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AN IMPROVED, REPRODUCIBLE METHOD OF PREPARING RAT LIVER PLASMA CELL MEMBRANES IN BUFFERED ISOTONIC SUCROSE

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SUMMARY

Improvements are described for a method of isolating rat liver plasma cell membranes from a liver homogenate prepared in a 0.25 M sucrose–0.5 mM $CaCl_2$–5 mM Tris buffer (pH 7.4). This method, introduced by TAKEUCHI AND TERAYAMA[5], is reproducible and the preparation contains no nuclei, 5% mitochondria and 9% microsomes, as determined enzymatically. No DNA and 31.6 μg RNA per mg plasma membrane protein were found. This amount is considerably less than has previously been reported. The yield is 0.4 mg plasma membrane protein per g wet weight liver for adult male or female rats. Rats 3–4 weeks old yield about 1/4–1/3 this amount. A discussion of the impurities in the preparation is appended. A comparison with similar methods indicates that from the point of view of reproducibility and impurities, the preparation described is the best available presently for cell homogenates made in isotonic sucrose.

INTRODUCTION

The first isolation method for rat liver cell membranes was proposed in 1960 by NEVILLE[1] and later modified by EMMELOT *et al.*[2,3] in 1962 and 1964. It involved homogenization of rat liver in ice-cold water buffered with 1 mM bicarbonate to pH 7.5. Because of the possibility that water might alter the physical structure or biochemical and biophysical properties of the membranes, similar isolations were attempted in 1962 by HERZENBERG AND HERZENBERG[4], in 1965 by TAKEUCHI AND TERAYAMA[5] and later by COLEMAN *et al.*[6] in 1967, all these groups starting with liver homogenates made in isotonic sucrose. The HERZENBERGS[4] homogenized their mouse livers in buffered isotonic sucrose but then switched to a strongly hypotonic solution to lyse nuclei during the preparation of their cell membrane fraction. Their final product (approx. 3 mg protein per g wet wt. of liver) was composed mostly of liver cellular and nuclear membranes, these authors stated. No photographs were shown. COLEMAN *et al.*[6] also admitted considerable though variable contamination of their membrane preparations with nuclear elements as well as microsomes. Since they failed to compare the glucose-6-phosphatase (EC 3.1.3.9) activity of their fractions with microsomal glucose-6-phosphatase activity from the same liver homogenates, no adequate assessment of microsomal contamination could be made. Only occasional desmosomes were seen in their electron micrographs. The method of TAKEUCHI AND

TERAYAMA[5] seemed more promising, since no DNA was present in the membrane fraction and probably little or no nuclear membranes because of the precautions taken against the lysing of nuclei. However, a number of improvements on their method seemed in order. Firstly, the sucrose had not been buffered. Secondly, no assays for known plasma membrane enzymes had been performed. Finally, a finer density gradient would have been advantageous. The experiments which follow report the results obtained with the Japanese method after changes were instituted to take care of the three above-mentioned factors. It is of interest that mitochondrial and microsomal contaminations could not be eliminated in spite of extensive purification procedures.

