THE DISTRIBUTION OF ALKALINE PHOSPHATASE IN THE MUCOSAL CELLS OF THE SMALL INTESTINE OF THE RAT, CAT AND DOG

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INTRODUCTION

Among the many investigators who have demonstrated histochemically the presence of alkaline phosphatase in the mucosa of the small intestine, only a few have observed a bilaminar distribution of this enzyme in the superficial zones of the mucosal cells, for example, Bourne (1944) in the guinea-pig, Deane & Dempsey (1945) and Hébert (1950) in a large number of mammalian species, and Moe (1952) in the mouse and cat. Since such a distribution might have considerable physiological importance in relation to the process of absorption of some of the products of digestion, the present study was undertaken to determine whether this phenomenon is constant, and to investigate the variations in the standard technique that are necessary for its demonstration.

MATERIALS AND METHODS

The material used comprised pieces of small intestine removed from ten cats, seven rats and two dogs immediately after the animals had been killed by coal gas. From each animal nine pieces of intestine were removed, the distance of each piece from the pylorus being noted. The first piece consisted of the first centimetre of the duodenum, and the last included the terminal part of the ileum, the other pieces being taken at regular intervals from the intervening part of the small intestine. Different fixatives were used, such as varying concentrations of alcohol, formalin, formalin and sodium chloride, formalin vapour, and acetone, and it was found that fixation in 80 % alcohol for 18 hr. gave the most satisfactory results for the present purpose. In order that a strict comparison could be made between the various parts of the intestine, and between the results of variations in the techniques employed, all nine pieces from each intestine were embedded in the same paraffin block. Serial sections, 3μ thick, were cut and mounted in distilled water, instead of the usual glycerine-albumen mounting medium, as the latter was found to give marked variations in the intensity of the reaction from one part to another of the same histological section. The method used for revealing the site of the alkaline phosphatase was the modification by Kabat & Furth (1941) of the Gomori (1939) technique, in which Mg ions are added as an enzyme activator. The sections were incubated in a substrate solution containing sodium-β-glycerophosphate at a constant pH of 9·4 for periods varying between 5 min. and 24 hr., after which they were placed in a 2 % solution of cobalt nitrate for 5 min. A few sections were left in the cobalt nitrate for times up to 30 min. to test if a diffusion of cobalt phosphate might occur during the carrying out of this part of the technique.

During the final step of the technique the sections were placed in solutions of ammonium sulphide of varying strengths and 'maturity' for periods ranging from 2 to 50 min. It was found that this step in the technique was the critical one for the demonstration of a bilaminar reaction at the free surface of the mucosal cells of the intestine. Gomori (1939), in his original description, suggested that the ammonium sulphide should be dark yellow in colour and that a few drops should be added to a Coplin jar of distilled water. In the present work three different ammonium sulphide solutions were used. Solution 'A' consisted of freshly prepared ammonium sulphide which was lemon in colour and smelt strongly of hydrogen sulphide; solution 'B' consisted of ammonium sulphide which had been allowed to age until it had become deep yellow and smelt of ammonia; and solution 'T' was prepared by adding sodium iodate to freshly prepared ammonium sulphide, a procedure which artificially ages the solution in a few hours. It is probable that the solution 'B' described above corresponds to the ammonium sulphide used by Gomori. Each solution was tested at strengths ranging between 0·04 and 1 % for varying times.

RESULTS

Throughout this work care has been taken to ensure that differing results in each of the various trials could have been produced by a difference in only one step of the technique. As far as possible, when variations in any one step were being studied, all the sections, including controls, were taken through the other steps of the procedure at the same time.

A. *Variations in the last step in which the sections are placed in the ammonium sulphide solution*

It was found that irrespective of the particular ammonium sulphide used three different types of reaction could be produced at the free surfaces of the mucosal cells of the small intestine. The first reaction (Pl. 1, fig. 1), consisted of two dark bands, one of which was situated at the free surface of the striated border and the other in the region of the apical cytoplasm. Between these two bands there was a clear, non-staining zone. The second reaction (Pl. 1, fig. 2), which occurred when the period during which the sections were placed in the ammonium sulphide solution was increased, differed from the first in that the clear zone between the two bands had partly disappeared. On further increasing the length of this final step of the technique the third reaction appeared (Pl. 1, fig. 3). This differed from the previous two reactions in that the two dark bands became blended into one broad band and the clear zone between them completely disappeared. The length of time which was required to produce these reactions varied with the ammonium sulphide used and with its strength (see Table 1). Decreasing the age and increasing the strength of the ammonium sulphide solution increased the rate at which any reaction occurred.

