the sharp increase in F-phosphatase between the last day *in ovo* and the 3rd–4th day after hatching. Before this hypothesis can be substantiated, however, it will be necessary to demonstrate that the post-hatching decrease of S-phosphatase is not an artifact, perhaps caused by the action of endogenous proteases or other factors; preliminary investigations have indicated that the decrease is not produced during the course of enzyme isolation[11].

It has recently been reported that the alkaline phosphatase of whole chicken intestine comprises 4 isozymes that are separable on DEAE-Sephadex and differ in their susceptibility to inhibition by EDTA salts[33]. The fact that we were not able to obtain 4 peaks with DEAE-Sephadex[16] may be due to the use of duodenum only in our work. The fact that SCHUSSLER[33] obtained virtually no counterpart of our S-phosphatase by filtration on Sephadex G-200, however, suggests that this component may have been removed during the preliminary purification of the commercial preparation she used.

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