BBA 66483

ALKALINE PHOSPHATASES OF THE CHICKEN DUODENUM

II. ENZYMIC DISSOCIATION OF A LARGE PHOSPHATASE COMPLEX PREDOMINANT IN THE DUODENUM BEFORE HATCHING

CHIN-HAI CHANG AND FLORENCE MOOG
Department of Biology, Washington University, St. Louis, Mo. 63130 (U.S.A.)

(Received July 20th, 1971)

SUMMARY

1. The duodenal alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) of the chicken is extracted by *n*-butanol in the form of a large, butanol-resistant complex (S) and two smaller forms (F_1 and F_2) that differ in surface sialic acid content. The S-phosphatase can be dissociated into 2 F-like components by trypsin and possibly other proteases. The F-phosphatases thus released appear to be equivalent to native F_1 and F_2 in electrophoretic mobilities, apparent molecular size, neuraminidase sensitivity, various catalytic properties and heat stability. The results suggest that the S-complex comprises F-phosphatase molecules bound together by a protease-digestible material that may be instrumental in integrating the enzyme into the microvillus membrane.

2. Embryonic duodenum yields the greater part of its butanol-extractable phosphatase as S-complex, but the reverse is true for the adult. This situation might reflect a difference in proportions of S and F pre-existing *in vivo*; or it might result from abundant hydrolases in the adult intestine acting to dissociate S-complex in the course of preparation of the tissue. To examine this question, embryonic and adult duodenal fragments were homogenized and/or extracted in combination. The proportion of F-phosphatase appearing in the combined extracts was close to the arithmetical average of the proportions in the component extracts.

INTRODUCTION

The duodenum of the chicken contains alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) that appears in three forms when extracted with *n*-butanol. One form is a high molecular weight complex that migrates slowly in starch gel; the other two are of relatively low molecular weight and migrate rapidly[1]. Although the three forms are virtually indistinguishable catalytically, they change in

Abbreviation: TAME, *p*-toluenesulfonyl-L-arginine methyl ester.

relative abundance during development. In the adult chicken the greater part of the duodenal phosphatase is extracted as the fast-moving forms (F); but in the embryo at hatching, the duodenum yields most of its phosphatase in the slow-migrating form (S)[1]. Before the developmental significance of this difference can be understood, it is necessary to determine how the S- and F-phosphatases are related to each other.

We have now found that the high-molecular-weight complex can be broken down by enzymatic action to yield two fast-moving phosphatases. These F-like forms are very similar to the native F-phosphatases according to several physical and chemical criteria, suggesting that the S-phosphatase is a complex of F-forms associated with butanol-resistant materials. Since the enzymes that can dissociate the S-complex are hydrolases of the types normally present in the functioning intestine of vertebrates, the greater quantity of S in the embryonic intestine might simply reflect a paucity of pancreatic or lysosomal hydrolases in the pre-functional period. This hypothesis is however not supported by the attempts we have made to test it.

MATERIALS AND METHODS

Phosphatase preparation and assay

The butanol extracts and S- and F-phosphatases used in this study were prepared according to methods previously described[1]. Chromatographic and electrophoretic methods, assay procedures, and methods for determining catalytic and other properties were also the same as those reported in the preceding paper[1].

Enzymic digestion of phosphatase

A preparation of S-phosphatase containing 257 μg protein per ml was used in all tests of the effects of hydrolase action. The reaction mixture consisted of 50 μl phosphatase preparation, 50 μl buffer and 50 μl of the hydrolase dissolved in the buffer. Controls contained 50 μl buffer in place of the hydrolase solution. Except with trypsin, the mixtures were incubated for 12–24 h at 37.5°, then subjected to starch gel electrophoresis[1] without further treatment. The hydrolases used, their sources, specific conditions of incubation initially employed, and references from which the conditions were adapted, are as follows:

(1) Cellulase, 100 mg/ml, from *Aspergillus niger* (Type I, Sigma Chemical Co.); 0.05 M sodium phosphate buffer, pH 5.3 (ref. 2).

(2) Lysozyme, 50 mg/ml, from egg white (crystalline, Grade I, Sigma Chemical Co.); 0.05 M sodium phosphate buffer, pH 7.0 (ref. 3).

(3) α-Amylase, 47.5 mg/ml, from hog pancreas (crystalline suspension, Worthington Biochemical Co.); 0.05 M sodium phosphate buffer, pH 6.9, containing 0.006 M NaCl[4].

(4) Hyaluronidase, 3000 NF units/100 μl, from bovine testis (Type VI, Sigma Chemical Co.); 0.05 M sodium phosphate buffer, pH 5.3, containing 0.15 M NaCl (ref. 5).