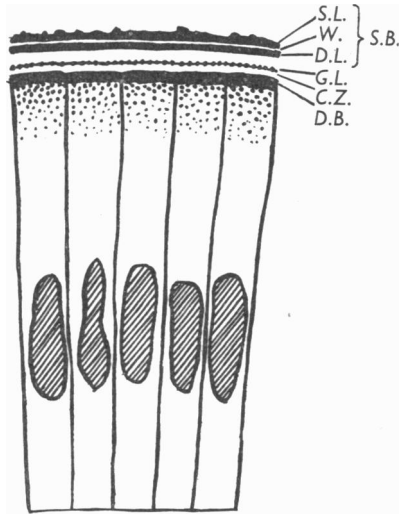
The exact localization of these bands becomes more evident when the section is examined under high magnification (Pl. 3, fig. 16), and when it is compared with a similar section stained with haematoxylin and eosin and magnified to the same extent (Pl. 3, fig. 17). It will be noted that at the higher magnification a third band

(*G.L.* in Pl. 3, fig. 16, and Text-fig. 1), intermediate in position, can be recognized, that appears to correspond in position to the granular layer (Baker, 1944) which can be plainly seen in the section stained with haematoxylin and eosin (*G.L.* in Pl. 3, fig. 17). The superficial band, which is situated at the free edge of the striated border, shows on close examination, a thin pale line (*W.* in Pl. 3, fig. 16, and Text-fig. 1), dividing it into two longitudinal layers, the superficial of which (*S.L.* in Pl. 3, fig. 16,

Table 1. *The effect of using three different ammonium sulphide solutions, for varying times, on the appearance of the striated border of the small intestine of the cat*

(The incubation time in the substrate in all cases was 60 min. at 37° C. All times are in minutes.)

Ammonium sulphide	Strength (%)	Appearance at various times		
		Double layer	Double layer closing up	Single layer
A	0·04	7	25	45
	0·1	2	6	10
B	0·2	8	30	45
	0·5	2	4	6
T	0·4	12	30	50
	1·0	5	9	12



Text-fig. 1. Diagrammatic representation of the localization of alkaline phosphatase at the free surfaces of the mucosal cells of the small intestine.

and Text-fig. 1) appears to be loosely applied to the border, whilst the deeper layer (*D.L.* in Pl. 3, fig. 16, and Text-fig. 1) seems to be part of the free edge of the border. The deepest band (*D.B.* in Pl. 3, fig. 16, and Text-fig. 1) appears to be situated in that portion of the cytoplasm lying below the granular layer, and the clear zone immediately external to it represents the thin clear zone of cytoplasm (*C.Z.* in Pl. 3, figs. 16, 17, and Text-fig. 1), lying deep to the granular layer, these sites being determined by comparison with the haematoxylin and eosin section.

In the opinion of the authors, the third or intermediate band (*G.L.* in Pl. 3, fig.16) does not represent a true area of phosphatase activity but is produced by the opacity of the granular layer at the base of the striated border.

B. *Variations in the period of incubation*

During this stage of the work the sections were incubated for differing times, and during the final step of the technique they were all placed in the same ammonium sulphide solution for the same time, it having been determined beforehand which ammonium sulphide solution at a given strength, and used for a given time, gave a bilaminar reaction in the region of the striated border.

When the sections were incubated for 5 min. in the substrate solution a definite bilaminar reaction was seen at the free surfaces of the intestinal cells (Pl. 1, fig. 6). The cytoplasm and nuclei were faintly positive but the Golgi zone appeared negative. With an incubation period of 25 min. there was still the same bilaminar reaction at the free surfaces of the cells, whilst the cytoplasm and nuclei were slightly more positive than at 5 min., and in some cells there was a faint reaction in the Golgi zone (Pl. 1, fig. 7). Incubation for 60 min. caused an increase in the reaction of the cytoplasm, especially in the region of the Golgi apparatus. With this period of incubation the nuclei of the mucosal cells gave a strongly positive reaction and some of the nuclei of the submucosa became positive (Pl. 1, fig. 8). At the end of 120 min. incubation there was a further general increase in the intensity of reaction, especially in the nuclei of the cells of the submucosa. The bilaminar reaction appeared the same as with the shorter periods of incubation (Pl. 1, fig. 9). It was noted that the bilaminar reaction was the same, no matter how long the period of incubation, and even periods up to 24 hr. produced only a general increase in the intensity of the reaction.

C. *Variations in sectioning and mounting*

For the demonstration of the site of alkaline phosphatase activity in the intestine the authors have found that sections cut at 3μ give a more easily interpreted picture than do sections of greater thickness. The thickness of the section is of prime importance when the exact localization in the striated border is being studied, as owing to the cylindrical nature of the villi thick sections tend to obscure the bilaminar reaction due to the overlapping of the various bands. Even with thin sections it was quite often found that the tips of the villi were cut obliquely and that the bilaminar reaction present in the sides of the villi appeared to become a single band at the apices (Pl. 2, fig. 15).