MATERIALS AND METHODS

Preparation of the liver cell membranes

Male rats, Sprague–Dawley descent, and approx. 1 year old, were decapitated, their livers removed and perfused with ice-cold 0.9 % NaCl. All subsequent procedures were carried out at 4°. The livers were weighed and minced in 8 vol. of 0.25 M sucrose–0.5 mM $CaCl_2$ and 5 mM Tris buffer (pH 7.4). The liver was then homogenized with 25 gentle up and down strokes (no twisting) of a loose-fitting Dounce Homogenizer (Blaesig Glass Co., Rochester, N.Y.) in approx. 6-g aliquots, to prevent any plasma membrane from breaking up too thoroughly. The homogenate was then centrifuged twice at 150 × *g* for 10 min and each time the pellets were discarded. All reported *g* values are maximum figures. The supernatant was then centrifuged at 2000 × *g* for 20 min and it and the pellet separated and retained. The latter was washed 6 or 7 times, rehomogenized each time, resuspended in the same volume of sucrose–Ca^{2+}–Tris buffer and centrifuged at 2000 × *g* for 20 min. The final pellet was homogenized gently in a wide-bore syringe and 5-ml portions layered over a discontinuous sucrose gradient prepared in a Spinco rotor SW 25.1 tube as follows: first the addition of 5 ml of sucrose, density 1.20 (45 %), then the layering of 9 ml of sucrose, density 1.18 (41.5 %) and finally the layering of 8 ml sucrose, density 1.16 (37 %). These sucrose solutions all contained 0.5 mM $CaCl_2$ and 5 mM Tris buffer (pH 7.4). The membranes were then centrifuged at 90000 × *g* for 2 h in the Spinco SW 25.1 rotor. The plasma membrane fraction at the sucrose density 1.16–1.18 (37–41.5 %) interface was collected by a syringe with polyethylene tube attached, diluted with ice-cold glass-distilled H_2O until the sucrose concentration was 10 %, and then centrifuged at 2000 × *g* for 20 min in a refrigerated centrifuge at 4°. The pellets were combined and gently homogenized in 2.5 × their volume of density 1.32 (65 %) sucrose containing 0.5 mM $CaCl_2$ and 5 mM Tris buffer (pH 7.4), and equal portions placed into three Spinco SW 65 rotor tubes. On top of this suspension was layered carefully a discontinuous gradient of 1 ml of sucrose, density 1.20 (45 %), then 1.5 ml sucrose, density 1.18 (41.5 %) and finally 1 ml of sucrose, density 1.16 (37 %). The tubes were then centrifuged at 250000 × *g* for 30 min in the Spinco SW 65 rotor or at 90000 × *g* for 2 h in the SW 39 rotor. The membrane fraction between densities 1.16 and 1.18 (37–41.5 %) was removed, diluted again until the sucrose concentration was 10 %, treated as above and recentrifuged at 250000 × *g* for 30 min. The same fraction at density level 1.16–1.18 was again removed and washed with buffered isotonic sucrose, not containing calcium, by centrifugation for 10 min at 20000 × *g* several times. The final preparation was

diluted with the same solution to about 1 mg protein per ml. Enzyme analyses were performed within 24 h.

Preparation of microsomes and mitochondria

Rat liver microsomes and mitochondria were prepared from the supernatant of the 2000 × *g* (20 min) centrifugation in the following manner. The supernatant was centrifuged at 10400 × *g* for 15 min and the mitochondrial pellet was resuspended in 0.25 M sucrose–5 mM Tris (pH 7.4) and recentrifuged at the same speed for 15 min. The mitochondrial pellet was resuspended in the same medium and used for succinate oxidase activity. The supernatant from the first mitochondrial centrifugation was centrifuged at 23500 × *g* for 1 h. The supernatant was discarded and the microsomal pellet was suspended in a small volume of 0.25 M sucrose–5 mM Tris (pH 7.4) and recentrifuged for 15 min at 10400 × *g*. This supernatant was then used as the microsomal preparation.

Preparation of membranes for electron microscopy

Membranes were prepared for electron microscopy by a rapid embedding procedure that permits observation in the microscope within 8 h. 0.1 ml of the final membrane suspension was mixed with 1.0 ml of 2.0 % osmic acid and fixed for 1 h. Dehydration followed careful removal of the fixative by centrifugation and washing of the suspension. The final membrane pellets were embedded in Epon 812. Thin sections, cut on the LKB Ultratome equipped with a diamond knife, were stained with 2 % uranyl acetate, counter stained with lead citrate[7] and examined in a Phillips EM 100 B electron microscope.

Enzymatic tests for membranes and other subcellular fractions

Glucose-6-phosphatase tests were carried out according to the method of HÜBSCHER AND WEST[8]. Succinate dehydrogenase (EC 1.3.99.1) activity was determined according to GREEN, MU AND KOLIOUT[9]. Mg^{2+} and (Na^+-K^+-Mg^{2+})-ATPase (EC 3.6.1.3) activity was followed by the method of EMMELOT *et al.*[3]. (Na^+-K^+)-ATPase activity figures were obtained by subtracting Mg^{2+}-ATPase activity from that of (Mg^{2+}-Na^+-K^+)-ATPase. 5′-Nucleotidase (EC 3.1.3.5) methodology was also that of EMMELOT *et al.*[3]. Protein concentrations of the various subcellular fractions were determined by the method of LOWRY *et al.*[10]. Inorganic phosphates were determined according to FISKE-SUBBAROW[11] and NADPH: cytochrome *c* oxidoreductase (EC 1.6.99.1) activity by the PHILLIPS–LANGDON technique[12].