(5) Pronase, 100 mg/ml, from *Streptymyces griseus* (Grade B, Calbiochem Co.); 0.05 M sodium phosphate buffer, pH 6.9 (ref. 6).

(6) Protease, 100 mg/ml, from bovine pancreas (crude powder, Schwarz Bio-Research Inc.); 0.1 M Tris–HCl buffer, pH 8.0, containing 0.1 M $CaCl_2$ (ref. 7).

(7) Trypsin, from bovine pancreas (Grade V, 3 times crystallized, Miles Labora-

tories, Ind.); buffer as for protease[7]. Special conditions used in working with trypsin will be described under RESULTS. To verify the effect of trypsin, soybean trypsin inhibitor (Type I-S, Sigma Chemical Co.) was incorporated into the incubation mixture in some cases.

Determination of trypsin activity

The rate of tryptic hydrolysis of *p*-toluenesulfonyl-L-arginine methyl ester (TAME, Sigma Chemical Co.) was measured by the increase in absorbance at 247 nm, according to a modification of the method of HUMMEL[7]. The assay mixture contained 0.3 ml of 0.01 M TAME, 2.6 ml of 0.046 M Tris–HCl, pH 8.1, with 0.0115 M $CaCl_2$ and 0.1 ml of enzyme. The reaction was run for 5–30 min at room temperature, and the rate of increase of absorbance was recorded with a Beckman DB spectrophotometer.

RESULTS

Dissociation of S-phosphatase into F-like components

The apparently high molecular weight of S-phosphatase[1] suggested that it might be an aggregate that could be reduced to its component molecules by simple chemical or physical treatment. Accordingly S-phosphatase in 0.01 M Tris–HCl, pH 8.0, with 0.005 M $MgCl_2$, was exposed to the following conditions, and then subjected to electrophoresis in starch gel to determine whether any fast-moving phosphatase had been released[8]: (1) freezing and thawing more than 50 times; (2) incubation in 2.5% sodium deoxycholate or 5% Triton X-100 for 2.5 h at room temperature, followed by dialysis against distilled water for 48 h at 2–4°; (3) storage at 4° for 6 months. None of these treatments led to the appearance of any F-like components demonstrable in starch gel. Incubation of S-phosphatase with 8 M urea or 8 M LiCl at room temperature inactivated the enzyme.

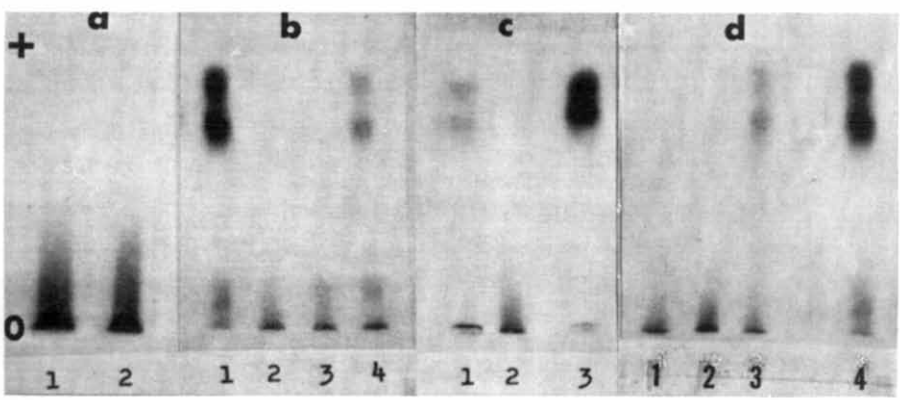

Fig. 1. Zymograms showing digestion of S-complex with carbohydrases and proteases. (a) Lysozyme: Slot 1, S-enzyme after treatment with incubation buffer; Slot 2, after treatment with lysozyme. (b) Cellulase: Slot 1, native F-enzymes; Slot 2, S-enzyme after treatment with incubation buffer; Slot 3, after boiled cellulase; Slot 4, after active cellulase. (c) α-Amylase: Slot 1, S-enzyme after treatment with α-amylase; Slot 2, after treatment with buffer; Slot 3, native F-enzymes. (d) Pronase: Slot 1, S-enzyme after treatment with incubation buffer; Slot 2, after boiled pronase; Slot 3, after active pronase; Slot 4, native F-enzymes. Boiled hydrolases were held in a boiling water bath for 5 min.

Since intestinal phosphatase is bound into a membrane[9–11] that appears to be linked to a carbohydrate-rich coat[12], the resistance of the S-enzyme to butanol might be due to complexing with proteins or carbohydrates. When S-phosphatase was incubated with large amounts of carbohydrases or proteases for prolonged periods to assure maximal hydrolytic action, some of the S-complex was converted into 2 fast-moving phosphatases electrophoretically equivalent to native F_1 and F_2 (Fig. 1). Cellulase, α-amylase, hyaluronidase, pronase, and pancreatic protease all had this effect; only purified lysozyme was ineffective. Phosphatase activity retained after incubation ranged from 60 to 89% (Table I), except with pancreatic protease; this enzyme preparation released F-like components in less than 5 h, but destroyed all phosphatase activity in 24 h.

