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DISTRIBUTION OF (Na^+-K^+)-STIMULATED ATPase ACTIVITY IN RAT INTESTINAL MUCOSA

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SUMMARY

The isolation in high yield of a membrane-bound high specific activity (Na^+-K^+)-stimulated ATPase from rat intestinal mucosal cells is described. The activity is originally associated with the mitochondrial subcellular fraction but can be isolated by sucrose gradient centrifugation after aging of the crude mitochondrial fraction. The final (Na^+-K^+)-stimulated ATPase membrane is relatively free of brush border, mitochondria, nuclear and microsomal contamination and would appear to be plasma membrane. The activity is recovered in greater than 50% yield from the original homogenate and with a 25–35-fold increase in specific activity. A small, but consistent amount of (Na^+-K^+)-stimulated ATPase is also found in the brush border fraction. These findings are discussed in relation to active transport in the intestine.

INTRODUCTION

Evidence for the involvement of (Na^+-K^+)-stimulated ATPases in active transport across biological membranes has been accumulating since the activity was first reported in crab nerve by SKOU[1]. BONTING, CARAVAGGIO AND HAWKINS[2] found the activity to be present in 21 tissues, isolated from ten different species, which had been found to possess a cardiac glycoside-sensitive transport system. POST *et al.*[3] and DUNHAM AND GLYNN[4], by showing a close correspondence of several enzyme and transport properties, concluded that the (Na^+-K^+)-stimulated ATPase was directly associated with the active transport of Na^+ and K^+ across the erythrocyte cell membrane.

The intestinal epithelial cell actively transports not only Na^+ and K^+, but also certain sugars and amino acids. Maximum active uptake of sugars and certain amino acids occurs in the presence of Na^+ (refs. 5, 6) and the uptake of Na^+ has been found to accompany the transport of such sugars and amino acids[7]. An interaction between sugar and amino acid transport has been demonstrated in the intestine in several species[7–10].

A sodium-mediated common carrier, directly or indirectly linked to the (Na^+-K^+)-stimulated ATPase, has been postulated[5,6,11]. CRANE[12] views the net intestinal movement of sugar against a concentration gradient as a consequence of a coupled downhill movement of Na^+ into the cell. The Na^+ gradient is maintained by

the operation of an energy-dependent Na^+ pump, of which the (Na^+-K^+)-stimulated ATPase is presumably a part. Such a mechanism would place no restrictions on the ATPase being directly associated with the carrier mechanism in the brush border of the intestinal mucosal cell. More specific knowledge of the subcellular distribution of the sodium-stimulated ATPase in the intestinal mucosal cell would be of help in evaluating such mechanisms.

MILLER AND CRANE[13] have succeeded in isolating the brush border membrane from hamster intestinal mucosal cells and have shown a number of enzymes to be located preferentially in this brush border fraction. TAYLOR[14], using the method of MILLER AND CRANE, has found a (Na^+-K^+)-stimulated ATPase in the brush border. BERG AND CHAPMAN[15], using a different isolation procedure, have found the (Na^+-K^+)-stimulated ATPase to be widely distributed among various membrane fractions.

The present study reports the isolation of a high specific activity, membrane-bound, (Na^+-K^+)-stimulated ATPase from rat intestinal mucosal cells. This membrane fraction is essentially free of brush border, mitochondrial and microsomal membrane and contains most of the (Na^+-K^+)-stimulated ATPase present in the original cellular homogenates. A small, but constant amount of (Na^+-K^+)-stimulated ATPase is also found in the brush border.

MATERIALS AND METHODS

Preparation of fractions

Male Sprague–Dawley rats (CFE type, Carworth, New York) were fed *ad libitum* and weighed 200–250 g when sacrificed. The rats were killed by decapitation and the small intestine removed and irrigated with cold 0.9% NaCl–5 mM EDTA (pH 7.4). All further operations were carried out at 4°. The intestine was everted with a glass rod, washed again with saline–EDTA and the mucosa removed by gentle scraping with a glass slide. The scrapings were homogenized as a 5% homogenate in 5 mM EDTA (pH 7.4) for 25 sec using a Powerstat variable transformer to decrease the blade speed of the Waring blendor, as suggested by EICHHOLZ[16]. The homogenate was filtered through coarse and fine nylon and then through No. 25 bolting silk (Reeve-Angel Co., New York). Crude brush border was sedimented in a clinical centrifuge at 700 × *g* for 10 min. The supernatant was decanted and saved for further fractionation. The brush border was washed 3 times in 2.5 mM EDTA (pH 7.4) and the washings were saved for enzyme assays. The final brush border pellet was resuspended in 50 mM Tris (pH 7.1), free of EDTA, and a gelatinous and flocculent material appeared which could be removed by centrifugation at 200 × *g* for 1 min in a clinical centrifuge. This material contains most of the DNA present in the crude brush border fraction and appears to be similar to the viscous contaminant which FORSTNER, SABESIN AND ISSELBACHER[17] remove from their brush border preparation and which HARRISON AND WEBSTER[18] remove by treatment with glass fiber paper. The original supernatant from the 700 × *g* spin was then centrifuged at 10000 × *g* for 10 min and the pellet resuspended in 2.5 mM EDTA (pH 7.4) (or 50 mM Tris (pH 7.1)) and is referred to below as Fraction II. The resulting supernatant was centrifuged at 105000 × *g* for 60 min and the pellet resuspended in 2.5 mM EDTA (pH 7.4) (or 50 mM Tris (pH 7.1)) and is referred to as Fraction III. The remaining

supernatant is designated as the Final Supernatant. Enzyme distribution studies were performed with fractions suspended in 2.5 mM EDTA or 50 mM Tris.