In the early stages of this work many sections were found to show marked variations in the intensity of reaction between one area and another (Pl. 2, fig. 14). This was found to be due to the use of a glycerine-albumen mounting medium, the glycerine of which inactivated the enzyme during the drying of the sections. Subsequently, mounting with distilled water was carried out, a process which gave a uniform intensity of the reaction throughout the section.

D. *Variations in the reaction in different parts of the small intestine, using a constant technique*

The pieces of intestine from each animal were treated by the technique which had been shown, in an earlier stage of the work, to give a constant demonstration of the bilaminar reaction at the free surfaces of the mucosal cells.

Dog. Throughout the length of the small intestine the cytoplasm of the mucosal cells gave a positive reaction. The cytoplasm between the nuclei and the free surface of the cells (superficial cytoplasm) gave a more dense reaction than that deep to the nuclei. The reaction was also noted to be stronger in the cells near the apices of the villi than in those nearer their bases. When different segments were compared it was found that there was a gradual decrease in intensity along the length of the intestine from the pylorus to the ileo-caecal valve.

The striated border was positive over the entire height of the villi, except in the last tenth (15 cm.) of the intestine where the striated border of the cells at the bases of the villi was negative. In the proximal half of the intestine the reaction in the striated border was bilaminar (Pl. 1, fig. 4) throughout the whole height of the villi. The extent of distribution of this bilaminar reaction in the villi gradually decreased in succeeding portions of the intestine, and in the terminal ileum it was present only at their apices. Where the bilaminar reaction was decreased in extent in the villi the striated border in other parts of these villi showed a positive reaction in the form of a single band (Pl. 1, fig. 5), except in the terminal ileum where the border was negative at the bases of the villi.

Cat. As in the dog, the cytoplasm of the mucosal cells of the cat's intestine was more strongly positive in the superficial regions of the cells (Pl. 1, fig. 7) and at the tips of the villi; it also showed a gradually decreasing intensity of reaction from the duodenum to the terminal ileum.

The striated border was positive throughout the villi only in the proximal half of the intestine; the distal half (Pl. 2, fig. 12) showed increasing negative areas at the bases of the villi until in the terminal ileum the striated border on the basal third of each villus was negative. The ratio of the extent of a bilaminar reaction to a single-line reaction varied along the length of the intestine. In the first part of the duodenum only the striated border on the apices of the villi showed the bilaminar reaction; this was followed by a length of intestine, approximately half of the total, in which the reaction was bilaminar for two-thirds of the depth of the villi; then followed a portion in which the extent of the bilaminar reaction gradually decreased until in the terminal third of the intestine the reaction consisted of a single band over the whole extent of the villi.

Rat. Although the technique used in investigating the localization of alkaline phosphatase in the small intestine of the rat was exactly the same as that used for the dog and cat, the reaction in those areas which were positive was much stronger than in the other two animals (Pl. 2, fig. 10). This was particularly noteworthy in the cytoplasm, which in the proximal third of the intestine gave a dense reaction with no variations between the cells at the tips and bases of the villi. The reaction of the cytoplasm of the middle third of the intestine was similar to that of the dog and cat in that the superficial portions 'stained' more densely than the basal portions and the cells at the apices of the villi were more strongly positive than those at their bases (Pl. 2, fig. 13). In the terminal third of the intestine there was a rapid falling off of the intensity of the reaction of the cytoplasm, and the cytoplasm of the cells in the lower 10 cm. or so of the ileum gave a negative reaction.

The reaction of the striated border was positive throughout the villi in the upper half of the intestine, but in the lower half less and less of the border was positive,

only small scattered areas being positive at the tips of the villi in the distal third of the intestine (Pl. 2, fig. 11). The reaction was bilaminar in form throughout the greater part of the intestine, except at the lower end where the small positive areas at the tips of the villi consisted of a single band.

DISCUSSION

Although a few investigators have noted the presence of a bilaminar reaction at the cuticular surface of the mucosal cells of the intestine, no detailed study has been made of the distribution of this reaction or of the factors which may be involved in its demonstration. Bourne (1944) and Deane & Dempsey (1945) merely mention the presence of a bilaminar reaction. Hébert (1950) and Moe (1952) both considered it to be produced by diffusion during prolonged periods of incubation.

The present work shows that, during the final step of the Gomori technique, in which the sections are placed in ammonium sulphide, three different reactions can be produced at the free surfaces of the mucosal cells of the intestine. There appears to be two possible explanations of this variation in the histological picture. First, the positive bands may represent regions with a high concentration of enzyme and the clear zones between them might be enzymically inactive regions. If this be so, then the disappearance of the clear zone, when the period in which the sections are placed in ammonium sulphide is increased, may indicate a diffusion of some product of reaction during the carrying out of the technique. Martin & Jacoby (1949), Gomori (1950, 1951), Yokoyama, Stowell & Mathews (1951), Novikoff (1951) and Moog (1951) have shown that a process of diffusion does occur during the incubation of sections in the substrate, and, whilst there seems to be some doubt as to whether it is the enzyme or the calcium phosphate that diffuses, most investigators consider that the latter substance is the diffusing agent. This problem of diffusion has thrown some doubt on the validity of the Gomori technique for the demonstration of the exact localization of alkaline phosphatase; however, when short periods of incubation are used, it does seem to be much more sensitive in demonstrating sites of low enzymic concentration than the azo-dye method of Manheimer & Seligman (1948).

