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(Na^+, K^+)-activated ATPase and Transintestinal Transport in Rat Intestine Incubated in vitro at different Temperatures

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Fisiologia. — (Na^+, K^+)-*activated ATPase and Transintestinal Transport in Rat Intestine Incubated* in vitro *at different Temperatures* (*). Nota di ALIDE FAELLI, GIOVANNI ESPOSITO, MIRELLA SIMONETTA e VITTORIO CAPRARO, presentata (**) dal Corrisp. V. CAPRARO.

RIASSUNTO. — Si è determinato il trasporto transintestinale di fluido nell'intestino di ratto incubato *in vitro* per un'ora sia a 28 °C sia a 38 °C. Detto trasporto è risultato sorprendentemente più elevato alla temperatura minore. Si è anche rilevato negli esperimenti *in vitro* un rigonfiamento dell'enterocita più cospicuo a 38 °C.

È stata determinata inoltre l'ATPasi (Na^+, K^+) dipendente nell'intestino di ratto, sia nell'animale *in vivo* sia nel preparato incubato *in vitro* alle stesse temperature. L'attività di tale enzima è stata misurata non solo nell'omogenato mucosale, ma anche in due frazioni di esso: orletto a spazzola e frazione-membrane. Detta attività è risultata essere più bassa a 38 °C che a 28 °C, anche se non significativamente.

La diminuita capacità di trasporto alla temperatura maggiore potrebbe pertanto essere dovuta, ma solo in parte, alla minore attività ATPasica (Na^+, K^+) dipendente, riscontrata a quella temperatura. Non è da escludere che il rigonfiamento dell'enterocita possa anch'esso agire negativamente sull'efficienza funzionale del medesimo.

INTRODUCTION

The first data concerning fluid transport through the intestine were obtained roughly a century ago by using both *in vivo* and *in vitro* techniques. This subject has been reviewed by Parsons [18]. At first, *in vitro* experiments showed a lower transport capacity than *in vivo* ones, but by everting the intestine [28] results improved. Recently good results have been also obtained by perfusing *in vitro* the uneverted intestine with a segmented flow of slugs of liquid separated by bubbles of oxygen-carbon dioxide [9, 10].

It has to be pointed out that the *in vitro* technique not only allows the determination of the absorption of substances from the mucosal side, but also their metabolic transformations and their transport into the serosal side can be determined.

Conversely, longer lasting experiments show better results by using the *in vivo* than by using the *in vitro* technique and less hydration of the tissue can be observed in the first case.

It is generally assumed that sodium concentration is critical for many transport activities. A sodium pump, located at the serosal border of the enterocyte [2, 24], maintains a low cell concentration of sodium by continuously

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pumping this cation towards the subepithelial space. By decreasing the sodium pump activity, cell sodium concentration increases and a swelling of the intestinal wall takes place. It seems that this pump derives its energy from hydrolysis of ATP by (Na^+, K^+)-dependent ATPase.

The present work deals with the determination of intestinal (Na^+, K^+)-dependent ATPase; it may be important to see if the level of this enzyme varies during *in vitro* perfusion under different incubation temperatures (28 °C and 38 °C).

As a matter of fact, this enzyme seems to be associated with the sodium pump [27]. In this work we have determined the (Na^+, K^+)-dependent ATPase (E.C. 3.6.1.3) of the entire mucosal cell as well as of two fractions of it, by using the Quigley and Gotterer technique [19, 20]. Such a tecnique has been criticized because it utilizes a mucosal scraping [3]; however the enzyme activity recovered in the baso-lateral membrane fraction is as high as 50 % of the original homogenate with a 30-fold increase in specific activity. More recent and sophisticated techniques give lower recovery [4, 12].

MATERIALS AND METHODS

Intestinal incubation.

Male albino rats (Wistar strain, Charles River Italiana) weighing 200–300 g, were used. Under barbituric narcosis a tract of jejunum 10 cm long was removed and everted.