When aliquots of the enzymic digests were chromatographed on Sepharose 6B under conditions previously employed[1], each experimental sample yielded a large included peak in approximately the same position as the native F-enzyme peak (Figs. 2b and 2d); the control samples, which contained no F-forms detectable in starch gels, yielded principally an excluded peak, with only a trace of activity in the F-enzyme region (Figs. 2a and 2c). The V_e/V_0 of the new peaks generated by enzymic digestion of the S-phosphatase indicate that the F-forms released are low-molecular-weight enzymes like the native F-phosphatase (Table I).

Comparing the proportion of activity in the F-peak in the enzymic digests with that in the controls reveals that a substantial part of the S-complex can be converted to F-forms under the conditions employed (Table I). If one assumes that the activity

TABLE I

ENZYMIC DISSOCIATION OF S-COMPLEX

	Hydrolase tested			
	Cellulase	*Pronase*	*α-Amylase*	*Hyaluronidase*
% F* after incubation with hydrolase	80.2	73.2	66.3	31.9
% F after incubation with buffer	9.5	7.0	11.2	5.3
% Activity retained after incubation with hydrolase	83.9	89.0	71.1	60.4
V_e/V_0 of F peak in hydrolase digests**	1.81	1.88	1.83	1.83

* Conditions for determining amount of F activity in each sample are given in Fig. 2.
** With the same column native F-phosphatase had $V_e/V_0 = 1.84$.

lost during digestion represents S- and F-forms equally, it appears that about 2/3 of the surviving S-complex is converted to F by the action of cellulase and pronase.

Under the severe conditions of digestion reported above, carbohydrases and proteases were equally effective in dissociating S-phosphatase. Since the carbohydrase preparations used may have been contaminated with protease activity, we studied the breakdown of S-complex further by employing dilute solutions of a 3 times crystallized trypsin preparation with a specific activity of 1125 absorbance units/mg per 10 min. After only 2 h incubation at 37°, trypsin at a final concentration of 9 μg/ml was still effective in converting S-phosphatase into 2 F-like components (Fig. 3, Slot 2). After treatment with 166 μg trypsin per ml, chromatography of the digest

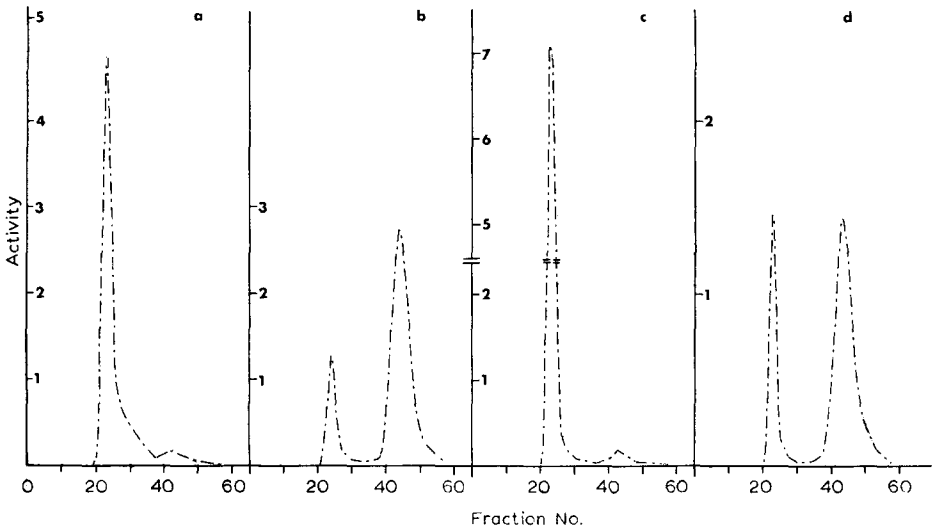

Fig. 2. Sepharose 6B chromatograms of hydrolase-digested S-complex. 50 μl of diluted digest was applied to the top of 0.9 cm × 28.5 cm column and eluted at 1.8–2.0 ml/cm^2 per h. Activity in each 0.26 ml fraction was expressed as Klett units per 0.01 ml per 30 min. Dextran peak was eluted at Fraction 23. The sum of activity of Fractions 20–35 is taken as S-activity, that of Fractions 36–58 as F-activity. (a) Buffer-treated control for cellulase digestion; S = 90.5% of total activity recovered from column, F = 9.5%. (b) Cellulase digest; S = 19.8%, F = 80.2%. (c) Control for pronase digestion; S = 93.0%, F = 7.0%. (d) Pronase digest; S = 26.8%, F = 73.2%.