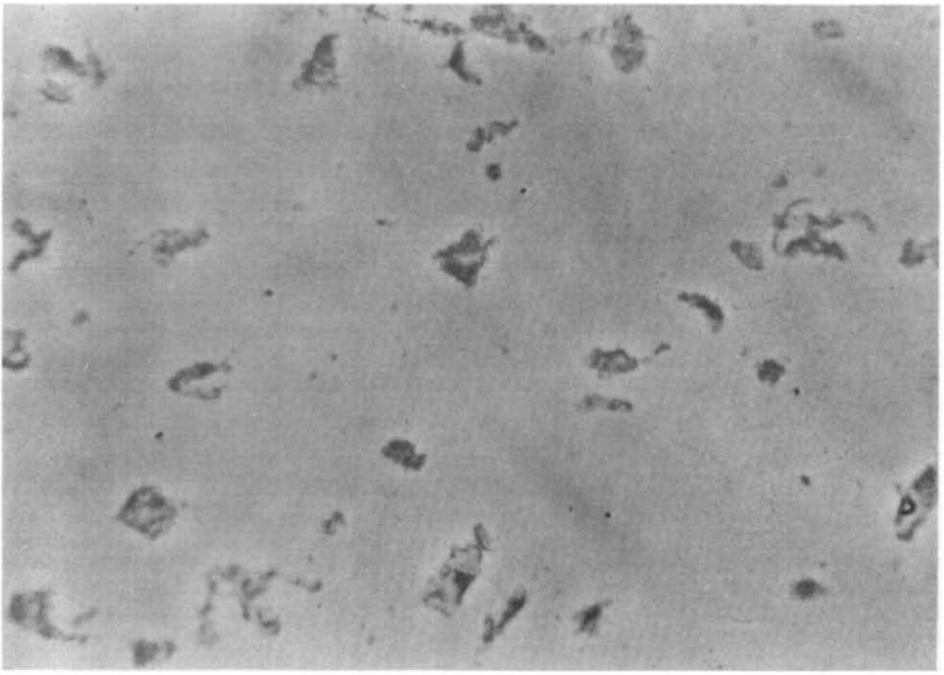
Miscellaneous

RNA was determined by the orcinol method of CERRIOTI[13]. DNA was determined by the diphenylamine procedure of BURTON[14].

All chemicals used were ACS or CP grade. Spectrophotometry was carried out on a Beckman Model B or DU photometer.

RESULTS

Fig. 1 represents a photograph of the final plasma membrane fraction obtained by the method described and made from a phase microscopy image at an enlargement



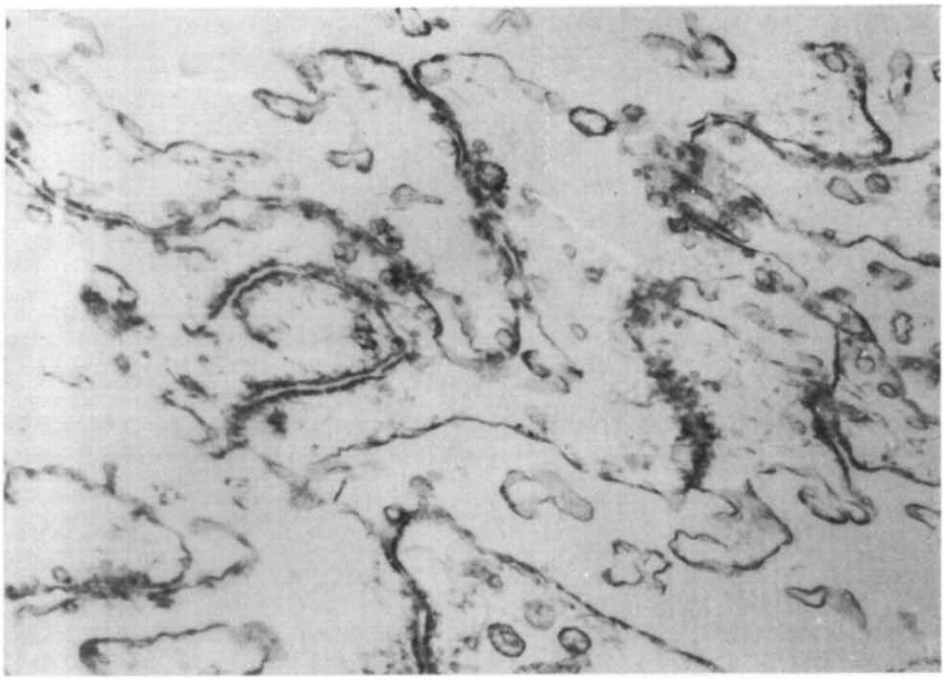


Fig. 1. "Corn flake" appearance of liver plasma membrane fraction as seen under phase microscopy at an enlargement of 700 ×. Occasional mitochondria-like dots are seen.