Sucrose gradients were used to further subdivide Fraction II. EDTA (1 mM) was used as the suspending medium in these fractionation studies. Discontinuous gradients were prepared by successive additions of 30 and 20 % sucrose to centrifuge tubes. Centrifugation was carried out for 90 min at 25 000 rev./min in a Spinco Model L ultracentrifuge using an SW 25.1 swinging bucket type rotor. The bands were removed from the gradient, diluted 3–4-fold with 2.5 mM EDTA (pH 7.4) (or 50 mM Tris (pH 7.1)) and then recentrifuged at 30 000 rev./min for 30 min. The sedimented membranes were then resuspended in EDTA or Tris before subsequent assays. The non-membranous, soluble protein was found to have no ATPase activity and was not further studied. Continuous sucrose gradients were formed by mixing 20 and 35 % sucrose over a 0.50% sucrose cushion, according to the method of MARTIN AND AMES[19]. The tubes were centrifuged overnight at 39 000 rev./min using an SW39 swinging bucket rotor.

Enzyme assay

Invertase (EC 3.2.1.26) was measured as described by HÜBSCHER, WEST AND BRINDLEY[20]. Glucose was measured by the glucose-oxidase method using Glucostat Special, obtained from Worthington Biochemical Corp. (Freehold, N.J.). Cytochrome oxidase (EC 1.9.3.1) was measured by the method of SMITH[21]. The cytochrome *c* was reduced by treatment with excess ascorbate and then reisolated by passage over a Sephadex G-25 column.

Alkaline phosphatase (EC 3.1.3.1) was determined with β-glycerophosphate as substrate. The assay system contained 30 mM glycine buffer (pH 9.1), 10 mM β-glycerophosphate, 5 mM $MgCl_2$ and 1 mM $ZnCl_2$, in a total volume of 1.0 ml. Incubation was for 15 min at 37°. The reaction was terminated by the addition of 0.25 ml of 2.5 M perchloric acid and immersion of the tubes in an ice-bath for several minutes. Perchlorate and protein were then coprecipitated by the addition of 0.25 ml of 2.5 M KCl. The tubes were centrifuged for 10 min in a clinical centrifuge, and an aliquot of the supernatant was assayed for phosphate by the method of MARTIN AND DOTY[22] and/or the method of CHEN, TOREBARA AND WARNER[23]. The pH and the concentrations of Mg^{2+} and Zn^{2+} were found to be optimal in separate experiments.

Glucose-6-phosphatase (EC 3.1.3.9) was measured by the method of HUBSCHER AND WEST[24] in the presence of 1 mM EDTA and the absence of divalent cations in order to minimize the activity of alkaline phosphatase. Glucose (0.4 M) inhibition of glucose-6-phosphatase also served to distinguish the enzyme from nonspecific phosphatases[17,25].

ATPase activity was measured under three conditions: in the presence of Mg^{2+}, Na^+ and K^+ ("total ATPase"), in the presence of Mg^{2+} alone ("Mg^{2+}-ATPase") and in the presence of Mg^{2+}, Na^+, K^+, and ouabain ("ouabain-insensitive ATPase"). The (Na^+-K^+)-stimulated ATPase was computed by subtracting either the Mg^{2+}-ATPase or the ouabain-insensitive ATPase from the total ATPase. Within experimental error, the Mg^{2+}-ATPase and the ouabain-insensitive ATPase were always the same. For the assay of total ATPase, the system contained 30 mM Tris (pH 7.1), 5 mM ATP (pH 7.1), 7.5 mM $MgCl_2$, 120 mM NaCl and 20 mM KCl. For the measurement of the ouabain-insensitive ATPase, 1 mM ouabain was added to this system. The Mg^{2+}-ATPase was

measured in a system containing 30 mM Tris (pH 7.1), 5 mM ATP (pH 7.1) and 7.5 mM $MgCl_2$. The total volume was 1.0 ml and the incubation was for 10 min at 37°. Termination of the reaction and phosphate analysis were carried out in the same manner as used for the alkaline phosphatase determination. Na^+-free ATP, neutralized with Tris, was obtained from Sigma Chemical Co. and used in all experiments.

DNA was measured by BURTON's[26] modified diphenylamine reaction, after extraction with cold trichloroacetic acid, ethanol at room temperature and hydrolysis in 5% perchloric acid at 90° for 15 min[27]. Highly polymerized calf-thymus DNA (Sigma type I) was used as standard.

Protein determination was by the method of LOWRY *et al.*[28] with bovine serum albumin used as standard.

Fractions for electron microscopy were fixed in 3.1% glutaraldehyde by centrifugation in a Microfuge, according to the method of SCHNAITMAN, ERWIN AND GREENAWALT[29]. The pellets were removed from the Microfuge tubes by cutting off the tips of the tubes and were washed and stored overnight in 0.25 M sucrose in 0.1 M phosphate buffer (pH 7.2). Pellets were then post-fixed for 1.5–2 h in 1% OsO_4 in 0.1 M phosphate buffer (pH 7.2). The pellets were dehydrated through a graded series of alcohol washings and embedded in epon by the procedure of LUFT[30]. Sectioning was performed with a diamond knife on an LKB ultramicrotome, and the sections were collected on unsupported 400-mesh grids. Sections were stained with 1% uranyl acetate for 30 min at 60° and then with lead citrate by the procedure of REYNOLDS[31]. They were then examined in a Siemens Elmiskop I operated at 30 kV and photographed at plate magnifications of 4000–20 000.