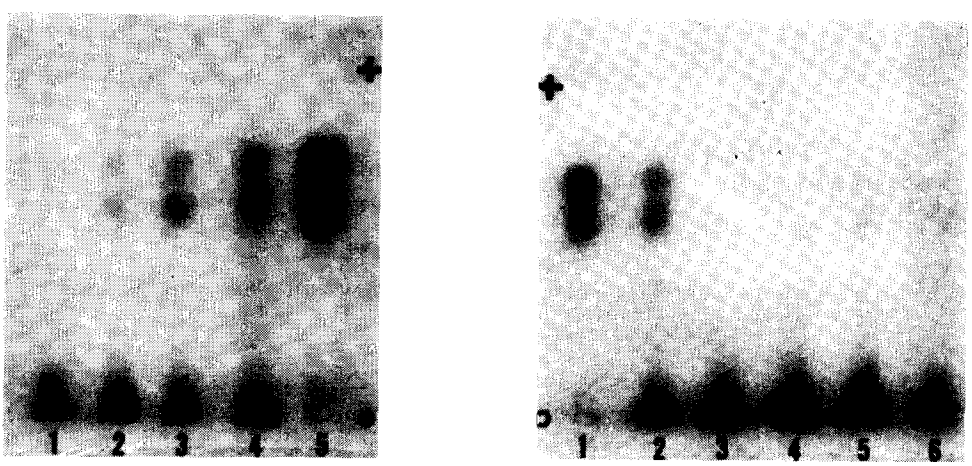

Fig. 3. Zymograms showing effect of trypsin on S-complex. 50 μl of S preparation (257 μg protein/ml) was incubated with 50 μl trypsin at a concentration of 0.026 mg/ml (Slot 2), 0.16 mg/ml (Slot 3), or 1 mg/ml (Slot 4). Slot 1, buffer-incubated control, Slot 5, native F-enzymes.

Fig. 4. Zymograms showing influence of soybean inhibitor on trypsin digestion of S-complex. 50 μl of S-preparation (257 μg protein per ml) was incubated for 2 h with 50 μl of trypsin (0.5 mg/ml) with or without trypsin inhibitor. Slot 1, native F-enzymes; Slot 2, tryptic digest of S-enzyme; Slots 3–5, tryptic digests with 1.0 mg/ml, 1.5 mg/ml and 2.0 mg/ml soybean inhibitor; Slot 6, untreated S-enzyme.

on Sepharose 6B showed that 12% of the S-complex was dissociated into F-forms in 2 h (Fig. 4, Slot 2); 80–85% of the phosphatase activity was retained under these conditions. The trypsin-released F-enzyme had a V_e/V_0 of 1.91, almost identical with that of native F-enzyme (1.89) determined with the same column. When soybean trypsin inhibitor[13] was added at a concentration twice that of the trypsin used, the effect of trypsin was virtually eliminated (Fig. 4, Slots 3–5).

When trypsin concentration was raised to 1.6 mg/ml and digestion continued for 4 h, the amount of S-phosphatase converted to F was increased to about 21%; this effect was also prevented by soybean trypsin inhibitor. Under these severe conditions 40–50% of the starting activity was lost.

To determine whether the carbohydrase preparations which had been found effective in dissociating S-complex were contaminated with trypsin, these preparations were assayed with TAME. This substrate was actively hydrolyzed by α-amylase (1.05 absorbance units/mg per 10 min) and by hyaluronidase (1.82 absorbance units/mg per 10 min). Moreover, the effect of α-amylase on the dissociation of S-complex was significantly reduced in the presence of soybean trypsin inhibitor. On the other hand, the cellulase preparation, which was highly effective in dissociating S-complex (Fig. 1), had virtually no effect on TAME (0.007 absorbance unit/mg per 10 min).

Influence of neuraminidase on released F-phosphatase

The 2 naturally occurring F-phosphatases differ in the presence of neuramini-

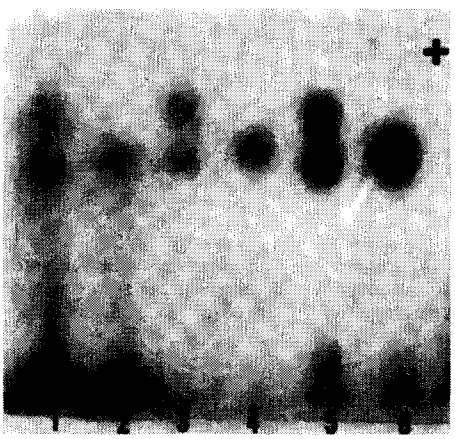

Fig. 5. Zymograms showing effect of neuraminidase on F components released from S-complex by cellulase, α-amylase or pronase. Aliquots of hydrolase digests were treated with neuraminidase as previously described[1]. Slots 1 and 2, α-amylase digest of S-complex treated with incubation buffer (1) or neuraminidase (2); Slots 3 and 4, pronase digests treated with buffer or neuraminidase; Slots 5 and 6, cellulase digests treated with buffer or neuraminidase.

dase-removable sialic acid on one of them[1]. When aliquots of cellulase, pronase, or α-amylase digests were further treated with neuraminidase as previously described[1], the component corresponding to the native F_1 was lost in each case, and the component corresponding to native F_2 increased in activity as compared to the controls (Fig. 5).