In this investigation the appearances suggest that diffusion occurs into the submucosal cells of the intestine and possibly to the nuclei of the mucosal cells when the period of incubation is increased beyond 25 min. However, with incubation periods up to 24 hr. the bilaminar reaction always remains constant, a fact which appears to indicate that the replacement of the two bands by a single band is not due to diffusion of either the enzyme itself or the product of incubation, namely, calcium phosphate. Neither did prolonged periods in the cobalt nitrate have any influence on the appearance of the bilaminar reaction. Thus, as variations in the technique before the final step do not affect the bilaminar reaction, any change from a bilaminar to a single reaction, if due to diffusion, must be produced by a diffusion of the cobalt sulphide. This, however, is unlikely since there is no evidence of diffusion of the cobalt sulphide into the subjacent cytoplasm and nuclei of the epithelial cells during prolongation of this step of the technique (Pl. 1, fig. 6).

The second explanation may be that there are areas of high and low enzymic concentration in the free surfaces of the cell. If this were so it would be expected that the reaction between the cobalt phosphate and the ammonium sulphide would

occur more quickly and give a more intense reaction in those regions with the highest concentration. Those regions with low enzymic activity, that is the clear zones in the neighbourhood of the striated border, would need a longer time in the ammonium sulphide to give a positive reaction, since the amount of cobalt phosphate formed would be proportionate to the amount of enzyme present.

No matter which of these explanations is accepted the findings of this work are highly suggestive of a distribution of alkaline phosphatase in a bilaminar manner at the free surfaces of the mucosal cells.

In the intestines of the three species examined there appears to be a definite difference in the distribution of alkaline phosphatase in the mucosal cells. There is a difference not only between one species and another, but also between different parts of the intestine of one and the same animal. Along its length, each intestine shows a gradient of intensity of 'staining' and also a gradient of distribution of the bilaminar reaction at the striated border, the gradient of the bilaminar reaction falling more rapidly than the gradient of intensity.

In the cat and dog both gradients show a gradual decline along the length of the intestine from the duodenum to the terminal ileum, in contrast to which the duodenum and upper jejunum of the rat show relatively constant reactions which are followed by steep gradients in the terminal jejunum and upper ileum, the last 20 cm. of the ileum being completely negative.

There has been much speculation as to the functional significance of alkaline phosphatase in the small intestine. Cori (1925) showed that the larger molecules of the hexoses (galactose and glucose) were more rapidly absorbed from the intestine than the smaller molecules of the pentoses (xylose and arabinose), a fact which suggested an active absorption of the former sugars. Bárány & Sperber (1939) gave definite proof of the active absorption of glucose by demonstrating that it is capable of being absorbed against a concentration gradient existing between the intestinal contents and the portal blood. To explain the different rates of absorption of sugars Lundsgaard (1933) and Verzár and his co-workers (Verzár & McDougall, 1936) postulated that during the absorption of those sugars which are rapidly absorbed a phosphorylation process occurs. In support of this theory of phosphorylation Verzár showed that the rate of absorption of glucose and galactose is independent of their concentration, and that these two sugars undergo phosphorylation *in vitro* and *in vivo*. On the other hand, the pentoses, xylose and arabinose, do not undergo phosphorylation, and their rates of absorption are dependent on their concentration in the intestine. Verzár further showed that when animals are treated with the metabolic poisons, monoiodoacetic acid and phloridzin, the absorption of glucose and galactose is markedly decreased whilst the absorption of arabinose and xylose remains constant. He believed that monoiodoacetic acid and phloridzin inhibited the phosphorylation of glucose and galactose, thus abolishing their selective absorption.