RESULTS

Enzyme distribution

The distribution of various marker enzymes was examined to evaluate the effectiveness of the separation of the various subcellular components. These results are presented in Table I. It is seen that alkaline phosphatase and invertase are concen-

TABLE I

DISTRIBUTION OF MEMBRANE MARKER ENZYMES IN RAT INTESTINAL MUCOSAL CELLS

Per cent represents the distribution of enzyme activity recovered in each fraction based on the total amount initially present in the homogenate. Specific activities, given in parentheses, are expressed as μmoles of product/h per mg protein. Values are the average computed from 4–8 experiments, with a S.D. of ± 5%.

Fraction	*Protein* (%)	*Invertase* (%)	*Alkaline phosphatase* (%)	*Cytochrome oxidase* (%)	*Glucose-6-phosphatase* (%)	*Inhibition by glucose* (%)
Total homogenate	100	100 (3.0)	100 (12)	100 (7)	100 (0.42)	25
Brush border (700 × *g*)	3	60 (43)	60 (120)	<1 (<0.5)	4 (0.36)	17
Brush border washings	8	17 (23)	14 (60)	10 (8)	3 (0.16)	—
II (10000 × *g*)	19	9 (1.5)	13 (4.4)	>75 (30)	26 (0.33)	18
III (105000 × *g*)	13	9 (2.1)	17 (7.1)	—	35 (0.57)	41
Final supernatant	55	—	—	—	28 (0.30)	13

trated in the brush border fraction and that the specific activities in this fraction are increased at least 10-fold over that present in the total homogenate. These results confirm the studies of MILLER AND CRANE[13] and HOLT AND MILLER[32] on brush border. The washed brush border fraction contains essentially no cytochrome oxidase. This mitochondrial marker enzyme is found almost entirely within Fraction II. The interpretation of studies with the microsomal marker, glucose-6-phosphatase, are complicated by the presence of the nonspecific, high specific activity alkaline phosphatase, which can hydrolyze glucose 6-phosphate. Conditions for assay of glucose-6-phosphatase have been chosen to minimize any contribution by alkaline phosphatase: divalent cations are not present, EDTA has been added and the pH of the assay medium is 6.5. Table I shows that there is little if any glucose-6-phosphatase activity in the brush border fraction. The bulk of the glucose-6-phosphatase is found in Fractions II and III and the Final Supernatant. Inhibition by glucose confirms the fact that the activity measured is in fact glucose-6-phosphatase[25,17]. An increase in specific activity over that of the initial homogenate is found only in Fraction III, which would be expected to contain most of the microsomes. This fraction also shows greatest inhibition by glucose. It may be concluded that the brush border fraction has little if any contamination with mitochondria or microsomes, that the mitochondria are isolated in a discrete fraction, but that the microsomes are probably randomly distributed throughout all the fractions but the brush border. In experiments not shown in Table I, DNA analyses indicate that less than 6 % of the DNA initially present in the homogenate is found in the brush border fraction and none in Fraction II and suggest that the brush border fraction and Fraction II are free of significant nuclear contamination.

Table II shows the distribution of ATPase activity in these subcellular fractions. The Mg^{2+}-ATPase is seen to be distributed through all the fractions, with the largest amount found in Fraction II. The (Na^+-K^+)-ATPase was found in all the membranous fractions, but the majority of the activity was confined to Fraction II. The brush border fraction is seen to contain about 15 % of the total (Na^+-K^+)-stimulated ATPase. This small amount of activity was found consistently in numerous preparations examined and could not be separated from the brush border membrane by centrifugation in sucrose gradients.

As detailed in an accompanying paper[33], the Mg^{2+}-ATPase shows no specificity among the various nucleotide triphosphates, whereas the (Na^+-K^+)-stimulated ATPase

TABLE II

DISTRIBUTION OF ATPase ACTIVITY IN RAT INTESTINAL MUCOSAL CELLS

For details see legend from Table I.

Fraction	*Mg^{2+}-ATPase* (%)	*(Na^+-K^+)-ATPase* (%)
Total homogenate	100 (7)	100 (5)
Brush border (700 × *g*)	11 (17)	15 (16)
Brush border washings	10 (14)	14 (15)
II (10000 × *g*)	38 (15)	60 (16)
III (100000 × *g*)	19 (9)	13 (4)
Final supernatant	5 (10)	<1 (<0.5)

is highly specific for ATP. The (Na^+-K^+)-stimulated ATPase is inhibited by the steroid ouabain, a potent inhibitor of all (Na^+-K^+)-stimulated ATPases and of Na^+ and K^+ transport.

Since mitochondria isolated from other sources have not been shown to possess a (Na^+-K^+)-stimulated, ouabain-sensitive ATPase and since in most isolation procedures the (Na^+-K^+)-stimulated ATPase is found in either the low-speed sediment or the microsomal fraction, the presence of the (Na^+-K^+)-stimulated ATPase in the mitochondrial fraction was studied further.