Properties of converted enzymes

F-like enzyme released from S-complex by the action of cellulase, hyaluronidase, pronase, or α-amylase was isolated after Sepharose 6B chromatography by pooling the fractions within the F-peak (Fig. 2). Kinetic analyses were performed on these samples by techniques previously applied to native F-phosphatase[1]. With phenyl phosphate as substrate, K_m values ranged from $2.3 \cdot 10^{-2}$ to $2.9 \cdot 10^{-2}$ M, native F_1 and F_2 yielding values of $2.6 \cdot 10^{-2}$ and $2.4 \cdot 10^{-2}$ M in parallel tests. Phenyl phosphate/β-glycerophosphate ratios[1] were 2.1–2.2, identical with those for the native forms. Activity retained in the presence of 20 mM L-phenylalanine was 30.0–31.9%, and 29.8–30.4% for native F. Energy of activation was close to that of native F (Fig. 6). Desialo-F_1 also gave results almost identical with those of native F. Hence the released or converted F-phosphatases are catalytically indistinguishable from those occurring spontaneously in butanol extracts.

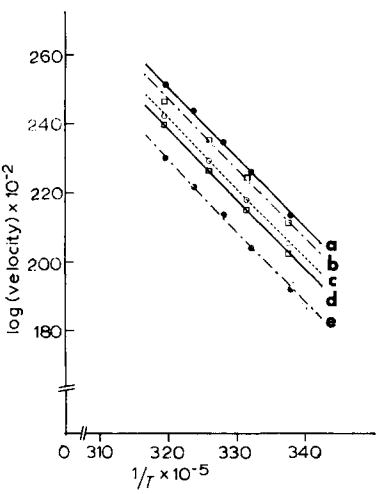

Fig. 6. Activation energy of native and converted isozymes. a, pronase-released F-enzyme; b, native F_1-enzyme; c, F_1-enzyme treated with incubation buffer; d, native F_2-enzyme, native S-enzyme and neuraminidase-treated F_1; values for these 3 fell on the same line; (e) cellulase-released F-enzyme.

When the F_2-phosphatase was treated with neuraminidase, it retained the same sensitivity toward Na_2EDTA as the buffer-treated control (Fig. 8); treatment of F_1 with neuraminidase however increased its sensitivity to that of F_2 (Fig. 7). Both pronase-and cellulase-released F-enzymes are more heat-stable than the native S-complex, being in fact somewhat more stable than native F-phosphatase (Fig. 8).

Co-extraction of adult and embryonic tissues

The fact that S-phosphatase can be converted to F-phosphatase by exogenous hydrolases suggests that the decrease in extractable S-enzyme with age might be due to the action of endogenous hydrolases that may become abundant in the intestine after hatching. To test this possibility, embryonic and adult tissues were extracted in combination according to two different schemes:

