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Isolation of Microvillus Plasma Membranes from Suckling-Rat Intestine

THE INFLUENCE OF PREMATURE INDUCTION OF DIGESTIVE ENZYMES BY INJECTION OF CORTISOL ACETATE

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1. Cortisone administration to suckling rats leads prematurely to induction of enzymes of the intestinal microvillus plasma membrane and lengthening of the intestinal microvilli. To investigate the membrane changes that might be involved, a method for the isolation of a fraction enriched with microvillus plasma membrane was developed in suckling rats. Plasma-membrane fractions were compared from 13-day-old control rats and from 13-day-old rats given cortisol acetate by subcutaneous injection for 3 days. 2. After cortisol injection, the activity of maltase, trehalase, sucrase and leucyl β-naphthylamidase increased markedly, and to the same extent, in intestinal homogenates and plasma-membrane preparations. Purification, and recovery of five marker enzymes with respect to homogenate activity, and recovery of protein, were similar for both membrane preparations, particularly after correction for non-membrane activity, which was high in suckling rats and affected by cortisol. 3. In material released from the plasma membrane by digestion with papain, maltase protein was increased after cortisol injection at least as much as maltase activity. Sucrase activity increased at least 200-fold, and this increase was associated with the appearance of a new sucrase band on polyacrylamide-gel electrophoresis. 4. Sodium dodecyl sulphate electrophoresis of plasma-membrane proteins revealed at least four additional macromolecules after cortisol injection. Concurrently several proteins disappeared from the plasma membrane. The added proteins appeared in the main to be removed from the plasma membrane by papain, whereas the deleted proteins were in the papain-resistant fraction. 5. Enzymic stimulation induced by cortisol acetate in the suckling-rat plasma membrane therefore appears to involve the addition of new proteins, rather than activation of proteins *in situ*. Deletion of proteins from the membrane during induction of hydrolytic enzymes may reflect other phenomena such as protein reorganization associated with the change in microvillus shape.

During postnatal development, the small-intestinal epithelial cell undergoes important changes in membrane structure and function which radically affect its capacity for digestion and absorption. The microvilli become elongated and narrow (Overton & Shoup, 1964), pinocytosis ceases (Clark, 1959), the intestine becomes relatively sealed to the passage of proteins (Halliday, 1959; Jones, 1972) and the activity of a number of membrane-bound enzymes, including duodenal alkaline phosphatase, maltase, sucrase and trehalase, is dramatically increased (Moog, 1951; Rubino *et al.*, 1964). These changes can be induced prematurely by cortisone (Clark, 1959; Halliday, 1959; Doell & Kretchmer, 1964; Overton, 1965) and some are retarded by adrenalectomy (Moog *et al.*, 1954; Koldovský *et al.*, 1965; Galand & Jacquot, 1970).

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The cortisone-evoked increase in the activity of membrane-bound enzymes has been studied extensively but the mechanism has eluded clarification. Within 24h of parenteral cortisone administration sucrase can be demonstrated by immunofluorescence for the first time at the brush-border surface (Doell *et al.*, 1965), as if cortisone had induced synthesis of the enzyme *de novo*. However, at least in mice and chicks, activation of enzyme has been demonstrated after the administration of actinomycin D, puromycin and cycloheximide (Moog, 1966; Grey & Moog, 1966; Brown, 1971), suggesting that the ability to synthesize new enzyme protein may not be necessary to the induction process. Overton (1965) and Moog (1966, 1971) have suggested that activation may occur instead, as part of a general process of membrane reorganization.

To study these developmental changes with more precision we undertook to isolate microvillus plasma

membrane from small intestine of suckling rats before and after exposure to cortisol injections for 3 days. This report documents the suitability of our method of membrane preparation for comparing these isolated plasma membranes and correlates induction with the appearance of new plasma-membrane sucrase and maltase. Also it includes evidence that cortisol induces relatively complex changes in protein composition of the plasma membrane.

A portion of this work has been presented to the Gastroenterology Research Group Meeting of American Gastroenterological Association, San Francisco, 1974 (Galand & Forstner, 1974*a*).

Materials and Methods

Materials

General reagents were of analytical grade from Sigma Chemical Co. (St. Louis, Mo., U.S.A.), Fisher Scientific (Montreal, P.Q., Canada) and BDH Chemicals Ltd. (Poole, Dorset, U.K.). TEMED (*NNN'N'*-tetramethylethylenediamine), acrylamide and bisacrylamide were obtained from Eastman Kodak Co., Rochester, N.Y., U.S.A., sodium dodecyl sulphate from BDH Chemicals Ltd. Molecular-weight markers for sodium dodecyl sulphate–polyacrylamide-gel electrophoresis calibration were IgA (immunoglobulin A) dimer, IgG (immunoglobulin G) (gifts from B. Underdown, University of Toronto), α_1 and α_2 collagen from rat tail (gift from F. Keeley, University of Toronto), β-galactosidase (Worthington Biochemical Corp., Freehold, N.J., U.S.A.), bovine serum albumin (BDH Chemicals Ltd.), ovalbumin (Worthington Biochemicals Co.), papain and trypsin (Sigma Chemical Co.).

Animals

White Wistar rats were used without regard to sex. Litters of suckling rats were decreased to eight animals 2 days after birth. Standard lab chow and water were given *ad libitum* to the mother and to weaned rats.

Induction protocol

Each litter was divided into two groups of four rats. One group was untreated and served as control; the other four rats were given subcutaneous injections of cortisol acetate (Merck, Sharp and Dohme Ltd., Kirkland, P.Q., Canada), on postnatal day 10 (5.0mg/100g body wt.), day 11 (2.5mg/100g) and day 12 (2.5mg/100g). Both control and injected rats were killed at 13 days.