Three groups of experiments were performed: in the control group the intestine was immediately processed as in " Preparation of fractions ", whereas in the other two experimental groups the intestine was incubated and perfused for 1 hr at 28 °C or at 38 °C respectively. The mucosal side of the intestine was incubated in 50 ml of Krebs–Henseleit-bicarbonate solution with 5.56 mM glucose added and gassed with O_2 95 % and CO_2 5 %. The serosal side was previously washed for 5 min and then perfused with 3 ml of the same mucosal fluid with a trace amount (0.5 μcuries/ml) of ^{14}C–PEG (poly[^{14}C]ethylenglycol, New England Nuclear Corp.) added. To determine fluid transport, ^{14}C-PEG was used since it is known that this substance does not enter the cell [8, 11].

The serosal perfusing fluid was recirculated by a peristaltic pump (2.5 ml/min, E. Bühler, Tübingen). After 5 min equilibration and at the end of the experiment, samples of the serosal and mucosal fluid were taken and assayed for ^{14}C-PEG (liquid scintillation spectrometry, Tri-Carb Packard mod. 3385). ^{14}C-PEG was practically never detected in the mucosal fluid. From the dilution of the isotope in the serosal medium, fluid transport was thus calculated.

For further details and for the scheme of the equipment, see Ref. [7].

Preparations of fractions.

Both incubated and non-incubated intestines were washed and irrigated with cold 0.32 M sucrose, 2.5 mM EDTA adjusted to pH 7.5 with Tris-OH.

All further operations were carried out at 4 °C. The mucosa was scraped off with a glass slide [5]; a portion of this scraped mucosa and the entire submucosal layer were separately weighed and dried overnight at 100 °C. The wet weight/dry weight ratio of both the mucosal and the submucosal layer were thus calculated. Furthermore, the mucosal and the total intestinal dry weights were calculated. The remaining part of the scraped mucosa was weighed and diluted in 9 vol. of 5 mM EDTA adjusted to pH 7.5 with Tris-OH and homogenized (M.S.E. Homogenizer, Crawley, England). After sampling for analysis, the homogenate was sedimented at 700×g for 10 min and the supernatant saved. Crude brush borders were then made up to the initial volume with 5 mM EDTA adjusted to pH 7.5 with Tris-OH, resuspended and centrifuged at 700×g for 10 min.

The latter procedure was repeated and brush border pellets were obtained. The three supernatants were mixed together and centrifuged at 10.000×g for 10 min (I.E.C. mod. B-50 ultracentrifuge, Needham Heights, Mass., USA) in order to separate baso-lateral membranes and mitochondria. The recovery of the three supernatants and the presence of a lower concentration of Tris in all solutions were the only modifications of Quigley and Gotterer's original technique [19]. It is known that only at a high concentration (1 M) does Tris cause disintegration of the brush borders [6].

In the original work by Quigley and Gotterer [19], this fraction presents a recovery of 60 % of the initial (Na^+, K^+)-dependent ATPase; we have obtained roughly the same recovery (see Table III).

By aging this fraction (37 °C for 4 hrs) and by centrifuging with a discontinuous sucrose gradient, the cited Authors have further purified the pellets and a practically pure fraction of baso-lateral membranes has been obtained, with a final recovery of more than 50 % of the initial (Na^+, K^+)-dependent ATPase. This means that most of the enzyme activity of unpurified pellets comes from the baso-lateral membranes. Thus, we have found a further purification of pellets unnecessary for our purpose, if it is taken into account that the (Na^+, K^+)-dependent ATPase is practically absent from mitochondria [15].

Pellets of brush border and of membranes were resuspended in a known volume of 2.5 mM EDTA adjusted to pH 7.5 with Tris-OH. The initial homogenate and the two fractions, i.e. brush border and membranes, were analyzed for ATPase activity (see below) and for total protein by a biuret method [14], using bovine serum albumin as a standard and deoxycholate for prior clarification.

ATPase assay.

The enzyme activity was measured under two conditions: both in the presence of Mg^{++}, Na^+ and K^+ (" Total ATPase ") and in the presence of the same ions plus ouabain (" ouabain-insensitive ATPase "). The (Na^+, K^+)-dependent ATPase was computed by subtracting the ouabain-insensitive ATPase from total ATPase. The assay system of total ATPase contained:

110 mM NaCl, 20 mM KCl, 5 mM ATP.Na_2, 7.4 mM $MgCl_2$ and 50 mM Tris-OH (pH 7.5). The assay system of ouabain-insensitive ATPase contained the same ions plus 1 mM ouabain. The total volume of the assay system was 2.5 ml and the temperature 37 °C. Reaction was started by adding ATP. Samples were taken at zero and 10 min; the reaction was stopped by adding 50 % trichloroacetic acid (*w/v*). Phosphate (Pi) released was determined by the method of Zilversmit and Davis [29]. The data were statistically analyzed by Student's "*t*" test for paired data.