On theoretical grounds, phosphorylation should be capable of increasing the rate of glucose absorption by the formation of concentration gradients across the intestinal epithelium. Glucose will diffuse into the intestinal cells as long as its concentration in the lumen of the intestine is higher than that in the interior of the cell. Should the glucose accumulate in the interior of the cell then its concentration would soon equal that in the intestinal lumen and further diffusion into the cell

would cease. However, if glucose, on entering the cell, is rapidly phosphorylated or esterified to form glucose phosphate the concentration gradient of glucose across the superficial membrane of the cell will be maintained and diffusion will continue. Since glucose exists only in its free form in the portal blood the glucose phosphate formed in the superficial part of the cell must undergo hydrolysis at some deeper part of the cell before passing into the blood stream. This hydrolysis or dephosphorylation of glucose phosphate will of itself cause a concentration gradient of glucose phosphate in the interior of the cell and also cause a sufficiently high concentration of glucose in the cell to produce a diffusion of this latter substance across the inner cell membrane into the portal blood. This system of esterification and hydrolysis would necessitate the presence of phosphatase in two layers in the mucosal cells. One of the chief objections to this theory of phosphorylation as the mechanism of active absorption of glucose in the intestine has been that, although phosphatase is capable of causing rapid hydrolysis, it is incapable of synthesizing biological phosphate esters which are essential for the formation of concentration gradients. Recently, however, Meyerhof & Green (1950) have shown that in the presence of high-energy phosphate donors, which are present in practically all tissues of the body, transphosphorylation by alkaline phosphatase can occur.

If some of the products of digestion are phosphorylated during absorption, then it might be postulated that the two bands of phosphatase which have been described would be capable of carrying out the processes of esterification and hydrolysis at the luminal surfaces of the mucosal cells.

It is unfortunate that in the literature there do not seem to be comparable figures for the rates of absorption of sugars from the intestines of the three animals which have been studied. However, Davidson & Garry (1940) found that glucose is not so rapidly absorbed from the intestine of the cat as from that of the rat, and Fisher & Parsons (1950) have shown that in the rat, even when the gradient of mucosal surface area in the small intestine and the gradient of utilization of glucose by the tissues of the intestinal wall are taken into account, there is a gradient of 'translocation' of glucose across the mucosa. These two findings would seem to favour the theory of phosphorylation when they are considered in the light of the present observations that the upper portion of the rat's intestine gives a much more positive reaction for alkaline phosphatase than the cat's, and that in the rat's intestine there is a gradient of intensity of reaction and also a gradient of distribution of the bilaminar reaction.

Ancillary evidence, derived from a study of the distribution of alkaline phosphatase in the kidney, of the localization of this enzyme in a bilaminar manner in the proximal convoluted tubules, where glucose is actively absorbed, will be presented in another paper by one of the present authors (Johnson).

SUMMARY

1. The presence of a bilaminar reaction at the free surfaces of the mucosal cells of the intestine has been confirmed.

2. This reaction can be constantly demonstrated by a modification of the Gomori technique which is described.

3. The effect of varying the period of incubation has been studied from the point of view of the bilaminar reaction and the phenomenon of diffusion.

4. In the small intestine of the rat, cat and dog a gradient of intensity of the general reaction and a gradient of bilaminar reaction have been observed.

5. The observations made in this work are discussed in relation to the process of phosphorylation.

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EXPLANATION OF PLATES

All the photomicrographs, except fig. 17, are of sections demonstrating the presence of alkaline phosphatase. No counterstaining was used.

Plate 1

Fig. 1. Jejunum of cat. Incubation time 60 min. Bilaminar reaction in the striated border, positive Golgi zone and positive nuclei. × 530.

Fig. 2. Jejunum of cat. Incubation time 60 min. Bilaminar reaction closing. × 530.

Fig. 3. Jejunum of cat. Incubation time 60 min. Reaction at the striated border in the form of a single layer. × 530.

Fig. 4. Jejunum of dog. Incubation time 5 min. Bilaminar reaction in the striated border, faintly positive superficial cytoplasm. × 530.

Fig. 5. Ileum of dog. Incubation time 5 min. Single reaction in the striated border at the base of a villus. × 530.

Fig. 6. Jejunum of cat. Incubation time 5 min. Bilaminar reaction, faintly positive superficial cytoplasm. × 530.

Fig. 7. Jejunum of cat. Incubation time 25 min. Bilaminar reaction. Cytoplasm, nuclei and Golgi zone faintly positive. × 530.

Fig. 8. Jejunum of cat. Incubation time 60 min. Bilaminar reaction. Positive cytoplasm, Golgi zone, and mucosal and submucosal nuclei. × 530.

Fig. 9. Jejunum of cat. Incubation time 120 min. Bilaminar reaction. Strongly positive cytoplasm, Golgi zone, and mucosal and submucosal nuclei. × 530.

Plate 2

Fig. 10. Duodenum of rat. Incubation time 20 min. Bilaminar reaction in the striated border; entire cytoplasm of the mucosal cells strongly positive. × 530.

Fig. 11. Lower ileum of rat. Incubation time 20 min. Scattered positive areas in the striated border at the tips of the villi. Submucosal cells positive. × 130.

Fig. 12. Ileum of cat. Incubation time 20 min. Negative reaction in the striated border at the bases of the villi, with single layer on their sides and bilaminar reaction towards their apices. × 130.

Fig. 13. Jejunum of rat. Incubation time 20 min. Bilaminar reaction in the striated border; superficial cytoplasm and Golgi zone positive. × 530.