Membrane preparation

A modification of the method of Welsh *et al.* (1972) was used. Rats were killed by a blow on the head. The whole small intestine was removed and rinsed with cold 150mM-NaCl. Slices of whole intestine from three suckling rats, or one adult rat, were homogenized in 160ml of 5mM-EDTA, pH7.4, in a Waring blender for 15s. The homogenate was filtered through cheesecloth. Of the filtrate 10.0ml was retained for analysis and 150ml was centrifuged at 40000*g* for 30min. The supernatant was decanted and retained for analysis. The sediment was suspended in 15ml of 1.0M-Tris–HCl buffer, pH7.4, and stirred in a Vortex mixer for 5min. A 5.0ml batch of the mixture was placed on the top of a discontinuous sucrose-density gradient containing 5.0ml each of 10, 20, 30, 40 and 50% (w/v) sucrose in 0.5M-$MgCl_2$. The gradient was centrifuged for 15min at 63000*g*. Six portions (5.0ml each) were removed sequentially from the top of the gradient with a Pasteur pipette. Then 40.0ml of 5mM-EDTA was added to each fraction and mixed. The fractions were either centrifuged for 1 h at 40000*g* or dialysed against three changes of 100vol. of water and freeze-dried. In each case the pellet or the freeze-dried product was resuspended in 5.0ml of water. All operations were performed at 4°C. Fractions rich in microvillus membranes were identified by appropriate marker enzymes as described in the text.

Hydrolysis of membrane fraction with papain

Membrane preparations were incubated for 30min with papain at 37°C. Each incubation mixture contained (per ml) 0.5mg of membrane protein, 10μg of papain (crystalline, 17 units/mg of protein; Sigma Chemical Co.), 4.5μmol of EDTA, 12.5μmol of potassium phosphate buffer, pH7.0 and 3.0μmol of cysteine. At the end of the digestion period the mixture was cooled in ice and centrifuged at 40000*g* for 30min. The supernatants were dialysed against three changes of water for 3 days at 4°C and then freeze-dried. The freeze-dried fractions were then suspended in 0.5ml of ice-cold water.

Polyacrylamide-gel electrophoresis

For electrophoresis of material released from the plasma membrane by papain, 4% (w/v) polyacrylamide gels were prepared and used as described by Forstner (1971). For electrophoresis of membrane proteins solubilized by sodium dodecyl sulphate, the method of Neville (1971) was used, without addition of 2-mercaptoethanol or dithiothreitol as recommended by Maestracci *et al.* (1973). Molecular weights were determined from logarithm of molecular weight versus relative mobility (R_F) plots.

Protein bands were fixed for 1h in 5% (w/v) trichloroacetic acid, stained with 0.25% Coomassie Blue overnight and destained with 25% (v/v) methanol–10% (v/v) acetic acid. Glycoproteins were stained with a periodic acid–Schiff technique (Zacharius *et al.*, 1969). For quantitative densitometry, gels were stained with Fast Green (Gorovsky *et al.*, 1970) and scanned with a Gilford 2400 spectrophotometer

equipped with a linear transport gel scanner at 625nm.

To determine the location of specific enzymes, gels were sliced with a razor blade by hand at 2.0mm intervals. Gel slices were crushed with a tuberculin syringe fitted with a 21-gauge needle in 0.5ml of water and left overnight at 4°C. The solution overlying the gel fragments was then analysed for enzyme activities as described below.

Assays

Disaccharidases were determined as described by Dahlqvist (1964), except that lactase was measured at pH6.0. Alkaline phosphatase was determined with *p*-nitrophenylphosphate as substrate under conditions described by Forstner *et al.* (1968). Leucine β-naphthylamidase was determined by the procedure of Goldbarg & Rutenberg (1958) as modified by Forstner (1971). Succinate dehydrogenase was determined as described by Pennington (1961). Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. Glycoprotein hexosamine was determined as described by Forstner (1970). DNA and RNA were determined after extraction with cold 5% trichloroacetic acid and 95% (v/v) ethanol at room temperature and hydrolysis in 5% (w/v) trichloroacetic acid at 90°C for 15min. DNA was determined by the method of Croft & Lubren (1965) with calf thymus DNA as standard. RNA was determined by the orcinol method of Dische (1953).

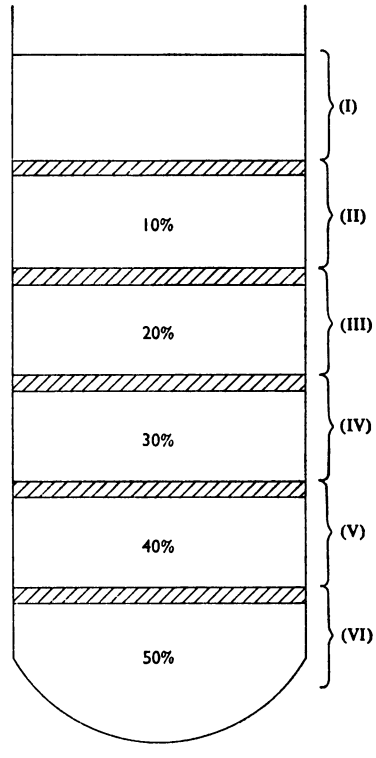


Fig. 1. *Density-gradient separation of membrane fragments, illustrating the source of fractions removed by Pasteur pipette*

Percentages refer to the sucrose concentration (w/v) in each zone. As noted in the Materials and Methods section membrane fragments were prepared from the precipitate obtained after centrifugation of the intestinal homogenate at 40000*g* for 30min, and by stirring vigorously in 1.0M-Tris–HCl, pH7.4.

Results

In preliminary experiments the activities of three enzymes, trehalase, maltase and sucrase, were followed in the intestinal mucosa of suckling rats at regular intervals from the beginning of the cortisol induction protocol. In each case a sigmoidal increase in activity occurred, which reached a maximum value between 84 and 96h. For convenience in subsequent experiments rats were killed at 72h, at which time the activity of the three enzymes ranged from 63 to 96% of the maximum value.

Initial attempts to prepare intestinal brush-border plasma membranes by conventional techniques (Forstner *et al.*, 1968) from suckling rats were unsuccessful. Few recognizable brush-border organelles were obtained and membrane yields were extremely low (<1%) and variable. A modified approach, based on the technique of Eichholz (1968) and similar to the method of Welsh *et al.* (1972) for isolating microvillus membrane from frozen human intestine, was therefore adopted. The 40000*g* precipitate obtained after centrifugation of an homogenate of rat intestine in 5.0mM-EDTA buffer, pH7.4, was disrupted in 1.0M-Tris–HCl buffer, pH7.4, and placed on a discontinuous sucrose density gradient as described in the Materials and Methods section. After centrifugation for 5min at 63000*g*, a number of bands formed at the sucrose interfaces. These were collected and labelled as shown in Fig. 1.