Isolation of (Na^+-K^+)-stimulated ATPase

When freshly isolated Fraction II is subjected to sucrose density gradient (20–35%) centrifugation and the collected fractions then assayed for (Na^+-K^+)-stimulated ATPase and cytochrome oxidase, no separation of the activities is found. If, however, Fraction II is first aged at 0–2° for 5 days and then placed on a sucrose gradient, a distinct separation of the two activities occurs, as seen in Fig. 1.

Comparable results can be obtained on a preparative scale by using a discontinuous sucrose gradient, prepared by layering 20% sucrose over 30% sucrose and centrifuging for 90 min at 25 000 rev./min in a Spinco SW 25.1 rotor. Using this technique the (Na^+-K^+)-stimulated ATPase activity is found in a membrane fraction which bands at the 20–30% interface, while the cytochrome oxidase activity sediments to the bottom of the tube. Fig. 2 schematically illustrates the major bands obtained in the discontinuous gradient and the accompanying bar graph indicates that the separation by this technique is nearly complete. The band designated M-1 has very little cytochrome oxidase activity and contains the (Na^+-K^+)-stimulated

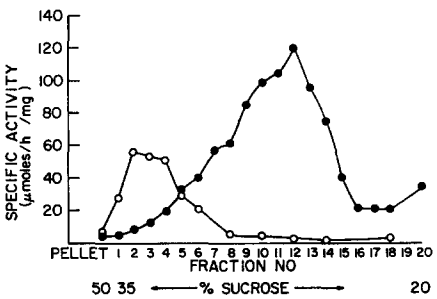

Fig. 1. Separation of (Na^+-K^+)-stimulated ATPase from cytochrome oxidase activity by continuous sucrose density gradient centrifugation. 4 mg protein of Fraction II, aged at 0° for 5 days, were placed on gradient as described in MATERIALS AND METHODS. ●—●, (Na^+-K^+)-stimulated ATPase; ○—○, cytochrome oxidase.

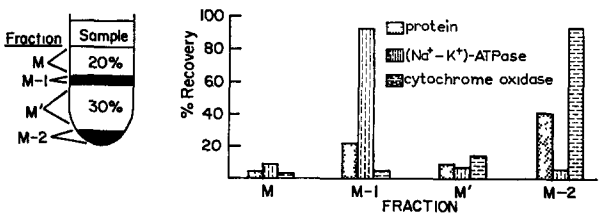

Fig. 2. Separation of (Na^+-K^+)-stimulated ATPase from cytochrome oxidase activity by discontinuous sucrose density gradient centrifugation. On left, diagrammatic representation of tube after centrifugation, demonstrating nomenclature used for collected fractions.

ATPase activity with a very high specific activity. Isolation of the M-1 fraction from Fraction II results in a 5–6-fold purification of the (Na^+-K^+)-stimulated ATPase activity and represents a 25–30-fold purification over the original homogenate.

Isolation of the (Na^+-K^+)-stimulated ATPase-containing membrane fraction can be accomplished after incubation of Fraction II for relatively short periods of time at 37°. Fig. 3 shows that the increase in the specific activity of the M-1 fraction reaches a maximum after Fraction II has been incubated 3–5 h at 37°. The Mg^{2+}-ATPase remains low and accounts for only about 10% of the total ATPase activity.

The increase in (Na^+-K^+)-stimulated ATPase found in the M-1 fraction after aging could be due to the activation of the membrane material which separates as the M-1 band even without aging or it could be due to the specific release from the mitochondrial fraction of a membrane containing a high specific activity, (Na^+-K^+)-stimulated ATPase. This question was further examined. Fresh and aged aliquots of

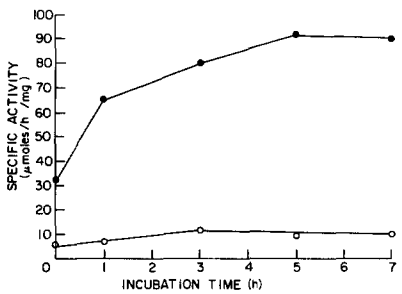

Fig. 3. Effect of incubation of Fraction II at 37° on the ATPase activity isolated in M-1 fraction. Freshly isolated Fraction II was incubated at 37°, and at the indicated time, an aliquot was removed and the M-1 fraction isolated by discontinuous sucrose density centrifugation. ●—●, (Na^+-K^+)-stimulated ATPase; ○—○, Mg^{2+}-ATPase.

TABLE III

EFFECT OF AGING ON THE DISTRIBUTION OF ENZYMATIC ACTIVITY ON SUCROSE GRADIENTS

Isolated Fraction II was divided into 2 parts. One half was placed immediately on a sucrose gradient prepared by layering 7 ml of 20% sucrose over 15 ml of 30% sucrose. The gradient was spun in an SW 25.1 Spinco rotor at 25000 rev./min for 90 min in a Spinco Model L ultracentrifuge. The second half was incubated at 37° for 4 h and then placed on a similar gradient. The individual bands were removed from each gradient, the membranes isolated as described in METHODS and assayed for protein, Mg^{2+}-ATPase and (Na^+-K^+)-ATPase. The M fraction and all soluble protein was discarded. Values in parentheses are specific activities.