Fig. 14. Jejunum of cat. Incubation time 25 min. Showing irregular reactions due to the action of glycerine-albumen mounting medium. × 40.

Fig. 15. Jejunum of cat. Incubation time 60 min. Showing the change from a bilaminar reaction on the side of the villus to a single reaction at the tip, the appearance being due to obliquity of the section. × 530.

Plate 3

Fig. 16. Jejunum of cat. Incubation time 5 min. Bilaminar reaction with intermediate third (granular) layer. × 2600.

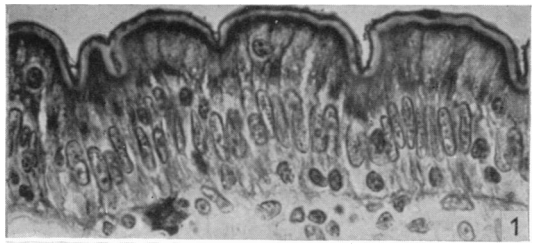
Fig. 17. Jejunum of cat. Stained with haematoxylin and eosin showing the granular layer at the base of the striated border. × 2600.

LIST OF ABBREVIATIONS

C.Z. Clear zone.
D.B. Deep band.
D.L. Deep layer of superficial band.
G.L. Granular layer.
S.B. Superficial band.
S.L. Superficial layer of superficial band.
W. Pale line dividing superficial and deep layers of superficial band.