Fig. 2 shows the specific activities of five microvillus plasma-membrane marker enzymes in density-gradient fractions obtained from control (Fig. 2*a*) and cortisol-treated rats (Fig. 2*b*). Except for sucrase, which was only detected as a trace in fraction (III) from control rats, the enzyme markers were present in all gradient fractions from both experimental groups, with a peak of activity in fractions (III) (Fig. 2*a*) or (IV) (Fig. 2*b*). A minor and isolated alkaline phosphatase peak in fraction (I) was obtained from both groups. In general the activity profiles exhibited by all of the enzyme markers were very similar within each experimental group. It is noteworthy, however, that the highest specific activity was not found in the

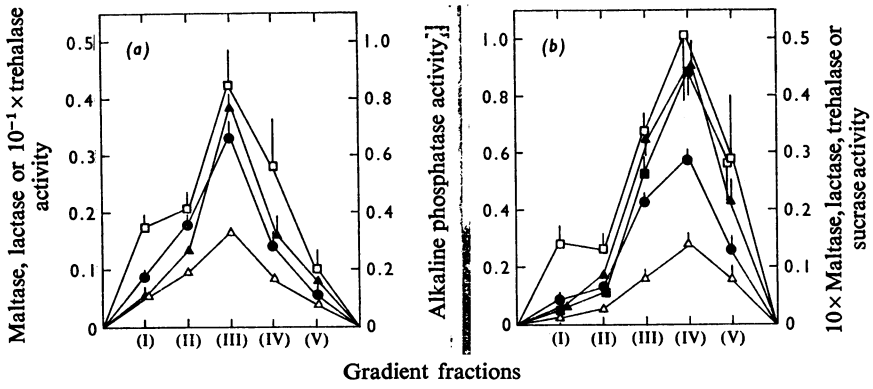


Fig. 2. *Specific activities of microvillus-membrane enzymes in density-gradient fractions*

Fractions were as described in Fig. 1. (*a*) 13-day-old suckling rats; (*b*) 13-day-old suckling rats after injection of cortisol acetate as described in the Materials and Methods section. □, Alkaline phosphatase; ■, sucrase; △, trehalase; ▲, lactase; ●, maltase. Points represent the mean units/mg of protein ±S.E.M. Number of experiments was as in Table 1.

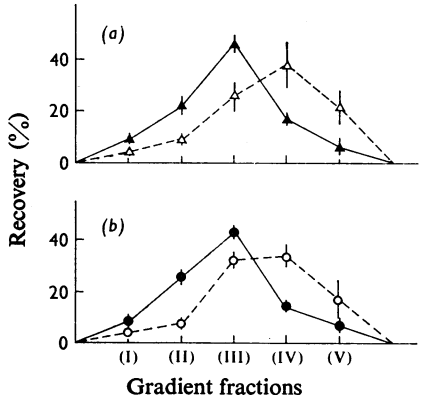


Fig. 3. *Recovery of trehalase and maltase in density-gradient fractions*

(*a*) Trehalase; percentage of total activity applied to the gradient, from 13-day-old suckling rats. ▲, Controls; △, injected with cortisol acetate as in the Materials and Methods section. (*b*) Maltase; percentage of total activity applied to the gradient, from 13-day-old suckling rats. ●, Controls; ○, injected with cortisol acetate. Points represent the mean±S.E.M. Number of experiments was as in Table 1.

same fraction in each group, but rather appeared above the 20% (w/v) sucrose zone ($d = 1.076$) in the control rats (i.e. in fraction III) and above the 30% sucrose zone ($d = 1.1128$) in the cortisol-injected rats (i.e. in fraction IV). Separate experiments (not shown) indicated that in adult rats the activity peak of several marker enzymes was shifted to zones of slightly higher sucrose concentration, suggesting that the isolated microvillus membranes from cortisol-injected rats were intermediate in density between those of the suckling and the adult animal.

Fig. 3 shows data obtained for trehalase and maltase, activity being expressed as a percentage of the total applied to the gradient. In each case there was a general shift of marker enzyme activity after cortisol injection to zones of greater density. Identical results were obtained with the remaining membrane markers. The change in fractions with the highest activity after cortisol treatment (Fig. 2) was associated therefore with an actual change in the density of membrane carrying the marker enzymes, and not with an unrelated shift in the distribution of contaminating protein. Fraction (III) in the control rats, and fraction (IV) in the injected rats, were therefore most representative of the plasma membrane in each group, since they contained the largest quantity of membrane marker as well as the highest specific activities of marker enzymes (Fig. 2). These fractions were always compared in subsequent experiments and are referred to as the plasma-membrane preparations or plasma membranes. The validity of this decision was supported by measurement of succinate dehydrogenase, DNA and RNA in gradient fractions as indices of mitochondrial, nuclear and ribosomal contamination (Fig. 4). Of all the fractions, fraction (III) (control rats) and fraction (IV) (injected rats) contained the smallest amount of contaminant and the greatest amount of trehalase. However, these preparations still contained 2–5% of the succinate dehydrogenase, DNA and RNA in the intestine, and therefore should be regarded as fractions enriched in plasma membranes but not pure.

Table 1 summarizes values obtained for total enzyme activity, protein and hexosamine in the whole mucosa and plasma-membrane preparations, in both

control and cortisol-treated groups. In most cases changes evoked by cortisol in the mucosa were paralleled by changes in the membrane. After cortisol treatment, both total mucosa protein and plasma-membrane protein were decreased to approximately the same extent (i.e. 12–15%), coinciding with a similar decrease in total body weight (21%; results not shown) and presumably part of a generalized catabolic response to the hormone. In contrast, mucosal and plasma-membrane acid-precipitable hexosamine increased slightly after cortisol treatment to approximately the same extent (19–21%). Both lactase and alkaline phosphatase were decreased in the whole mucosa of rats given cortisol, but increased slightly in the plasma membrane. Koldovský & Sunshine (1970) have shown that neutral β-galactosidase, which is known to be a membrane-bound enzyme, is weakly enhanced by cortisol in rats of similar age, and Moog (1953) showed that duodenal alkaline phosphatase is similarly responsive. The small increments in total lactase and alkaline phosphatase activity in the plasma membrane after cortisol treatment probably reflect such a response. Total leucine β-naphthylamidase, maltase and trehalase activites were markedly greater after cortisol treatment, each to a slightly greater extent in the membrane preparations than in the whole mucosa. Sucrase was not detected in mucosal homogenates from control rats, but weak sucrase activity was present in the plasma-membrane preparation from the control animals. As noted in Table 1, the stimulation in membrane sucrase activity produced by cortisol was immense in comparison with either maltase or trehalase.