It is usually accepted that (Na^+, K^+)-dependent ATPase is better activated in the presence of high sodium concentration in most biological substrates [25] including rodents' intestine [1, 20, 22, 23]. De Wolff however [3] claimed that (Na_+, K^+)-dependent ATPase is more activated in the presence of a low sodium concentration (25 mM) which is close to the cell sodium concentration. Therefore, in some preliminary experiments we have tested the enzyme activity in the presence of sodium at both 120 and 25 mM concentration. Our results, not reported in the present work, are in agreement with those of most Authors.

Electron microscopy.

Brush border and membrane pellets were fixed for 2 hrs at 4 °C; the fixing solution was a 3 % glutaraldehyde in 0.1 M sodium-cacodylate buffer (pH 6.9). After washing in the same buffer the pellets were postfixed for 1 hr with 1 % OsO_4 in sodium-cacodylate buffer. The pellets were then dehydrated through a graded series of ethanol washings and embedded in Epon by the procedure of Luft [17]. Sectioning was performed on a LKB Ultrotome III ultramicrotome; sections were stained with a semi-saturated solution of uranyl-acetate and then with lead-citrate by the procedure of Reynolds [21]. The preparations were then examined in a Hitachi 11 B electron microscope.

RESULTS AND DISCUSSION

Quigley and Gotterer [19] present not only electron micrographs of each fraction obtained from the mucosal homogenate, but also an enzymatic chaterization of each fraction [19, 20]. Since we have followed the first part of the same fractionating technique, we have only controlled our two fractions by electron micrographs.

In fig. 1 a practically pure preparation of brush border is presented. Figure 2 shows the baso-lateral membranes with few mitochondrial cristae and fragments of rough endoplasmic reticulum.

In Table I data concerning total proteins expressed in mg per g dry weight of scraped mucosa are presented in unperfused intestines (control) or in intestines incubated 1 hr at 28 °C or at 38 °C respectively. Data concerning the homogenate and the two fractions, i.e. brush border and membranes coming from perfused intestines are statistically compared with control intestines.

TABLE I

Protein content of the mucosa and its fractions.

Protein content of the homogenate and the two fractions brush border and membranes, given in mg per g mucosal dry weight, is reported. Values ± S.E.M. refer to untreated intestines (control) and intestines perfused one hour at 28 °C and 38 °C respectively. Single P values of each column refer to first datum of the same column.

	N° of expt.	Proteins (mg/g mucosal dry weight)		
		Homogenate	Brush border	Membranes
Control	9	550 ± 25	86 ± 5	100 ± 7
1 hr at 28 °C	5	478 ± 24	78 ± 6	120 ± 6
P		> 0.05	> 0.3	> 0.05
1 hr at 38 °C	5	535 ± 21	57 ± 6	115 ± 7
P		> 0.6	< 0.01	> 0.2

The 1 hr perfusion both at 28 °C and at 38 °C does not statistically affect the amount of proteins of homogenate, brush border and membranes in comparison with control experiments.

The only exception is the brush border fraction of the intestine incubated at 38 °C in which the diminution is statistically significant. This fact could signify a partial disruption of the brush border at 38 °C.

In Table II data concerning the specific activity of (Na^+, K^+)-dependent ATPase are presented as μmoles phosphate released per min and per g of pro-

TABLE II

(Na^+, K^+)-activated ATPase of the homogenate and its fractions.

(Na^+, K^+)–activated ATPase expressed in μmoles P_i per min and per g mucosal proteins is reported for the homogenate and the two fractions brush border and membranes. Values ± S.E.M. refer to untreated intestines (control) and to intestines perfused one hour at 28 °C and 38 °C respectively. Single P values of each column refer to first datum of the same column.