Fig. 2. Electron microscopic image of the same preparation at 26500 × enlargement. Numerous desmosomes are visible but no mitochondria, ribosomes or nuclei.

TABLE I

SOME ENZYME ACTIVITIES OF RAT LIVER PLASMA MEMBRANE AND OTHER SUBCELLULAR FRACTIONS

Values are given as mean ± standard deviation. All enzyme activities are expressed as μmoles per mg protein per h; phosphohydrolases at 37° and others at 25°. (Mg^{2+}-Na^+-K^+)-ATPase *minus* Mg^{2+}-ATPase = (Na^+-K^+)-ATPase. RSA, relative specific activity of the membrane fraction to one or the other of the isolated fractions. Numbers in parentheses indicate the number of preparations analyzed. —, not tested.

	Plasma membrane enzymes			*Microsomal enzymes*		*Mitochondrial enzymes*
	5′-Nucleotidase	*(Na^+-K^+)-ATPase*	*Mg^{2+}-ATPase*	*Glucose-6-phosphatase*	*NADPH:cytochrome c oxidoreductase*	*Succinate dehydrogenase*
A. Whole homogenate	3.8 ± 0.13 (3)	0.5 ± 0.5 (4)	7.0 ± 0.5 (4)	2.8 ± 0.4 (4)	—	—
B. Plasma membranes	44.3 ± 14.1 (5)	7.9 ± 3.7 (7)	43.5 ± 19.1 (7)	0.9 ± 0.2 (7)	6.9, 7.7 (2)	1.2 ± 0.6 (7)
C. Mitochondria	—	—	—	—	—	22.8 ± 11.6 (7)
D. Microsomes	—	—	—	9.6 ± 1.1 (7)	73.3, 87.3 (2)	—
RSA	B/A = 11.7	B/A = 15.8	B/A = 6.2	B/D = 0.09	B/D = 0.09	B/C = 0.05

of 700×. The individual elements appear to resemble "corn flakes". No nuclei or ribosomes are seen and only rarely what appears to be a mitochondrion-like dot. The picture is similar to that shown by NEVILLE[1] and TAKEUCHI AND TERAYAMA[5]. Fig. 2 is a photograph of an electron microscopic image at an enlargement of 26500×. Sections were prepared as stated in METHODS. Numerous darker desmosome areas can be seen. There is an absence of mitochondria, ribosomes and nuclei. Microscopically, no method of identifying either nuclear membrane or smooth reticulum fragments has been described; such elements could possibly be present.

Table I records the specific activities of various enzymatic activities found in the final plasma membrane fraction. Of these, 5'-nucleotidase and (Na^+-K^+)-ATPase activities are found only in plasma membrane fractions, Mg^{2+}-ATPase is present in plasma membranes and mitochondria, succinate dehydrogenase only in mitochondria, and glucose-6-phosphatase and NADPH:cytochrome *c* oxidoreductase in the endoplasmic reticulum (microsome) fraction. The relative specific activities of 5'-nucleotidase and (Na^+-K^+)-ATPase, both plasma membrane enzymes, with respect to the whole homogenates are of the same order of magnitude (11.7 and 15.8). Mg^{2+}-ATPase, being present in both mitochondria and plasma membranes, shows only a 6-fold increase in relative activity in the membrane fraction as compared with the whole homogenate. On the other hand, the microsomal enzymes, glucose-6-phosphatase and NADPH: cytochrome *c* oxidoreductase, have a relative specific activity of 0.091 and 0.092, when comparing membrane and microsomal activity. This indicates a microsomal contamination of 9.1 and 9.2 % respectively. Succinate dehydrogenase activity results in a relative specific activity of 0.053 or 5.3 % contamination of the plasma membranes with mitochondria.

No DNA was found to be present under conditions of the diphenylamine reaction, but 31.6 μg per mg membrane protein of RNA was found. Our yield for the plasma membrane fraction was tested in 11 rats and was found to average 8.7 mg protein per 20 g wet weight of rat liver, or 0.44 mg protein per g wet weight adult male or female rat liver.

DISCUSSION

Our plasma membrane fraction contained 31.6 μg RNA per mg membrane protein which compares favorably with the figure of 52 μg obtained by TAKEUCHI AND TERAYAMA[5].