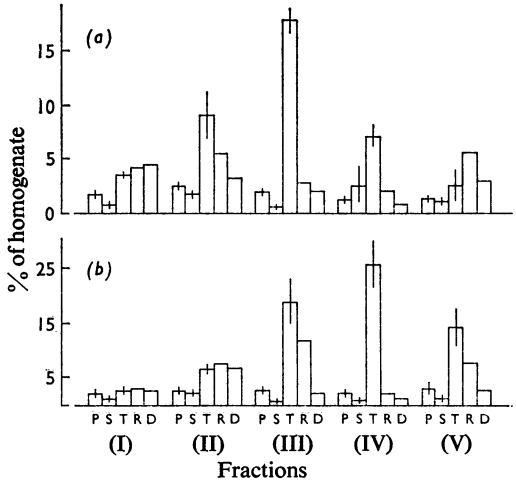


Fig. 4. *Protein, succinate dehydrogenase, trehalase, RNA and DNA in gradient fractions*

(*a*) Control 13-day-old suckling rats; (*b*) 13-day-old suckling rats given cortisol acetate as described in the Materials and Methods section. P, Protein; S, succinate hydrogenase; T, trehalase; R, RNA; D, DNA. Values for RNA and DNA are the mean of two experiments, for succinate dehydrogenase the mean±S.E.M. for four experiments, and for protein and trehalase the mean±S.E.M. for the number of experiments shown in Table 1.

Table 2 compares the specific activities and purification ratios of membrane-bound enzymes in whole mucosa and plasma-membrane fractions, in control and cortisone-treated rats. All enzyme specific activities were greater in the membrane than in the mucosa, and cortisone markedly increased the specific activity of maltase, sucrase and trehalase in both fractions. Purification of plasma-membrane enzymes was variable and ranged from 4.6- to 12.8-fold. Although purification ratios were generally similar for each enzyme in both experimental groups, in each

Table 1. *Total protein, hexosamine and enzyme in intestinal mucosa and microvillus plasma membrane from cortisol-treated and untreated 13-day-old rats*

The induction protocol is described in the Materials and Methods section. Rats were killed 72h after the first injection of cortisol acetate. Values given are the mean±S.E.M. per rat intestine. The number of separate preparations studied is in parentheses. Enzyme results are in EC units.

	Mucosa			Membrane		
Assay	−Cortisol	+Cortisol	% change	−Cortisol	+Cortisol	% change
Protein (mg)	86.4 ±4.7 (10)	72.98±4.2 (7)	−15	1.77 ±0.18 (10)	1.54 ±0.15 (7)	−12
Hexosamine (mg)	0.89±0.07 (4)	0.97±0.1 (4)	+21	0.049 ±0.012 (3)	0.058±0.012 (4)	+19
Lactase	4.37±0.36 (10)	3.5 ±0.33 (7)	−20	0.66 ±0.08 (10)	0.75 ±0.05 (7)	+13
Alkaline phosphatase	17.1 ±1.7 (5)	9.9 ±0.9 (3)	−42	1.58 ±0.26 (5)	1.93 ±0.5 (3)	+18
Leucyl β-naphthylamidase	2.56±0.39 (8)	4.62±0.51 (7)	+180	0.38 ±0.07 (6)	0.83 ±0.15 (5)	+218
Maltase	4.57±0.39 (11)	29.7 ±1.4 (9)	+650	0.56 ±0.05 (7)	4.78 ±0.61 (6)	+850
Trehalase	0.12±0.03 (8)	0.8 ±0.08 (7)	+666	0.029 ±0.01 (5)	0.233±0.05 (4)	+800
Sucrase		3.44±0.21 (7)		0.0039±0.001	0.88 ±0.09 (7)	+22500

Table 2. *Specific activities of marker enzymes in intestinal mucosa and microvillus plasma membrane*

Conditions are as described in Table 1. Values are expressed as means±S.E.M. The number of separate preparations assayed is shown in parentheses. n.d., Not determined. Enzyme results are in EC units/mg of protein.

	Without cortisol treatment			With cortisol treatment		
Enzyme	Mucosa	Membrane	Purification (fold)	Mucosa	Membrane	Purification (fold)
Lactase	0.050 ±0.003 (10)	0.399 ±0.06 (10)	8.0±0.8 (10)	0.049 ±0.003 (10)	0.480±0.045 (7)	10.0±0.7 (7)
Alkaline phosphatase	0.178 ±0.012 (5)	0.864 ±0.142 (5)	4.6±0.7 (5)	0.146 ±0.004 (3)	1.056±0.215 (3)	7.7±1.7 (3)
Leucine β-naphthylamidase	0.033 ±0.004 (8)	0.23 ±0.005 (6)	7.3±1.1 (6)	0.070 ±0.007 (7)	0.516±0.079 (5)	7.5±0.4 (5)
Maltase	0.057 ±0.004 (11)	0.310 ±0.02 (7)	5.8±0.4 (7)	0.45 ±0.02 (9)	3.10 ±0.3 (6)	7.1±0.6 (6)
Trehalase	0.0014±0.0002 (8)	0.0164±0.001 (5)	10.6±1.6 (5)	0.0125±0.001 (7)	0.146±0.02 (4)	12.8±1.1 (4)
Sucrase	n.d.	0.0023±0.0005 (7)	—	0.047±0.003 (9)	0.45 ±0.04 (7)	9.5±0.8 (7)

Fig. 5. *Supernatant enzyme activity in control and cortisol-injected suckling rats*