	N° of expt.	(Na^+, K^+)–activated ATPase (μmoles Pi/min/g mucosal proteins)		
		Homogenate	Brush border	Membranes
Control	9	91 ± 7	58 ± 9	232 ± 25
1 hr at 28 °C	5	89 ± 8	44 ± 17	197 ± 9
P		> 0.7	> 0.4	> 0.2
1 hr at 38 °C	5	62 ± 10	35 ± 15	167 ± 44
P		< 0,05	> 0.1	> 0.2

teins of homogenate, of brush border and of membranes. As usual, data coming from perfused intestines are statistically compared with the unperfused ones (control). Table II shows that the specific activity of (Na^+, K^+)-dependent ATPase, referred to that of the homogenate, diminishes in the brush border and increases from two to three times in the membrane fraction. Results of the membrane fraction are very similar to those reported by the original work by Quigley and Gotterer [19]; however in the brush border these Authors found a higher specific activity of (Na^+, K^+)-dependent ATPase. Furthermore, in Table II a not statistically significant diminution (see P values) of perfused intestines in comparison with the control is observable in the homogenate and fractions; this decrease, however, is statisticallys ignificant in the homogenate incubated at 38 °C.

TABLE III

(Na^+, K^+)-*activated ATPase of the homogenate and its fractions.*

(Na^+, K^+)-activated ATPase expressed in μmoles P_i per min and per g dry mucosal weight is reported for the homogenate and the two fractions brush border and membranes. Values ± S.E.M. refer to untreated intestines (control) and to intestines perfused one hour at 28 °C and 38 °C respectively. Single P values refer to first datum of the same column.

	N° of expt.	(Na^+, K^+)-activated ATPase (μmoles Pi/min/g dry mucosal weight)		
		Homogenate	Brush border	Membranes
Control	9	50 ± 4	5 ± 1	23 ± 3
1 hr at 28 °C	5	44 ± 3	3 ± 1	25 ± 2
P		> 0.3	> 0.1	> 0.6
1 hr at 38 °C	5	35 ± 5	2 ± 1	19 ± 4
P		< 0.05	< 0.05	> 0.3

In Table III data of (Na^+, K^+)-dependent ATPase are presented as μmoles phosphate released per min and per g dry weight of scraped mucosa. As usual, data coming from perfused intestines are statistically compared with the unperfused ones (control). Table III shows that, also here, the brush border recovery of the initial (Na^+, K^+)-dependent ATPase activity is lower than that found in the original work by Quigley and Gotterer [19].

On the contrary in all three experimental groups the recovery of the membrane fraction is similar to that found by the cited Authors [19].

From the data of Tables II and III we can see that throughout 1 hr incubation, both at 28 °C and 38 °C, the amount of (Na^+, K^+)-dependent ATPase decreases in the homogenate, in the brush border and membrane fractions in comparison with control experiments; but the decrease is not statistically significant except in the homogenate incubated at 38 °C ($P < 0.05$).

Furthermore, from Tables II and III a comparison of data on (Na^+, K^+)-dependent ATPase after 1 hr perfusion at 28 °C and at 38 °C respectively shows a non statistically significant decrease of the enzyme, due to the temperature (all single P values calculated and not reported in Tables II and III exceed 0.05).

A close correlation between the transport activity and the concentration of (Na^+, K^+)-dependent ATPase seems to be demonstrated in each intestinal tract [13].

These two parameters are high in the jejunum, but they decrease in the ileum. It is known that in the caecal tract the transport activity is drastically reduced and a drastic reduction of (Na^+, K^+)-dependent ATPase has been observed [26]. An increase of these two parameters has been found in the colon [13]. Let us now compare data concerning fluid transport in intestines incubated for 1 hr at 28 °C and 38 °C respectively. These data are reported in Table IV. We can see that fluid transport at 28 °C is higher than that found at 38 °C and the difference between the two data is statistically significant ($P < 0.01$, not reported in Table IV).

TABLE IV

Fluid transport and wet weight/dry weight ratios under different experimental conditions.

Fluid transport expressed in ml per g total dry weight of the intestine and per hour, refers to expreriments carried out at 28 °C and 38 °C respectively. Wet weight/dry weight ratios reported for mucosa (mucosal scrapes) and submucosa (submucosal and muscular layers) relate to unperfused (control) and perfused intestines. Values ± S.E.M. are reported. N° of experiments in parentheses. Single P values refer always to "control" datum of the same colum.