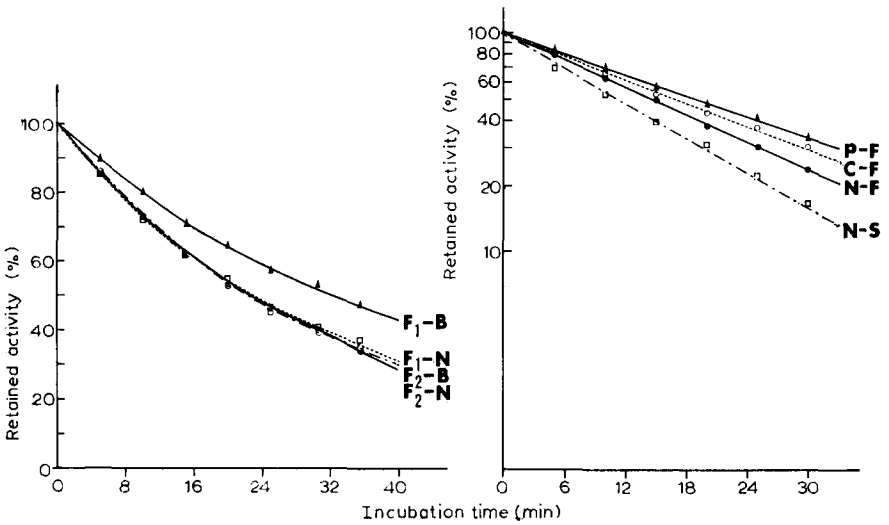

Fig. 7. Na_2EDTA sensitivity of F_1 and F_2 after neuraminidase treatment. At time zero 100 μl of 0.012 M Na_2EDTA in 0.01 M Tris–HCl, pH 7.8, was added to 800 μl of enzyme at 21.5° in a small capped test tube. 30 sec after addition of EDTA, 100 μl of the mixture was removed and assayed to determine initial (100%) phosphatase activity. At 5- or 6-min intervals thereafter 100-μl aliquots were removed and assayed to determine activity remaining. F_1-B and F_2-B mean F_1- or F_2-phosphatase treated with incubation buffer; F_1-N and F_2-N mean F_1- or F_2-phosphatase treated with neuraminidase.

Fig. 8. Heat stability of native and converted isozymes. The enzymes were incubated at 63° as previously described[1]. P-F, pronase-released F-phosphatase; C-F, cellulase-released F-phosphatase; N-F, native F preparation, containing both F_1 and F_2; N-S, native S-phosphatase.

(1) Duodenal loops totalling 12.1 g were collected from 2 pullets, and loops totalling 8.7 g from 87 embryos just before hatching. Each pool was homogenized in a glass homogenizer at 0° and diluted to 150 mg/ml with cold distilled water. The phosphatase activity of each homogenate was determined, and then a portion of adult homogenate was combined with a portion of embryonic homogenate containing an equal amount of activity (relative volumes of homogenates were 1:2.4). Combinations were made in duplicate. About 4 h elapsed between homogenization and mixing, the preparations being kept at 0° during this time. Original and mixed homogenates were frozen and thawed 10 times before being stirred with an equal volume of *n*-butanol at room temperature, as previously described[1]. Recovery of enzyme activity in the butanol extracts was 64–67%.

The butanol extracts were chromatographed on a 0.9 cm × 29 cm Sepharose 6B column under identical conditions, with the eluates being collected in 0.26-ml fractions. First traces of activity in all cases were detected in Fraction 20, with S-phosphatase then appearing as a peak centered at Fraction 24, F as a peak centered at Fraction 45.

Fractions 20–36 were pooled as S-phosphatase, 37–58 as F-phosphatase. For the duplicate adult extracts, F-phosphatase represented 55.2% and 55.9% of the activity recovered from the columns; for the duplicate embryonic extracts, the comparable values were 23.3% and 29.1%. Averaging the proportions of F-phosphatase in the adult and embryonic extracts leads to the prediction that the combined extracts

should have contained 41.2% of their activity as F, if no interaction had occurred between the homogenates. In fact, the duplicate co-extracts contained 39.4% and 45.0% of F-enzyme. Hence, combining the homogenates before extraction did not lead to any shift of activity from one form of phosphatase to the other.

(2) In the second test, embryonic and adult duodenal tissues were combined before homogenization. Duodenal loops from 2 pullets and 21 embryos near hatching were separately cut into 1–2-mm pieces at 2°, and 120 mg of randomly selected adult pieces and 330 mg of embryonic pieces were combined immediately in duplicate. Original and mixed pools of tissue were then homogenized and assayed for phosphatase activity. Although previous results had indicated that quantities of adult and embryonic tissue combined should have roughly equal phosphatase activities, the 120 mg of adult duodenum proved to have 5.3 times as much activity as the 330 mg of embryonic duodenum*. In addition, the activities of the duplicate co-homogenates averaged 14.7% lower than expected. After extraction with butanol, the extracts of the combined tissues and of the separate embryonic and adult samples were chromatographed on Sepharose 6B, as in the preceding case. The 2 adult extracts yielded 85.0% and 88.0% of the total recovered activity in the F region; the embryonic extracts yielded 28.3% and 45.3%. The theoretical proportion of F-phosphatase (F_p) in the combination extracts can be computed from the average results of the 2 embryonic extracts (F_e) and the adult extracts (F_a) by the following formula:

$$F_p = \frac{F_e + 5.3\,F_a}{1 + 5.3}$$

This formula predicts that 78.6% of the activity recovered after chromatography of the combined tissues should be in the F region if no interaction has occurred. The values actually obtained for the duplicate co-extracts were 67.2% and 74.0%. Obviously any endogenous factors that might have been associated with the adult duodenum did not act to increase the percentage of the extracted phosphatase in the F-form.