	Activity (total units) in			
	Fraction II	*Fraction M-1*	*Fraction M'*	*Fraction M-2*
Aging: none				
(Na^+-K^+)-ATPase (A)	340 (20)	50 (26)	130 (63)	200 (19)
Mg^{2+}-ATPase	370 (22)	30 (15)	40 (21)	350 (34)
Protein (mg)	17.0	1.9	2.1	10.3
Aging: 37° for 4 h				
(Na^+-K^+)-ATPase (B)	490 (29)	340 (135)	100 (72)	80 (10)
Mg^{2+}-ATPase	270 (16)	30 (11)	20 (10)	170 (20)
Protein (mg)	17.0	2.5	1.5	8.3
Difference (B − A)	+150	+290	−30	−120

Fraction II were placed on identical discontinuous sucrose gradients. The M-1, M' and M-2 bands were isolated, assayed for protein, Mg^{2+}-ATPase and (Na^+-K^+)-stimulated ATPase and the specific activities and recoveries were calculated. The results of such an experiment are shown in Table III. It is seen that aging causes an increase in the total (Na^+-K^+)-stimulated ATPase and a decrease in the Mg^{2+}-ATPase found in Fraction II. Isolation of the subfractions show that after aging, there is a loss of (Na^+-K^+)-stimulated ATPase from the M-2 and M' fractions and an increase in this activity in the M-1 fraction. The total increase of activity found in the M-1 fraction is numerically equal to the loss of activity from M-2 and M' *plus* the net increase in activity found in the total Fraction II. It would therefore appear that the aging process brings about both the specific release of a high specific activity membrane as well as the net activation of the (Na^+-K^+)-stimulated ATPase activity. This conclusion is supported by other experiments, not illustrated, in which the two processes are dissociated, *i.e.*, the transfer of activity from the M-2 and M' fractions to the M-1 fraction has occurred without a net increase in the total (Na^+-K^+)-stimulated ATPase.

The events taking place during the aging process would appear to depend upon an interaction between the ATPase-containing membrane and the mitochondrial fraction and to be independent of any soluble or less dense membranous material present in Fraction II. Freshly isolated Fraction II was immediately placed on a discontinuous sucrose gradient, centrifuged and separated into the various subfractions. The results are similar to those in Table III and are tabulated in Table IV. Less than 7 % of the recoverable (Na^+-K^+)-stimulated ATPase is found in the M-1 band. The majority of the (Na^+-K^+)-stimulated ATPase remains in the M-2 fraction. This isolated M-2 band was then incubated at 37° for 3 h, again placed on a sucrose gradient and the subfractions reisolated. The results show (Table IV) that the M-1

TABLE IV

EFFECT OF AGING OF ISOLATED M-2 FRACTION

Values in parentheses are specific activities.

Fraction	*(Na^+-K^+)-ATPase (total units)*	*Mg^{2+}-ATPase (total units)*	*Protein (mg)*
Isolated from Fraction II at zero time			
II	800 (19)	800 (19)	42
M-1	60 (17)	70 (22)	3.4
M'	130 (60)	60 (20)	2.8
M-2	700 (26)	700 (26)	27
Isolated from M-2 fraction, initially isolated at zero time, then aged at 37° for 3 h			
M-2	900 (33)	560 (21)	27
2nd M-1	560 (153)	80 (21)	3.7
2nd M'	250 (104)	50 (20)	2.5
2nd M-2	200 (16)	310 (24)	13
Isolated from Fraction II at zero time, then aged at 37° for 3 h			
M-1	57 (16)		3.4

band isolated after incubation of the previously purified M-2 band now contains about 55 % of the recoverable (Na^+-K^+)-stimulated ATPase and has a specific activity nine times greater than the M-1 band freshly isolated at zero time. On the other hand, when the M-1 band freshly isolated at zero time is separately incubated for 3 h at 37° no increase in specific activity occurs. The mitochondrial-containing fraction is therefore required for the appearance of the high specific activity (Na^+-K^+)-stimulated ATPase in the M-1 band.

Possible contamination of M-1 fraction with mitochondrial membranes

Despite the fact that the sucrose density gradient studies showed a clear separation of the M-1 (Na^+-K^+)-stimulated ATPase from the mitochondrial marker enzyme, cytochrome oxidase, further studies were undertaken to rule out the presence of mitochondrial membranes within the M-1 fraction. The effect of dinitrophenol and oligomycin on the ATPase activity of the M-1 fraction was examined. In fresh, but not aged mitochondria, dinitrophenol stimulates ATPase activity[34]. Oligomycin, on the other hand, inhibits ATPase of mitochondria[35]. As seen in Table V, dinitrophenol has no significant effect on either the Mg^{2+}-ATPase or the (Na^+-K^+)-stimulated ATPase of the M-1 fraction at concentrations up to 1.2 mM. Oligomycin, up to a concentration of 20 μg/mg membrane protein, had no effect on the Mg^{2+}-ATPase. High concentrations of oligomycin are required to obtain inhibition of the (Na^+-K^+)-stimulated ATPase. In independent experiments it was found that oligomycin, at a concentration of 1.5 μg/mg protein, causes 50 % inhibition of the Mg^{2+}-ATPase of the mitochondrial fraction (M-2) isolated from intestinal mucosa. The high concentrations of oligomycin required for the inhibition of the M-1 (Na^+-K^+)-stimulated ATPase are similar to those described for the inhibition of other (Na^+-K^+)-stimulated

TABLE V

EFFECT OF DINITROPHENOL AND OLIGOMYCIN ON M-1 ATPase

Specific activity of enzyme used, in μmoles P_i liberated per h per mg protein, was: (Na^+-K^+)-stimulated ATPase, 116, Mg^{2+}-ATPase, 10.