From SPECTOR's *Handbook of Biological Data*[15], in Table 52, it can be calculated that 1 g fresh rat liver contains 3.9 mg of microsomal RNA and 20 mg of microsomal protein. The microsomal RNA/protein ratio would then be 0.195. Since we found in our plasma membrane fraction 31.6 μg RNA per mg membrane protein, the RNA/protein ratio for the plasma membrane preparation is 0.0316. If the RNA were all microsomal, then maximally 0.0316/0.195 or 16.2 % of the plasma membrane fraction would be microsomal in origin.

However, it can also be estimated from our 5 % mitochondrial contamination how much RNA could derive from the mitochondria present. Again the same source[15], Table 52, states that mitochondrial total protein amounts to 37.5 mg per g fresh liver and mitochondrial RNA is 25 % of the total nucleic acid per g fresh liver. From these figures one can calculate that 3.67 μg RNA is the maximum which could be present

with a 5% mitochondrial contamination. This would leave us 31.6—3.7 or 27.9 μg RNA per mg plasma membrane protein as microsomal RNA, providing no nuclei were present. Since no DNA was found it can be assumed that this is the case. Our enzymatically determined microsomal contamination figure was 9%; *i.e.* 90 μg protein per mg of plasma membrane protein could be microsomal. Setting up proportional equations with the SPECTOR[15] microsomal data quoted above, it is evident that such a 9% contamination would be equivalent to the presence of 17.5 μg RNA per mg plasma membrane protein. Since we actually found 27.9 μg RNA per mg plasma membrane this would amount to 27.9/17.5 × 9% or 14.3% microsomal contamination. RAY *et al.*[16] indicate that total rat liver microsomal RNA ranges between 250 and 300 μg per mg protein. The 27.9 μg (or 31.6 μg, discounting the mitochondrial contamination) would again indicate a microsomal contamination of approx. 10%. Thus, the enzymatically determined microsomal contamination of 9% does not differ too much from the figure obtained from the RNA present in the plasma membrane fraction.

FRANKE[17] makes nuclear ghosts by adding water or 0.02 M sucrose to a nuclear suspension. It is most likely, therefore, that hypotonic media would promote the appearance of nuclear and mitochondrial ghosts in a tissue homogenate and that the addition of an isotonic medium containing Ca^{2+} in making a homogenate would prevent the breaking open of most nuclei. The addition of Ca^{2+} to our homogenizing medium leads to clumping and precipitation of nuclei, which also makes it less likely that nuclear contamination of the cell membrane fraction will occur. Recently, EMMELOT AND BOS[18] have also added Ca^{2+} to their water–bicarbonate homogenizing medium when preparing hepatoma cell membrane fractions. They state this maneuver prevents the breakage of hepatoma cell nuclei but that such breakage does not occur when normal liver homogenates are used. These considerations raise the question as to what percentage of the liver cell membrane preparation of COLEMAN *et al.*[6] is composed of nuclear membranes, especially since their yield of membrane material per g wet weight of liver is about 3× as great as our preparation (1.29 mg per g liver) and the amount of RNA and DNA in their membrane preparations is excessively high. Of course, our smaller yield may also be due to the fact that our purification procedure is more extensive and may, in itself, lead to loss of membrane material. TAKEUCHI AND TERAYAMA'S[5] yield of 0.3 mg membrane protein per g wet weight of liver is closer to the figures of the present authors of 0.44 (even though their purification procedure is far less extensive than ours). EMMELOT *et al.*[3], isolating their liver plasma cell membranes from a water homogenate of rat liver, record a yield of 0.4 mg of plasma membrane protein per g wet weight of liver.

Data from Table I of the article of COLEMAN *et al.*[6] indicate a very wide variability in the specific activities of the various enzymes measured in their membrane preparations. It can be seen that the variability in seven of our preparations is much less. For a measure of contamination of membrane enzymes with those of other fractions, it seems more desirable to compare the specific activities of succinate dehydrogenase, glucose-6-phosphatase and NADPH:cytochrome *c* oxidoreductase of the fractions in which these enzymes are found, with the activities of the same enzymes in the membrane fraction. This we have done in seven experiments and determined a 5% contamination for mitochondria and a 9% contamination for microsomes. Since microsomal contamination of our plasma membrane seemed not inconsiderable when tested by glucose-6-phosphatase content, this fact was checked by determining the