DISCUSSION

The large, slow-migrating S-phosphatase and the two smaller and more mobile F-phosphatases that are extracted from chicken duodenum with *n*-butanol are similar in numerous catalytic and other properties[1]. This similarity is now explained by the demonstration that S-phosphatase can be dissociated to yield two F-phosphatases that are virtually indistinguishable from those that appear spontaneously in butanol extracts. This result might mean that S is simply a random aggregation of F molecules, but the striking differences in proportions of S and F in pre- and post-hatching stages, and the failure of S to break up on prolonged storage, or repeated freezing and thawing, or treatment with detergent[1], make it more likely that the F-phosphatases are complexed with butanol-resistant materials that link phosphatase into the microvillus membrane, of which it is an integral part[9–11].

The bonds involved in the maintenance of S-complex are at least in part

* Eggs of the Babcock strain of White Leghorns were used in this experiment, because eggs of the Kimber strain, used in all other experiments in this series, were no longer available. The Babcock embryos were smaller at the hatching stage, and had lower phosphatase activity.

susceptible to the action of trypsin, though the destructive effect of this enzyme on phosphatase activity makes it impossible to determine whether the S-complex is solely dependent on trypsin-sensitive linkages. Since intestinal phosphatase is a glycoprotein[14–16], and the microvillus membrane is associated with a carbohydrate-rich coating[12], carbohydrates might also be involved in the binding together of F-phosphatases; but this possibility is not necessarily supported by the results obtained with hyaluronidase and α-amylase, both of which proved to be contaminated with significant trypsin-like activity. The cellulase preparation, which was highly effective in converting S to F, was almost trypsin-free, but may have contained a similar protease; animal tissues are generally devoid of the β-glucosidic links that would be the principal target of crude cellulase preparations[2]. The failure of purified lysozyme to break down S-phosphatase shows that the complex is not dependent on *N*-acetylmuramyl–*N*-acetylglucosaminic linkages; in addition, the ineffectiveness of lysozyme might be due to contamination with the trypsin inhibitor that is present in egg white[17], from which the enzyme is obtained.

Enzymic dissociation of S-phosphatase from both embryonic and adult intestines yields two smaller molecular types that appear to be identical with the F_1- and F_2-phosphatases found in undigested extracts. In both cases F_1 can be transformed into F_2 by desialation, indicating that the two forms may differ only in their sialic acid content. This view is supported by the fact that desialation eliminates the difference in Na_2EDTA sensitivity between the two forms. (F_1 may however be a class of molecules rather than a sharply definable structure, for a series of F_1's with slightly different electrophoretic mobilities can be eluted from DEAE-Sephadex by stepwise increases in the ionic strength of the elution medium[8].) Although the appearance of both F_1 and F_2 in extracts and enzymic digests suggests that they may co-exist *in vivo*, the relation between them is likely to remain obscure until more is known of the way in which phosphatase is differentiated. Addition of sialic acid residues might occur progressively as the epithelial cells that carry the phosphatase migrate from villus base to tip[18]; conversely, the desialation might occur as some molecules are freed from their binding to the microvillus membrane in the course of extraction. The latter possibility is the less likely one, since sialic acid is generally terminal[19], and the chicken intestine has very little endogenous neuraminidase[20].

Although the S-phosphatase of the chicken duodenum is not a unique phenomenon, as has been pointed out[1], such large complexes of low mobility generally represent only a small part of butanol-extractable phosphatase; the duodenum of the chick embryo is exceptional in yielding most of its phosphatase in the polydisperse S-form. The question arises whether the greater proportion of F-phosphatase in adult extracts is an artifact of preparation. This might be the case if the pre-hatching gut were deficient in pancreatic or lysosomal enzymes that could attack the S-complex on homogenization. The evidence of this point, though only fragmentary, militates against such an interpretation. In the chick embryo, pancreatic α-amylase activity is high at the time of hatching[21], and acid phosphatase, an indicator of lysozymal enzyme activity, is abundant in the intestinal epithelium[22,23]. Moreover, the embryonic intestine has an active protein-digesting function in birds, since it must break down and absorb the albumen, which is ingested halfway through the period of incubation[24]. These considerations do not rule out the possibility that the adult gut contains some factor that attacks S-complex in the course of preparation; but such a

factor, if it exists, did not act to dissociate embryonic S-phosphatase in the experiments reported in this paper. Admittedly these experiments are not conclusive, since pancreatic enzymes in the intestine might be rapidly inactivated[25], or might be bound to the epithelial brush border[26,27] in such a way as to exert a disruptive effect only at the site of binding. Cathepsin[28] and other hydrolases[22,23] of the epithelial lysosomes might similarly have a limited range of action. Nevertheless, the evidence presently at hand does not favor the view that the difference in proportions of S- and F-phosphatase in the pre- and post-hatching stages is artifactual.