Expt. No.	*Addition*	*Relative activity*	
		Mg^{2+}-ATPase	*(Na^+-K^+)-ATPase*
1	None	100	100
	Dinitrophenol, mM		
	0.012	93	99
	0.120	93	98
	0.300	102	103
	1.20	102	94
2	None	100	100
	Oligomycin, μg/mg protein		
	1.0	100	
	1.5		96
	5.0	103	
	7.5		86
	10	105	
	15		67
	20	100	
	30		60

ATPases[36,37] and contrasts with the relatively low concentrations of oligomycin required for inhibition of mitochondrial ATPase.

Monoamine oxidase is a mitochondrial enzyme which is recognized as a marker

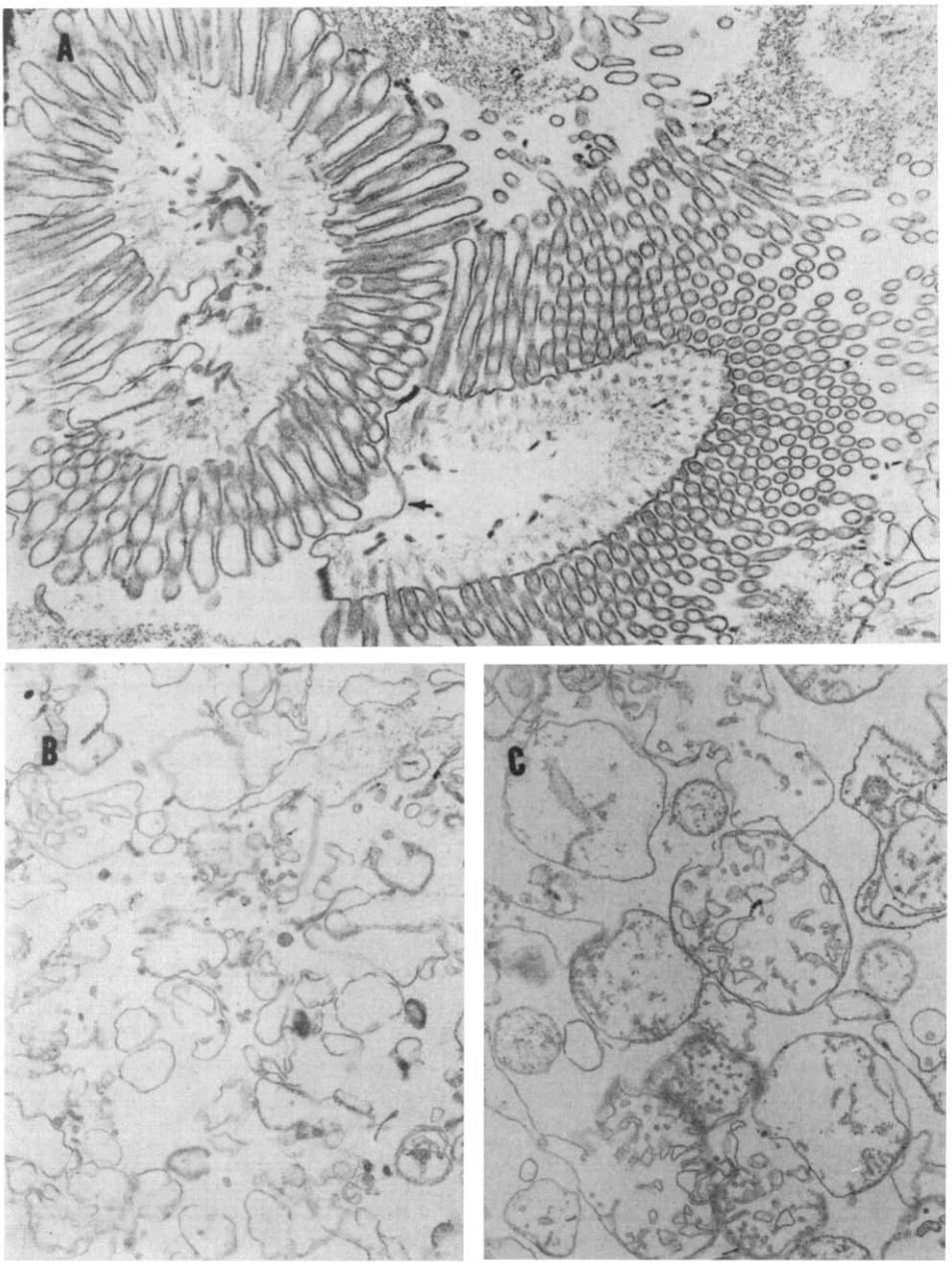

Fig. 4. Electron micrographs of isolated fractions. A. Brush border. Arrow points out lateral plasma membrane attached to brush border. Magnification 17000 ×. B M-1 fraction. Magnification 16000 ×. C. M-2 fraction. Magnification 16000 ×.

for the outer mitochondrial membrane by most workers in the field[38]. Sucrose gradient experiments, not illustrated, have shown that all the recoverable monoamine oxidase activity of Fraction II is found in the mitochondrial, M-2, fraction and that no detectable monoamine oxidase activity is found in the M-1 fraction. These studies would therefore tend to rule out the possibility of major mitochondrial contamination of the M-1 fraction.