The supernatants were obtained by centrifugation of the intestinal homogenate at 40000*g* for 30min. Percentages are the mean values±S.E.M. and the number of separate preparations are as given in Table 1. Open bars represent values for control 13-day-old suckling rats, and stippled bars those for 13-day-old suckling rats given cortisol acetate as described in the Materials and Methods section. (1) Maltase; (2) trehalase; (3) lactase; (4) alkaline phosphatase; (5) leucine β-naphthylamidase.

case purification was greater in the plasma membranes from the cortisol-treated rats. Comparison of the two membrane preparations was complicated, however, by the presence of a great deal of marker activity in the 40000*g* supernatant fraction from the suckling rat homogenates. This soluble fraction was, as shown in Fig. 5, 10–30% greater in the control rats than in the cortisol-treated rats depending on the marker studied. Soluble maltase activity has been examined in detail (Galand & Forstner, 1974*b*) in suckling rats and found to consist of two membrane-free maltases, one of which is derived from lysosomes. It seems likely that some of the soluble activity seen with the other markers has a similar derivation. Acid β-galactosidase (Alpers, 1969), for example, is very active in suckling-rat intestine (Koldovsky & Sunshine, 1970), and could contribute significantly to total soluble lactase activity if released from lysosomes. As with maltase (Galand & Forstner, 1974*b*) it is also possible that plasma-membrane-like enzymes may be much more soluble in suckling rats, before injection with cortisol or weaning, than afterwards. In any case it seemed probable that a more appropriate comparison of plasma membranes obtained in the two sets of experimental conditions would be achieved by eliminating soluble activity from consideration. Table 3 presents recovery and purification data for the plasma-membrane marker enzymes based only on activity found in the precipitate obtained by centrifugation of the homogenate at 40000*g* for 30min. These results show that the degree of purification of several enzyme markers in the

Table 3. *Recovery and purification of plasma-membrane enzymes after correction for marker enzyme activity in the 40000g supernatant*

Conditions are as described in Table 1. The number of separate preparations assayed is shown in parentheses. Precipitable activity was determined by subtracting activity in the 40000*g* supernatant from the activity of the total homogenate. Purification was determined by calculating the initial specific activity of membrane-bound enzyme in the homogenate, as precipitable activity/total homogenate protein.

	% of precipitable activity		Purification (fold)	
	−Cortisol	+Cortisol	−Cortisol	+Cortisol
Lactase	24.2±2.9 (10)	25.7±3.5 (7)	12.3±1.4 (10)	11.9±1.3 (7)
Maltase	22.4±2.5 (7)	19.4±2.3 (6)	10.7±1.1 (6)	9.2±1.2 (5)
Leucyl β-naphthylamidase	20.1±3.1 (6)	19.6±3.3 (5)	10.4±2.0 (6)	8.5±2.0 (5)
Alkaline phosphatase	28.7±6.1 (5)	41.5±9.6 (3)	15.0±1.8 (4)	17.0±2.7 (3)
Trehalase	21.1±1.9 (5)	27.7±5.2 (4)	13.0±2.4 (5)	13.5±1.1 (4)
Sucrase		27.9±2.7 (7)		10.4±1.0 (7)

plasma membranes from control and injected rats is very similar, in contrast with the data in Table 2. Recovery of alkaline phosphatase exhibited the largest variation, but the difference between the two membranes was not statistically significant. Taken together with the fact that the recovery of protein in the plasma-membrane preparations is virtually identical for control [2.0±0.1% (10)] and injected rats [2.1±0.1% (7)], these results provide strong evidence that the two membrane preparations are basically comparable.

In view of the fact that a great increase in marker enzymes was found in the plasma-membrane preparations from cortisol-treated rats, whereas the recovery of protein was unchanged, the preparations were examined to determine whether the increased specific activity of some of the marker enzymes might simply be due to activation *in situ* of proenzymes.

Plasma membranes were prepared from 13-day-old rats, both treated and untreated with cortisol, and the papain-soluble fraction which has been characterized with respect to enzyme content and electrophoretic behaviour in adult rats (Forstner, 1971) was examined by polyacrylamide-gel electrophoresis. Plate 1 compares simultaneous runs in which identical amounts of papain-solubilized protein were applied to 4% (w/v) polyacrylamide gels and stained for protein and glycoprotein. The position of maltase, sucrase and alkaline phosphatase was established by serial slicing of an identical gel and determination of enzyme activity. No sucrase activity was found in the gels from control rats, and it is significant that the sucrase area in these gels contained no protein or glycoprotein. In addition there was a marked increase in material staining for protein and glycoprotein in the maltase area after cortisol treatment, associated with a corresponding increment in maltase activity (see below). The periodic acid–Schiff stain also revealed a predominance of fast-travelling glycoproteins in the material solubilized by papain from control plasma membranes. The most advanced glycoproteins in the gels disappeared after cortisol treatment, whereas the periodic acid–Schiff stain became more intense at the upper or slower-moving boundary, forming a rather distinct band at this margin.

Table 4. *Maltase activity and maltase protein in papain-solubilized material from control and cortisol-injected suckling rats*

Papain-solubilized protein (60–280 μg) was applied to 4% (w/v) polyacrylamide gels, and electrophoresis and staining with Fast Green were performed as described in the Materials and Methods section. Maltase protein was estimated by densitometry, the area under the maltase peak being expressed as a fraction of total stained area, and multiplying by the total protein applied to the gel. Each experiment was performed independently with material derived from different litters.

	Protein (μg)	Activity (μmol/min)
Expt. 1		
(*a*) −Cortisol	6.0	0.16
(*b*) +Cortisol	27.0	0.70
(*b*)/(*a*)	4.5	4.3
Expt. 2		
(*a*) −Cortisol	30.0	0.67
(*b*) +Cortisol	69.0	1.70
(*b*)/(*a*)	2.3	2.5

The maltase band was separated clearly from other proteins (Plate 1), and unlike sucrase, it was also present in the control rats, so that it was possible to determine whether enzyme activity increased out of proportion to enzyme proteins after cortisol treatment. As Table 4 shows in two experiments the increment in maltase protein after cortisol treatment was proportional to the increased amount of maltase activity in the sample applied to the gel. The sample, after cortisol treatment, also contained some maltase associated with the sucrase–isomaltase complex (Galand &