	Fluid transport (ml $g^{-1} h^{-1}$)	Wet weight/dry weight	
		Mucosa	Submucosa
Control	—	4.43 ± 0.37 (5)	4.15 ± 0.14 (5)
1 hr at 28 °C	2.02 ± 0.24 (11)	6.58 ± 0.43 (4)	5.29 ± 0.18 (4)
P		< 0.01	< 0.01
1 hr at 38 °C	0.98 ± 0.24 (11)	8.13 ± 0.40 (5)	5.07 ± 0.33 (5)
P		< 0.001	< 0.05

If the previously cited correlation between ATPase and transport does exist, the higher transport activity at 28 °C could be due in part to the higher level of (Na^+, K^+)-dependent ATPase during the 1 hr experiments at 28 °C than that at 38 °C.

However, the difference of (Na^+, K^+)-dependent ATPase content, as evidentiated by our data, seems too small to explain the low transport activity

of the intestine at 38 °C in comparison with that at 28 °C. A metabolic dependent process, such as transintestinal transport, should be two to three times higher at 38 °C than at 28 °C.

The more pronounced decrease of (Na^+, K^+)-dependent ATPase at 38 °C than at 28 °C could be due to a higher disruption of the absorbing epithelial cells at the higher temperature. By using the everted sac technique Levine *et al.* [16] demonstrated, by a histological evaluation, that disruption of the intestinal epithelium at 37 °C is greater than at room temperature.

In Table IV the wet weight/dry weight ratio is reported for the scraped mucosa and the remaining submucosa for unperfused intestines (control) and after 1 hr perfusion at 28 °C and at 38 °C. It is possible to note from Table IV that the swelling after perfusion (always evident, see P data of Table IV) is practically the same for the submucosa (the two data after 1 hr perfusion at 28 °C and at 38 °C are statistically equal, $P > 0.6$, not reported in Table IV). On the contrary, in the scraped mucosa the high temperature causes a further swelling (the difference between the two data, i.e. 1 hr perfusion at 28 °C and at 38 °C respectively, is statistically significant, $P < 0.05$, not reported in Table IV).

This swelling of the mucosal cells may cause mechanical damage of the epithelium.

The intestinal swelling, observed in *in vitro* experiments in general, and the lower transport activity at 38 °C in comparison with that at 28 °C in particular, might be due moreover to low levels of ATP, ADP and AMP in the intestines incubated *in vitro*.

Preliminary results from this laboratory seem to be in keeping with this hypothesis.

We conclude that the lower (Na^+, K^+)-dependent ATPase activity at 38 °C in comparison with that at 28 °C can be due, in part, to the higher disruption of the epithelial cells.

This explanation does not seem to adequately support the reduced transintestinal transport at 38 °C.

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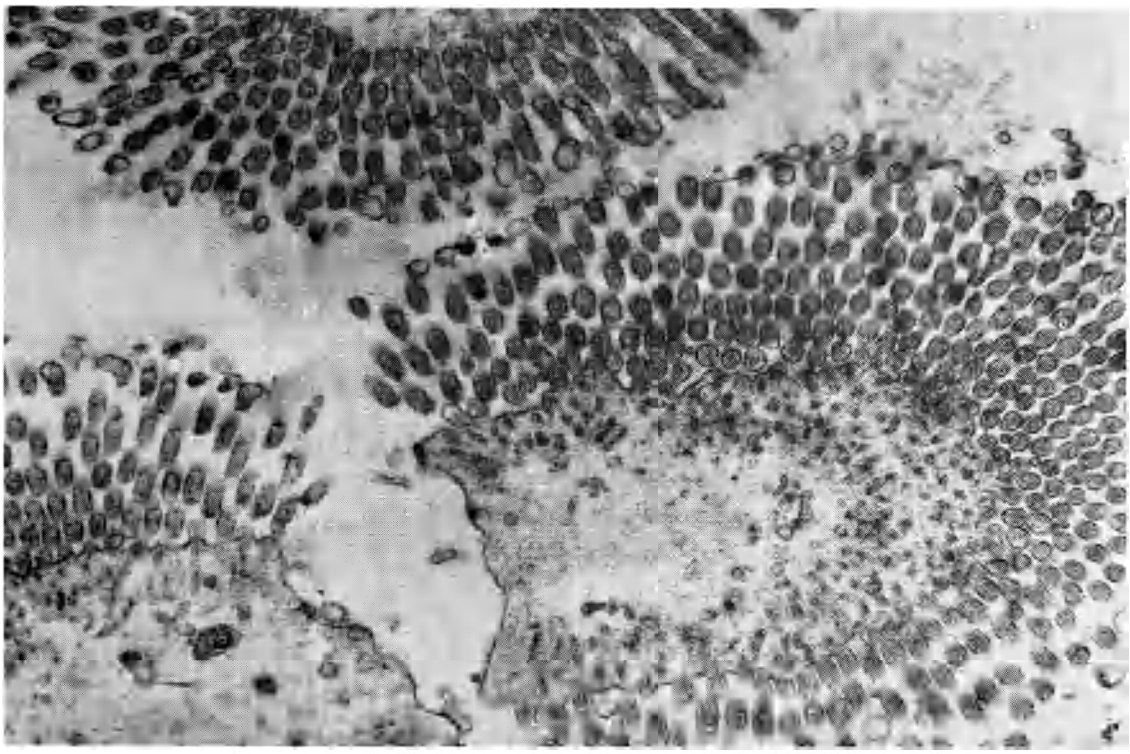