Possible microsomal contamination of M-1 fraction

The experiments shown in Table I indicate that about 26 % of the microsomal marker enzyme, glucose-6-phosphatase, was found in Fraction II. The M-1 fraction was analyzed for this activity and found to split 0.15 μmole of glucose 6-phosphate/h per mg protein. This specific activity is relatively low and more significantly, the activity was not inhibited by the presence of 0.4 M glucose, under which conditions the microsomal enzyme is markedly inhibited. It is likely, therefore, that this glucose-6-phosphatase activity found in the M-1 fraction is due to a nonspecific phosphatase and does not represent contamination by microsomal membrane.

Appearance of isolated fractions in the electron microscope

Freshly isolated brush border preparations and aged M-2 and M-1 fractions were examined in the electron microscope and are shown in Fig. 4. Brush border preparations were very homogeneous and contained only small amounts of a fibrous-appearing material. Morphologically this substance resembled the granular fibers seen along the terminal web at the base of the microvilli. Frequently portions of the cell membrane attached to the brush borders appeared to reseal and form closed membranes around the remaining terminal web (see Fig. 4A, arrow). Of interest also were densely staining areas on the membranes which resembled desmosomes. The aged mitochondrial fraction, M-2, clearly retains the morphological appearance of mitochondria, although much of the matrix has been lost and the mitochondria themselves appear swollen and disrupted (Fig. 4C). Cristae are clearly recognizable, and almost all profiles show that a large fraction of the outer mitochondrial membranes are retained in this fraction. These results support the biochemical data regarding the distribution of cytochrome oxidase and monoamine oxidase among the fractions following aging. The M-1 fraction (Fig. 4B) is a heterogeneous population of membrane and consists primarily of empty vesicles much smaller in diameter than the mitochondria in the M-2 fraction. Morphologically these two fractions appear quite distinct and together with the biochemical evidence presented above suggest that the membrane material in the M-1 fraction is not derived directly from the mitochondria.

There was no significant morphological difference between the M-1 and M-2 fractions examined after isolation following incubation of Fraction II at 37° for 3 h compared with those fractions isolated at zero time.

DISCUSSION

The major portion of the (Na^+-K^+)-stimulated ATPase in the rat intestinal mucosal cell has been isolated in a fraction (M-1) which contains less than 2 % of the total cellular protein. The enzyme is tightly bound to a membrane which is not mitochondrial, microsomal or brush border in nature.

The intestinal mucosal cell is somewhat unique in that as a columnar epithelial

cell it possesses an apical and basal pole which are morphologically distinct. Upon homogenization and cellular fractionation the apical pole can be isolated as a relatively pure membrane subfraction. This brush border membrane, originally isolated by MILLER AND CRANE[13], has subsequently been further purified and studied by numerous laboratories[17,39–41]. It has been shown to play a dominant role in the terminal hydrolysis of carbohydrate and peptides[42] and also shown to be the site of transport of biological metabolites across the intestine[7]. The basal pole or the remainder of the limiting plasma membrane of the intestinal mucosal cell has not been isolated in comparable purity.

The importance of the plasma membrane of the intestinal mucosal cell, exclusive of the brush border, as a regulator of the intra- and extracellular ionic environment has been discussed by CRANE[5] and others[6,11]. One might anticipate that this membrane would contain a (Na^+-K^+)-stimulated ATPase of high specific activity. EMMELOT *et al.*[43] have isolated the plasma membrane from rat liver and have shown that the membrane contains a number of marker enzymes, including the (Na^+-K^+)-stimulated ATPase. The membrane preparation contained in the M-1 fraction described in this paper contains a (Na^+-K^+)-stimulated ATPase with a specific activity which is seven to ten times higher than that previously reported from rat[15], hamster[14] and rabbit[44] intestinal mucosal cells. The specific activity of this preparation, isolated simply by differential and sucrose gradient centrifugation, in fact compares well with that reported for the most highly purified (Na^+-K^+)-stimulated ATPases isolated from other tissues[45].

Preliminary studies have shown that the M-1 fraction contains a considerable amount of the 5'-nucleotidase activity of the intestinal mucosal cell (unpublished observations). This enzymatic activity was also found in the plasma membrane preparations isolated from rat liver in the studies of EMMELOT *et al.*[43]. It is likely therefore that the M-1 fraction obtained after aging of Fraction II contains most of the plasma membrane, free of brush border. The degree of heterogeneity and the enzymatic characteristics of this fraction are under further investigation. The properties of the ATPase are reported in an accompanying paper[33].

The results of the present work indicate that the isolated brush border fraction satisfies the requirements of the apical membrane in that it is morphologically identifiable and contains the majority of invertase and alkaline phosphatase with little mitochondrial, nuclear and microsomal contamination. With the method of isolation used in these studies, the brush border fraction consistently was found to contain about 15% of the total cellular (Na^+-K^+)-stimulated ATPase. It is not yet possible to determine whether this activity is an integral part of the brush border membrane or whether the activity is present on small tags of the lateral plasma membrane which remain attached to the brush border during fragmentation of the cell. Because of the high specific activity of the membrane fraction tentatively identified as the plasma membrane (*i.e.*, M-1) it would appear that the tags of lateral membrane seen on electron microscopy of the brush border would be sufficient to account for the presence of the (Na^+-K^+)-stimulated ATPase found in the brush border fraction.