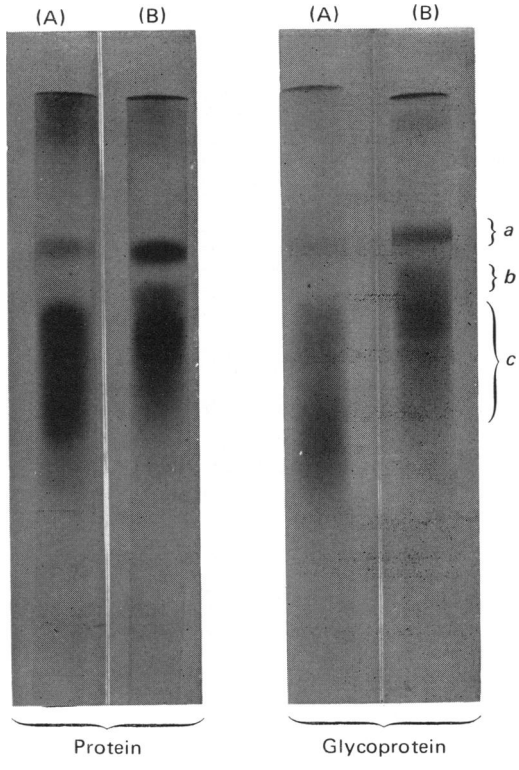
Forstner, 1974*b*), but since the presence of this component leads to slight over-estimation of the increase in the activity of the maltase band, it would not affect the essential conclusion that the increase in protein in the maltase band is at least as great as the increase in enzymic activity. Thus at least for maltase, and for sucrase which appears as a completely new band, the possibility that activation of a protein of similar molecular weight and electrophoretic mobility contributes to the increase in enzyme activity after administration of cortisol seems unlikely.

These experiments appear to suggest that new proteins are added to the plasma membrane as a result of cortisol injection. If so, other proteins must be deleted since the recovery of protein in the two membrane preparations was unchanged. Alternatively, it is important to exclude the possibility that differences might be confined to the papain-soluble fraction owing to differing susceptibility of the two membranes to the enzyme. Intact plasma membranes were therefore solubilized in 2% sodium dodecyl sulphate and subjected to polyacrylamide-gel electrophoresis (Plate 2, gels 1 and 2). The papain-solubilized proteins and the papain-resistant residue were treated similarly to determine whether these fractions truly reflected any change taking place in the whole membrane (Plate 2, gels 3–6). Solubilized proteins were not reduced in order to limit disaggregation of functional units, since several of the induced enzymes are known to be polymeric (Cogoli *et al.*, 1973; Maestracci *et al.*, 1973). Plate 2 (gels 1 and 2) clearly shows that the pattern of protein banding in plasma membranes from control and cortisol-treated rats is different. Further, there is evidence of deletion as well as of addition. Proteins of approximately 90000, 95000, 120000 and 150000 daltons disappear after cortisol injection, and bands of 140000, 170000 and 400000 daltons appear. Both the 140000- and 400000-dalton bands were present in material removed from the membrane by papain (Plate 2, gels 3 and 4), again predominantly in the cortisol-treated rats, indicating that these bands represent induced proteins removed in a relatively unchanged state by papain. The papain-soluble material was particularly distinguished by a very strong band of approx. 200000 daltons which appeared in the membranes from the cortisol-treated rats. This band was very faint in the intact membrane and its appearance in the papain-solubilized material suggests considerable concentration, as might be expected of sucrase which also has a molecular weight of 200000. Similarly the 400000-dalton band could represent maltase, which is known to have a high molecular weight (Forstner, 1971; Galand & Forstner, 1974*b*). Maestracci *et al.* (1973) have identified a similar 420000-dalton band with maltase activity in human intestinal membranes. Papain seemed to result in the disappearance of protein bands in the 180000-dalton region, whereas proteins in the 90000-dalton area were increased. These changes were approximately equivalent in membranes of both experimental groups and might therefore represent the result of proteolytic cleavage of macromolecules originally present in both membranes. Examination of the papain-resistant residue was less rewarding owing to the difficulty experienced with solubilization, but the patterns obtained suggest that the 95000- and 150000-dalton proteins prominent in the control plasma membranes may be principally confined to this portion of the membrane, since they were present in the residue from control rats and were absent after cortisol treatment. The papain-resistant residue contained no protein of molecular weight over 150000, and particularly no band at 180000 daltons. In the absence of the appearance of a new prominent band at lower molecular weight these results appear to indicate that all of the macromolecular material over 150000 daltons was released by papain, even though it was not subsequently always recognized in the soluble fraction.

Discussion

Our studies indicate that microvillus plasma membrane can be isolated reproducibly in good yield from suckling-rat intestine, and more importantly that the same method of preparation is applicable to membranes isolated after the induction of membrane-associated enzymes by cortisol. Comparative purification of the two membrane preparations was particularly difficult to assess because of the different activities of soluble enzymes in the suckling-rat intestine. When soluble activity was excluded from the calculations, however, the purification of the membrane-bound enzymes in both preparations was identical. Also the same percentage of protein and similar percentages of membrane-bound marker enzymes were recovered. The justification for excluding the activity of soluble markers rests with the assumption that the soluble enzymes exist independently of the plasma membrane in the cell. We have obtained evidence that this is true for maltase in the suckling rat (Galand & Forstner, 1974*b*). The close similarity between the results obtained for maltase and those of other markers suggests that the assumption may be universally applicable.