If the tendency of phosphatase to be extracted in the S- or F-form is intrinsic to the intestinal epithelium, the basis of the phenomenon is probably to be sought in the state of differentiation of the membrane in which the enzyme is invested. Around the time of hatching phosphatase activity undergoes an upsurge[29] that is enhanced by actinomycin D and cycloheximide[30], and hence may result from alteration of phosphatase molecules rather than from *de novo* synthesis. Concomitantly the surface membrane, in which the phosphatase is invested, expands dramatically as the microvilli increase 4-fold in length, with little change in volume[31]. These differentiative steps might bring about a change in the binding of phosphatase, with the result that butanol would extract it more readily in the F-form. If this is the case, further study of the material that binds phosphatase into the S-form may provide important clues to the molecular events that constitute the differentiation of the surface membrane of the small intestine.

ACKNOWLEDGEMENT

This work was supported by grant HD 03490 from the National Institutes of Health of the U.S. Public Health Service.

REFERENCES

1 C. H. CHANG AND F. MOOG, *Biochim. Biophys. Acta*, 258 (1972) 154.
2 K. W. KING AND R. W. SMIBERT, *Appl. Microbiol.*, 11 (1963) 315.
3 D. SHUGAR, *Biochim. Biophys. Acta*, 8 (1952) 302.
4 P. BERNFELD, *Adv. Enzymol.*, 12 (1951) 379.
5 S. TOLKSDORF, M. H. MCCREADY, D. R. MCCULLAGH, E. SCHWENK AND N. J. BLOOMFIELD, *J. Lab. Clin. Med.*, 34 (1949) 74.
6 M. NOMOTO, Y. NARAHASHI AND M. MURAKAMI, *J. Biochem. Tokyo*, 48 (1960) 906.
7 B. C. W. HUMMEL, *Can. J. Biochem. Physiol.*, 37 (1959) 1393.
8 C. H. CHANG, *The Nature of the Heterogeneity of Chick Duodenal Alkaline Phosphatase during Post-Hatching Development*, Ph. D. Thesis, Washington University, St. Louis, Mo., 1971.
9 J. OVERTON, A. EICHHOLZ AND R. K. CRANE, *J. Cell Biol.*, 26 (1965) 693.
10 J. HUGON AND M. BORGERS, *J. Histochem. Cytochem.*, 14 (1966) 629.
11 T. ODA, S. SEKI AND S. WATANABE, *Acta Med. Okayama*, 23 (1969) 357.
12 S. ITO, *Fed. Proc.*, (1969) 12.
13 M. KUNITZ, *J. Gen. Physiol.*, 30 (1947) 291.
14 P. PORTMANN, R. ROSSIER AND H. CHARDONNENS, *Helv. Physiol. Pharmacol. Acta*, 18 (1960) 414.
15 L. ENGSTRÖM, *Biochim. Biophys. Acta*, 52 (1961) 36.
16 P. K. SAINI AND J. DONE, *FEBS Lett.*, 7 (1970) 86.
17 M. LASKOWSKI AND M. LASKOWSKI, JR., *Adv. Protein Chem.*, 9 (1954) 203.
18 C. P. LEBLOND AND B. MESSIER, *Anat. Rec.*, 132 (1958) 247.
19 A. NEUBERGER AND R. D. MARSHALL, in A. GOTTSCHALK, *Glycoproteins*, Vol. V, Elsevier, Amsterdam, 1966, p. 235.
20 B. COOK AND G. L. ADA, *Biochim. Biophys. Acta*, 73 (1963) 454.

21 R. G. KULKA AND D. DUKSIN, *Biochim. Biophys. Acta*, 91 (1964) 506.
22 C. BUONANNO, V. BOTTE AND E. COTTINO, *Bull. Zool.*, 31 (1964) 479.
23 A. PENTILLA AND J. GRIPENBERG, *Z. Anat. Entwicklungsgesch.*, 129 (1969) 109.
24 M. N. RAGOZINA, *Trudy Inst. Morfol. Zhivotn. Akad. Nauk SSSR*, 14 (1955) 250; *Ref. Z. Biol.*, (1956) No. 77177.
25 M. H. KHAYAT AND J. CRISTOPHE, *Am. J. Physiol.*, 217 (1969) 923.
26 D. M. GOLDBERG, R. CAMPBELL AND A. D. ROY, *Scand. J. Gastroenterol.*, 4 (1969) 217.
27 D. H. ALPERS AND M. SOLIN, *Gastroenterology*, 58 (1970) 833.
28 L. HSU AND A. L. TAPPEL, *Biochim. Biophys. Acta*, 101 (1965) 83.
29 F. MOOG, *J. Exp. Zool.*, 115 (1950) 109.
30 F. MOOG AND R. D. GREY, *Biol. Neonat.*, 9 (1966) 10.
31 J. OVERTON AND J. SHOUP, *J. Cell Biol.*, 21 (1964) 75.