The M-1 membrane has been isolated from the mitochondrial fraction with little or no detectable mitochondrial contamination. The adherence of the ATP-requiring M-1 ATPase to the mitochondria, an ATP-synthesizing and ATP-binding organelle, is of interest. The possible physiological and biochemical significance of this association is now under investigation.

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REFERENCES

1 J. C. SKOU, *Biochim. Biophys. Acta*, 23 (1957) 394.
2 S. L. BONTING, L. L. CARAVAGGIO AND N. M. HAWKINS, *Arch. Biochem Biophys.*, 98 (1962) 413.
3 R. L. POST, C. R. MERRITT, C. R. KINSOLVING AND C D. ALBRIGHT, *J Biol. Chem.*, 235 (1960) 1796
4 E T DUNHAM AND I. M. GLYNN, *J Physiol. London*, 156 (1961) 274.
5 R. K CRANE, *Federation Proc*, 21 (1962) 891.
6 T Z CSAKY, *Federation Proc*, 22 (1963) 3.
7 P. F. CURRAN, *Federation Proc.*, 24 (1965) 993.
8 H. NEWEY AND D. H. SMYTH, *Nature*, 202 (1964) 400.
9 S. J SAUNDERS AND K. J. ISSELBACHER, *Biochim Biophys Acta*, 102 (1965) 397
10 F ALVARADO, *Science*, 151 (1966) 1010.
11 S G SCHULTZ AND R. ZALUSKY, *J. Gen. Physiol*, 47 (1964) 1043.
12 R K CRANE, *Federation Proc.*, 24 (1965) 1000.
13 D. MILLER AND R K. CRANE, *Biochim Biophys Acta*, 52 (1961) 293.
14 C B TAYLOR, *Biochim Biophys. Acta*, 60 (1962) 437.
15 G G. BERG AND B CHAPMAN, *J. Cellular Comp. Physiol.*, 65 (1965) 361.
16 A. EICHHOLZ AND R K. CRANE, *J Cell Biol*, 26 (1965) 687
17 G. G. FORSTNER, S. M. SABESIN AND K. J. ISSELBACHER, *Biochem. J.*, 106 (1968) 381.
18 D D HARRISON AND H. L WEBSTER, *Biochim Biophys. Acta*, 93 (1964) 662
19 R G. MARTIN AND B. N AMES, *J. Biol. Chem*, 236 (1961) 1372
20 G HUBSCHER, G R WEST AND D. N BRINDLEY, *Biochem. J.*, 97 (1965) 629.
21 L SMITH, *Methods Biochem. Anal*, 2 (1955) 427.
22 J B MARTIN AND D. M. DOTY, *Anal Chem*, 21 (1949) 965
23 P S. CHEN, T. Y TOREBARA AND H WARNER, *Anal. Chem*, 28 (1956) 1756.
24 G HUBSCHER AND G R. WEST, *Nature*, 205 (1965) 799.
25 J FIELD, S EPSTEIN AND T. EGAN, *J Clin Invest*, 44 (1965) 1240
26 K BURTON, *Biochem. J*, 62 (1956) 315
27 W C SCHEIDER, in S P. COLOWICK AND N O KAPLAN, *Methods in Enzymology*, Vol. 3, Academic Press, New York, 1957, p. 680.
28 O H LOWRY, N. J. ROSEBROUGH, A. L FARR AND R J RANDELL, *J. Biol Chem*, 193 (1951) 265.
29 C SCHNAITMAN, V. G. ERWIN AND J. W. GREENAWALT, *J Cell Biol.*, 32 (1967) 719.
30 J. H. LUFT, *J Biophys Biochem. Cytol*, 9 (1961) 409
31 E S REYNOLDS, *J. Cell Biol.*, 17 (1963) 208
32 J H HOLT AND D. MILLER, *Biochim Biophys. Acta*, 58 (1962) 239.
33 J. P QUIGLEY AND G. S. GOTTERER, *Biochim. Biophys. Acta*, 173 (1969) 469.
34 E. RACKER, *Advan Enzymol.*, 23 (1961) 323
35 H A. LARDY, D JOHNSON AND W. C. MCMURRAY, *Arch Biochem. Biophys*, 78 (1958) 587.
36 H E M VAN GRONINGEN AND E C SLATER, *Biochim. Biophys. Acta*, 73 (1963) 527.
37 F F JOBIS AND H. J BREMAN, *Biochim. Biophys. Acta*, 73 (1963) 346.
38 P. K TUBBS AND P. B GARLAND, *Brit Med Bull.*, 24 (1968) 158
39 C F JOHNSON, *Science*, 155 (1967) 1670
40 C. A R. BOYD, D. S PARSONS AND A V. THOMAS, *Biochim. Biophys. Acta*, 150 (1968) 723.
41 J W. PORTEOUS, *Federation European Biochem Soc Letters*, 1 (1968) 46
42 A. EICHHOLZ, *Biochim Biophys Acta*, 135 (1967) 475.
43 P. EMMELOT, C J BOS, E L BENEDETTI AND P. H. RÜMKE, *Biochim. Biophys. Acta*, 90 (1964) 126
44 S. H RICHARDSON, *Biochim. Biophys. Acta*, 150 (1968) 572.
45 W. SCHONER, C. VON ILBERG, R. KRAMER AND W. SEULERT, *J. Biochem.*, 1 (1967) 334.