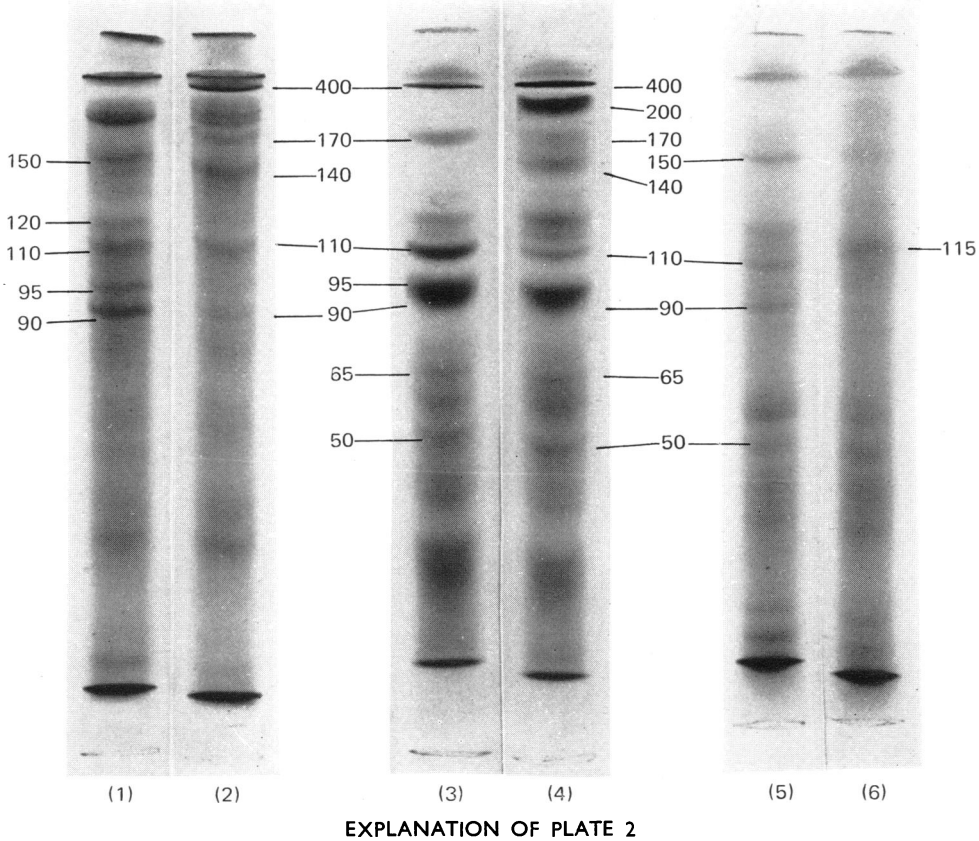
Enhancement of leucine β-naphthylamidase, maltase, trehalase and sucrase activities in the membrane was qualitatively similar to that produced by cortisol in the whole mucosa. Induced enzymes therefore appear rapidly on the surface membrane, in accord with previous evidence obtained for sucrase by immunofluorescence (Doell *et al.*, 1965). Trehalase has not previously been shown to respond to cortisol in the same manner as sucrase (Doell & Kretchmer, 1964), leucine β-naphthylamidase (Moog, 1971) and maltase (Moog *et al.*, 1973).



EXPLANATION OF PLATE 1

Polyacrylamide-disc-gel electrophoresis of papain-soluble protein and glycoprotein from microvillus plasma-membrane fractions

Material solubilized with papain from fraction (III) from control rats (gels B) was compared with similar material from fraction (IV) obtained from suckling rats given cortisol acetate (gels A). For this 4% polyacrylamide gels were used and stained as described in the Materials and Methods section. Identical quantities of protein were applied to each gel. *a*, Maltase band; *b*, sucrase band; *c*, location of lactase, alkaline phosphatase and leucyl β-naphthylamidase.



EXPLANATION OF PLATE 2

Polyacrylamide-gel electrophoresis of whole membrane, papain-sensitive and papain-insensitive fractions of membrane, solubilized with 2% sodium dodecyl sulphate

Electrophoresis was performed in 11% gels by using the sulphate–borate–sodium dodecyl sulphate buffer described by Neville (1971). Gels (1) and (2) contained 120μg of protein from complete plasma membrane, derived from control rats (gel 1) and cortisol-injected rats (gel 2). Gels (3) and (4) contained 80μg of protein from papain-solubilized membrane fraction. Gel (3) was from control rats and gel (4) from cortisol-injected rats. Gels (5) and (6) contained 150μg of protein from papain-resistant membrane fraction. Gel (5) was from control rats, gel (6) from cortisol-injected rats. Bands are labelled according to their estimated mass in thousands of daltons. Above 110×10^3 daltons the relationship between logarithm of molecular weight and R_F values is not linear, and therefore these estimates are approximate only.

Simple activation of existing enzyme accounts for at least part of the increased activity of alkaline phosphatase in cell culture exposed to cortisol (Griffin & Cox, 1966), and has been proposed (Moog, 1971) to account for the increased activity of membrane-bound enzymes in the intestine. Our studies make this possibility very unlikely for at least two enzymes, maltase and sucrase. The amount of protein in the isolated maltase band was increased substantially in response to cortisol (Table 4), and at least as much as the enzymic activity. Additional activity was therefore almost certainly accounted for by an increase in enzyme protein. Sucrase activity increased 200-fold in the plasma membrane under the same stimulus, and this increase coincided with the appearance *de novo* of an electrophoretic protein band with sucrase activity in papain-solubilized membrane material. Sucrase has a molecular weight of approx. 200000 (Kolinska & Semenza, 1967). The possibility that papain may not have solubilized such a molecule in the control rats was excluded by failure to find it in the papain-resistant residue. At the same time electrophoresis of sodium dodecyl sulphate-treated papain-solubilized material showed that a protein with a molecular weight of 200000 definitely appeared after cortisol treatment. The massive increase in sucrase activity therefore appears to be directly related to a great increase in the number of sucrase molecules and not to activation of pre-existing enzyme. The number of new or more prominent protein bands demonstrated by electrophoresis of sodium dodecyl sulphate-solubilized membrane was also roughly commensurate with the number of enzymes shown to be affected by cortisol. It seems reasonable to conclude therefore that induced activity is generally associated with the appearance of additional enzyme protein in the membrane. Whether the additional enzyme protein is initially derived by acceleration or initiation of ribosomal synthesis, by intra- or extra-membranous molecular transformation of inactive proenzymes, or by a significant decrease in the rate of enzyme destruction, remains unclear. The latter two possibilities can be explored with the use of isolated plasma membranes, however.

Since reducing agents were not used in the present studies with sodium dodecyl sulphate the molecular-weight estimates should be regarded as approximations (Fish *et al.*, 1970). The majority of the protein changes also occur in a molecular-weight range that cannot be calibrated easily on polyacrylamide-gel electrophoresis, and many involve glycoproteins which may behave anomalously (Segrest *et al.*, 1971). Nevertheless induced bands of estimated molecular weight appropriate to maltase and sucrase were seen, suggesting that the difficulties may be more apparent than real. The differences in the protein patterns of intact plasma membrane from control and cortisol-treated rats were consistently present in multiple preparations. Random aggregation of unrelated proteins to form new bands, or variable mobility of individual proteins are unlikely problems therefore, since the factors promoting these aberrations would apply equally to both membranes and should not account for reproducible differences.