ADDENDUM

Since this paper was written, Professor J. F. Danielli has kindly provided the authors with pieces of small intestine of the cat which had been freeze-dried. Sections of this material prepared and treated by the methods described in the present paper also revealed a bilaminar reaction in the mucosal cells.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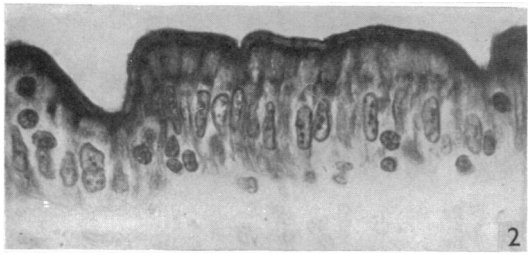
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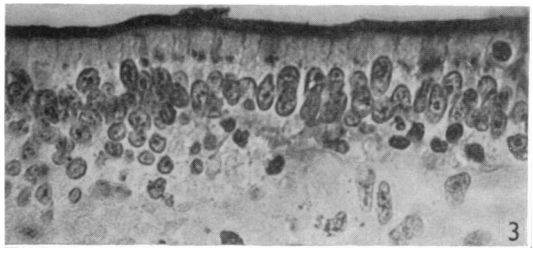
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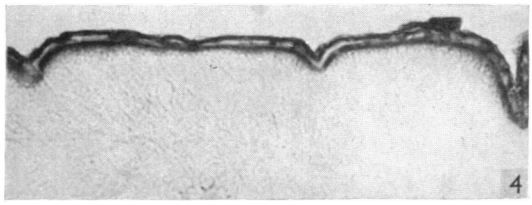
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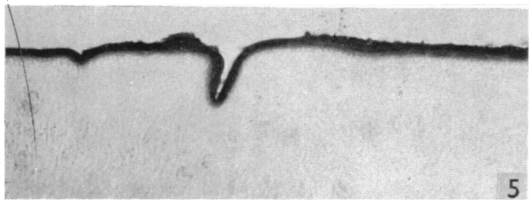
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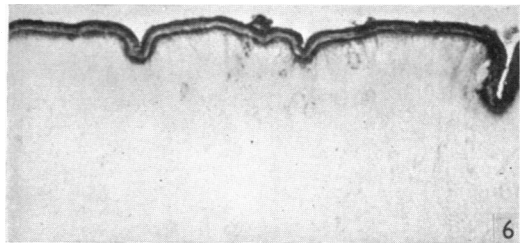
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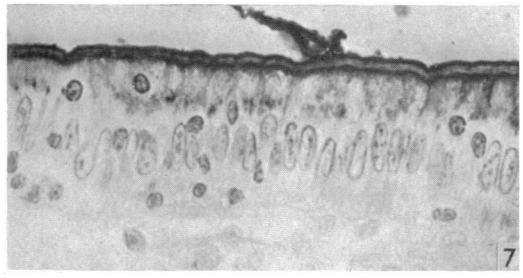
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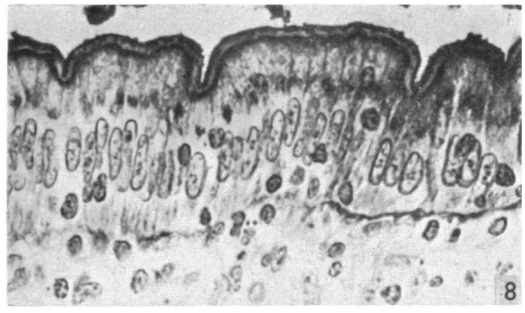
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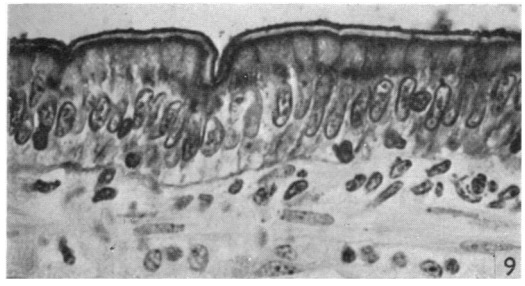
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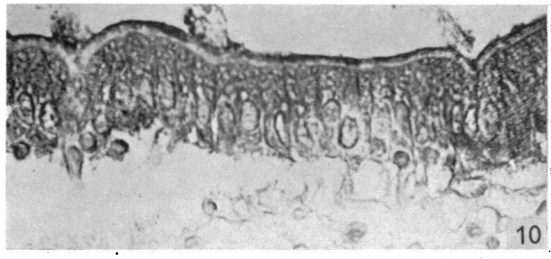
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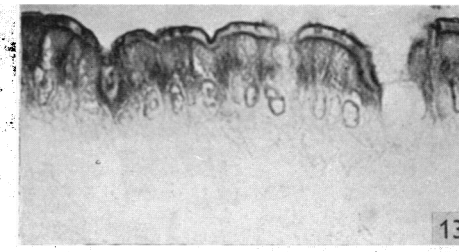
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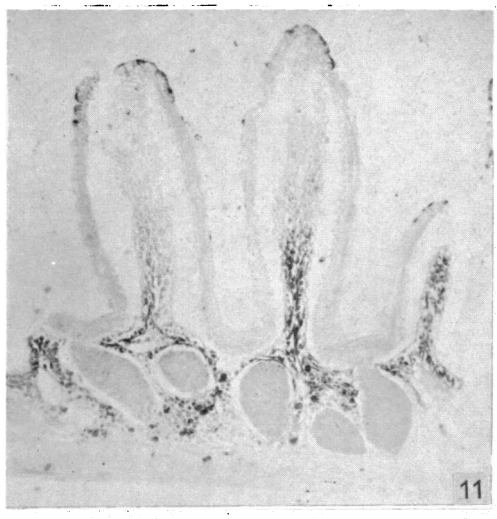
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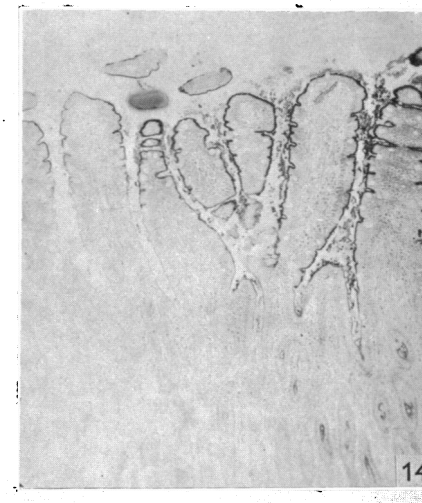
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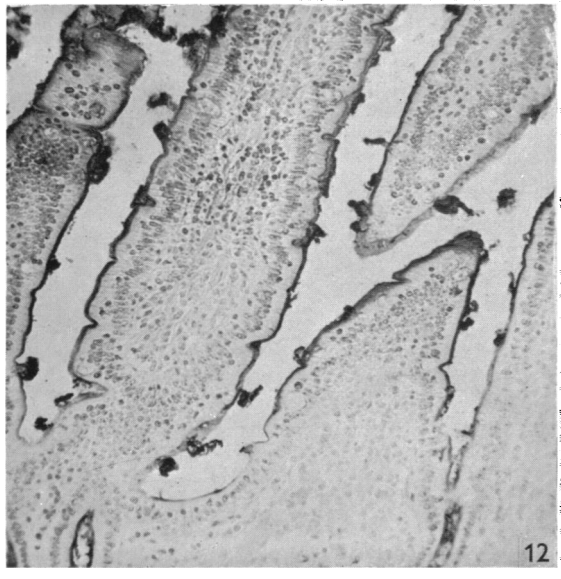
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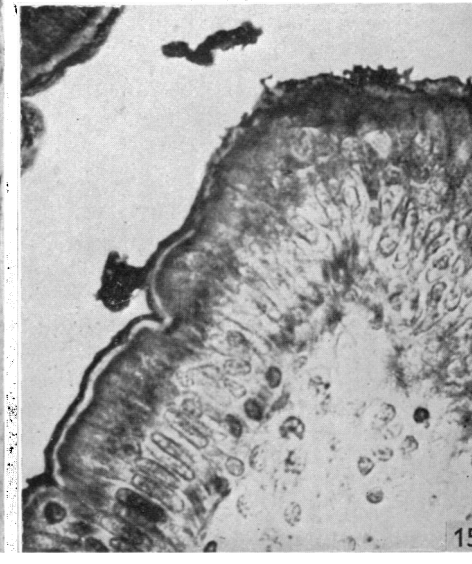
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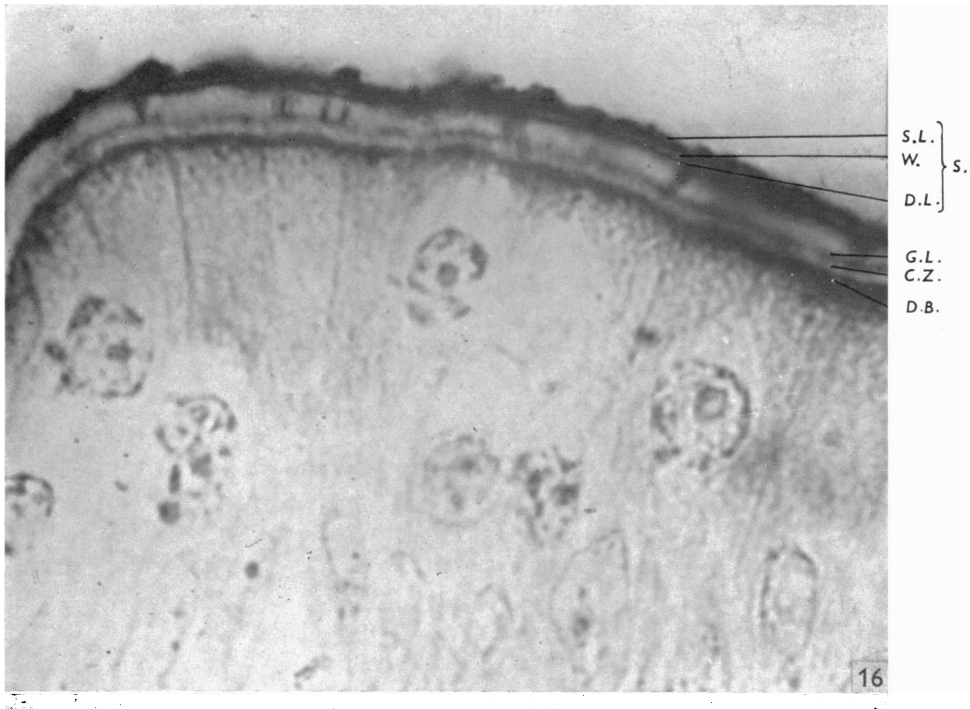