content of another microsomal enzyme, NADPH:cytochrome *c* oxidoreductase. As seen in Table I, the contaminating amounts of both enzymes are identical. Similar figures cannot be obtained from the article of COLEMAN *et al.*[6]. One cannot, for instance, estimate mitochondrial contamination since the specific activity of the succinate dehydrogenase in pure mitochondrial fractions is not recorded by this group. In their third preparation recorded in their Table I, there is actually an enrichment of glucose-6-phosphatase, generally considered to be a microsomal enzyme, in the membrane fraction as compared to the original homogenate. STEIN, WIDWELL AND STEIN[19] (Table I, p. 187) have recently reported on subcellular contaminations of a rat liver cell plasma membrane fraction made from a sucrose homogenate. The mitochondrial contamination determined by cytochrome oxidase activity studies of the mitochondrial and membrane fractions is 4.4 %, a figure which closely approximates the 5 % which we found. However, microsomal contamination as determined by glucose-6-phosphatase studies of the microsome and plasma membrane subfractions amounted to 20 % as opposed to our 9 %. Whether this difference is significant or not is difficult to state since the 20 % figure represents one "typical" analysis.

It is also of interest that the ratio of lipid: protein in the membrane preparation of COLEMAN *et al.*[6] is somewhat less than 1 and this is not too different from the ratio obtained by TAKEUCHI AND TERAYAMA[5] with a preparation very similar to that of the present authors. Despite this fact, the final membrane preparation of COLEMAN *et al.*[6] has a reported specific gravity of less than 1.13 in contrast to 1.16–1.20, as determined by the sucrose gradient technique of the Japanese authors[5] and by ourselves (1.16–1.18). Both the NEVILLE[1] and EMMELOT *et al.*[2,3] preparations, prepared from a water homogenate, also have a specific gravity agreeing with the latter figures. It is, at present, not possible to explain this discrepancy.

The question as to whether it is possible to obtain a plasma membrane preparation with little or no contamination with mitochondria and endoplasmic reticulum is still an open one. Certainly the present authors have not been able to attain this aim, although in several preparations the succinate dehydrogenase activity was zero. EMMELOT *et al.*[3] are of the opinion that low concentrations of microsomal enzymes should be expected in plasma membrane preparations, since there are multiple points of continuity between the endoplasmic reticulum strands and the plasma membrane. The 9 % contamination obtained by the present authors and the 20 % contamination as measured by glucose-6-phosphatase content indicated by STEIN, WIDWELL AND STEIN[19] cannot be considered as small in extent. However, the inability of all those who have attempted the task of plasma membrane separation to achieve this purpose constitutes no final proof that it cannot be achieved. Specifically, curtain electrophoresis has not yet been applied to the purification problem.

There is finally a question as to whether a plasma membrane preparation prepared from tissue homogenized in isotonic sucrose can be shown to be different from one prepared in water. Certainly, morphologically the two appear much the same, viewed electron-microscopically. From the point of view of contamination there appears to be some, although not a great difference. Our preparation has possibly more mitochondrial (5 %) but less microsomal (9 %) contamination. EMMELOT *et al.*[3] report no cytochrome *c* oxidase or succinate-cytochrome *c* reductase (p. 137) and 20 % glucose-6-phosphatase as well as 20 % antimycin-insensitive NADH:cytochrome *c* oxidoreductase (p. 142). Comparing values for plasma membrane enzymes in the two

types, figures indicate that 5′-nucleotidase activity is approximately the same in both, whereas the Emmelot preparation appears to be higher for (Na^+-K^+)-ATPase (specific activity of 12 as against 8 for the sucrose-treated membranes). Whether the enzyme specific activity values are meaningful is questionable, since (Na^+-K^+)-ATPase figures obtained from a different rat species range from 17 to 23 for sucrose-treated membranes. Not enough use of both types of plasma membrane preparations has been made biochemically or biophysically to detect any difference between the two as yet, except in two instances. Some results of the present authors, soon to be published, indicate that a doubling of alkaline phosphatase activity in the plasma membrane fraction can be achieved when the sucrose isolation is used instead of bicarbonate isolation. Using the same preparations, no difference was found between the two methods in the case of (Mg^{2+}-Na^+-K^+)-ATPase. Secondly, gel-electrophoresis of proteins from plasma membrane suspensions prepared from bicarbonate and isotonic sucrose liver homogenates, has resulted in markedly different protein patterns. We believe that further chemical differences will appear upon careful analysis and that eventually therefore, biophysical and biochemical differences will also emerge.

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