In spite of an apparent increase in density and in enzyme activities, our analysis failed to reflect a net increase in membrane protein in the cortisol-injected rats. In part this failure was a consequence of the deletion, after administration of hormone, of several proteins. The deleted proteins have not been definitely identified with the plasma membrane, but it is evident that two at least may be associated with the papain-resistant portion of the membrane, rather than the papain-sensitive fraction, which contains most of the induced enzymes. This observation invites the conclusion that cortisol has a complex effect, incompletely defined by the known changes in brush-border hydrolases. The change in the mobility of glycoproteins seen in the 4% sodium dodecyl sulphate–polyacrylamide gels also seems to involve proteins which have not been identified with known hydrolases. It is tempting to suggest that these non-enzymic changes may reflect alterations in both proteins integral to the membrane, and superficial glycoproteins at the surface, which are related to the major reorganization in membrane structure hinted at by elongation and narrowing of the microvilli (Overton & Shoup, 1964). At any rate it is evident that there are a sufficient number of alterations in protein composition to satisfy the requirements of the known functional and structural changes induced by cortisol. Clearly no single protein change can be held accountable for all other changes, although there is still a possibility that a single organizer protein might be induced as a primary event (Moog, 1971). Since the present studies indicate that changes initiated by hormonal action can be defined with relative precision in the plasma membrane, however, a systematic study of the mechanisms responsible for them may provide a much clearer understanding of the controlling factors operative in intestinal development.

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References

Alpers, D. H. (1969) *J. Biol. Chem.* **244**, 1238–1246
Brown, K. M. (1971) *J. Exp. Zool.* **177**, 493–506
Clark, S. (1959) *J. Biophys. Biochem. Cytol.* **5**, 41–50
Cogoli, A., Eberle, A., Sigrist, H., Joss, C., Robinson, E., Mosimann, E. & Semenza, G. (1973) *Eur. J. Biochem.* **33**, 40–48

Croft, D. N. & Lubren, M. (1965) *Biochem. J.* **95**, 612–620
Dahlqvist, A. (1964) *Anal. Biochem.* **7**, 18–25
Dische, Z. (1953) *J. Biol. Chem.* **204**, 983–997
Doell, R. & Kretchmer, N. (1964) *Science* **143**, 42–43
Doell, R., Rosen, G. & Kretchmer, N. (1965) *Proc. Nat. Acad. Sci. U.S.* **54**, 1268–1273
Eichholz, A. (1968) *Biochim. Biophys. Acta* **163**, 101–107
Fish, W. W., Reynolds, J. A. & Tanford, C. (1970) *J. Biol. Chem.* **245**, 5166–5168
Forstner, G. (1970) *J. Biol. Chem.* **245**, 3584–3592
Forstner, G. (1971) *Biochem. J.* **121**, 781–789
Forstner, G., Sabesin, S. & Isselbacher, K. J. (1968) *Biochem. J.* **106**, 381–390
Galand, G. & Forstner, G. (1974*a*) *Gastroenterology* **66**, 693
Galand, G. & Forstner, G. (1974*b*) *Biochem. J.* **144**, 281–292
Galand, G. & Jacquot, R. (1970) *C. R. Acad. Sci. Ser. D* **271**, 1107–1110
Goldbarg, J. A. & Rutenberg, A. M. (1958) *Cancer* **11**, 283–291
Gorovsky, M. A., Carlson, K. & Rosenbaum, J. L. (1970) *Anal. Biochem.* **35**, 359–369
Grey, R. D. & Moog, F. (1966) *Nature (London)* **211**, 418–419
Griffin, M. & Cox, R. (1966) *Proc. Nat. Acad. Sci. U.S.* **56**, 946–953
Halliday, R. (1959) *J. Endocrinol.* **18**, 56–66
Jones, R. (1972) *Biochim. Biophys. Acta* **274**, 412–419
Koldovský, O. & Sunshine, P. (1970) *Biochem. J.* **117**, 467–471
Koldovský, O., Jirsova, V. & Heringova, A. (1965) *Nature (London)* **206**, 300–301
Kolinska, J. & Semenza, G. (1967) *Biochim. Biophys. Acta* **146**, 181–195
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall R. J. (1951) *J. Biol. Chem.* **293**, 265–275
Maestracci, D., Schmitz, J., Preiser, H. & Crane, R. K. (1973) *Biochim. Biophys. Acta* **323**, 113–124
Moog, F. (1951) *J. Exp. Zool.* **118**, 187–208
Moog, F. (1953) *J. Exp. Zool.* **124**, 329–346
Moog, F. (1966) *J. Exp. Zool.* **161**, 353–368
Moog, F. (1971) in *Hormones and Development* (Hamburgh, M. & Barrington, E., ed.), pp. 143–160, Appleton–Century–Crofts, New York.
Moog, F., Bennett, C. & Thomas, E. (1954) *Anat. Rec.* **120**, 777–778
Moog, F., Denes, A. E. & Powell, P. M. (1973) *Develop. Biol.* **35**, 143–159
Neville, D. M. (1971) *J. Biol. Chem.* **246**, 6328–6334
Overton, J. (1965) *J. Exp. Zool.* **159**, 195–201
Overton, J. & Shoup, J. (1964) *J. Cell Biol.* **21**, 75–85
Pennington, R. J. (1961) *Biochem. J.* **80**, 649–654
Rubino, A., Zimbalatti, F. & Auricchio, S. (1964) *Biochim. Biophys. Acta* **92**, 305–311
Segrest, J. P., Jackson, R. L., Andrews, F. P. & Marchesi, V. T. (1971) *Biochem. Biophys. Res. Commun.* **44**, 390–395
Welsh, J., Preiser, H., Woodley, J. & Crane, R. K. (1972) *Gastroenterology* **62**, 572–582
Zacharius, R. M., Zell, T. E., Morrison, J. H. & Woodlock, J. J. (1969) *Anal. Biochem.* **30**, 148–152