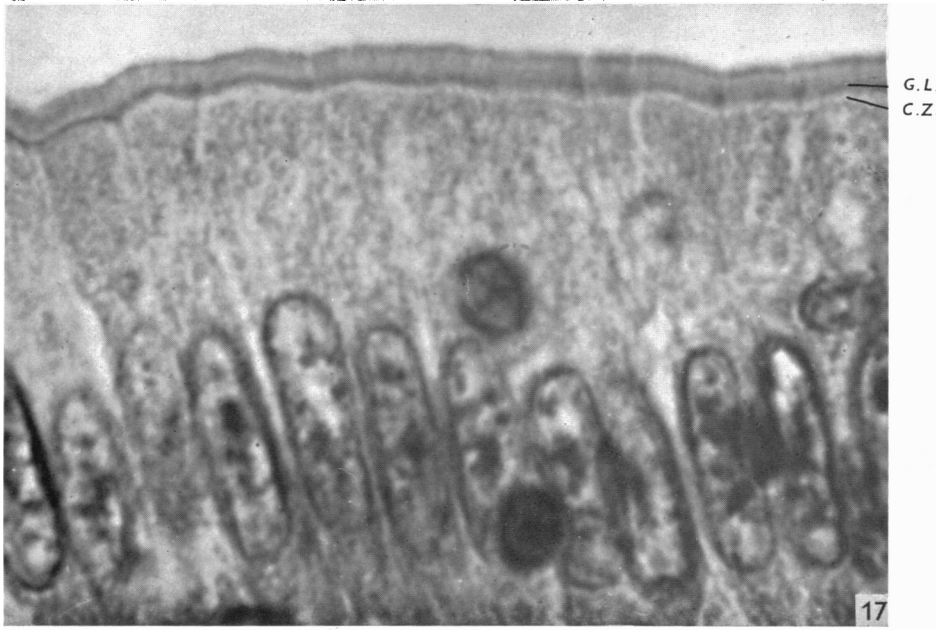

JOHNSON AND KUGLER—ALKALINE PHOSPHATASE IN THE MUCOSAL CELLS OF SMALL INTESTINE