Fig. 1. – Electron micrograph of brush border fraction. Magnification 15000×.

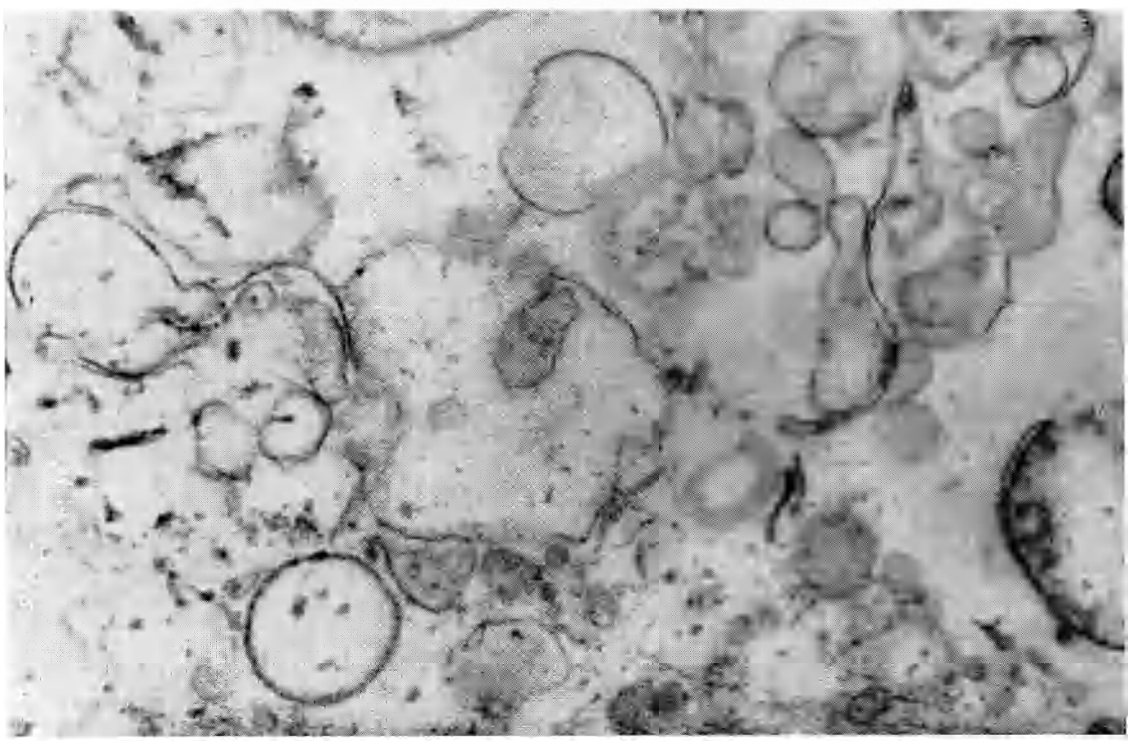

Fig. 2. – Electron micrograph of "membrane" fraction. Magnification 33000×.

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