A Comparison of Some Biochemical Properties of Dissociated Liver Cells and of Slices[1] (34340)

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The need for normal mammalian cells, especially liver cells, for many biochemical studies requires no lengthy discussion. In the absence of a method for culturing normal rat liver cells, many investigators have sought to develop methods for preparing freshly isolated cell suspensions, which are viable for a limited period of time, but which nevertheless give the investigator a chance to probe some normal metabolic functions. Most current methods have some limitations, even the use of slices. Thus Noda (1) found that trypsinization of muscle tissue causes a marked loss of intracellular K^+, and Friedmann and Epstein (2) found that protein synthesis of rat liver cells was greatly reduced. Howard and Pesch (3) found that respiratory activity of rat liver cells varied greatly with the method of preparation.

In our search for a method to prepare normal rat liver cells, we were attracted to the method of Rappaport and Howze (4), who reported the preparation of liver cells in good yields, without the necessity of perfusion, by using sodium tetraphenylborate (TPB) as the dissociating agent.

In preliminary experiments, following rigorously the method of Rappaport and Howze (4), in which 3×10^{-3} *M* TPB was used in the dissociating medium, we were able to obtain rat liver cells with good morphology and in excellent yields as they had reported. However, these cells showed abnormal respiratory activity, had very low content of K^+, and had greatly impaired ability to take up K^+. Because of these early results, we undertook a more thorough study of the biologic action of TPB on rat liver slices. In addition, we have compared the biochemical activity of rat liver cells prepared by several different methods, including the use of TPB at a low level (1, 3, 5).

Materials and Methods. Sodium tetraphenylborate (TPB) was obtained from either K and K Laboratories, Inc., or J. T. Baker Chemical Company. Collagenase (Type I) and hyaluronidase (Type I) were supplied by Sigma Chemical Company. Hanks' minimal essential medium (Hanks' MEM) was purchased from Schwarz Bioresearch, Inc.

Cox male rats, 2–4 months of age, were obtained from Laboratory Supplies, Inc., Indianapolis. The rats were housed in galvanized cages and fed a standard Purina Chow diet until used for an experiment. The rats were killed by decapitation, exsanguinated, and their livers were removed just prior to use in the experimental procedures.

Tissue slices were prepared in the usual manner using a long razor blade and guide as described by McIlwain (6). The slices were used either (a) directly to obtain control values, or (b) after leaching in cold saline to determine the unleachable potassium content according to McLean's procedure (7), or (c) after leaching in cold saline containing varying concentration of TPB to determine the effect of TPB on the unleachable potassium content and on the respiratory activity. Leached slices were incubated for 1 hr at 37° in Ringer's phosphate buffer (pH 7.4) to permit the accumulation of K^+ lost during the leaching process. The respiratory activity as well as K^+ content of the slices was determined in every instance.

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The saline leaching solution was 150 m*M* in NaCl while 150 m*M* NaCl containing varying amounts of TPB (3×10^{-5} to 3×10^{-3} *M*) was used to study the effect of this agent.

When a liver was to be dissociated into a cell suspension by any procedure other than the enzymatic procedure of Howard and Pesch (3), it was treated in the following manner: Liver slices, or pieces (1–2 mm^3) from 2.5 to 3.0 g of tissue were placed in 180 ml of the appropriate dissociating medium (cf. below) at 25° and stirred for 30 min with a magnetic stirrer. The mixture was then filtered through nylon marquisette cloth, and the undispersed tissue was again stirred in a fresh amount of dissociating medium as before. When using a medium other than one containing TPB or an enzyme, it was usually necessary to triturate the tissue gently on the filter cloth to obtain a large enough number of cells for the biochemical experiments. This was done with a rubber policeman and several 50-ml portions of the selected medium.

The various batches of cell suspensions were combined and centrifuged at 50*g* for 3–4 min, which was sufficient to cause a loosely packed pellet of cells free of red blood cells and cellular debris to form. The supernatant was removed and the cells were washed by several suspensions and centrifugations using cold buffered sucrose–salt solution at pH 7.4. Finally, the cells were suspended in buffered sucrose–salt solution or KCl-free Ringer's solution at pH 7.4 to form a stock solution, which was stored in an ice bath up to 3 hr before being used.

The solutions used for dissociation were the following: (a) buffered sucrose–salt solution—0.05 *M* sucrose, 0.14 *M* NaCl, 5×10^{-3} *M* sodium phosphate buffer (pH 7.4), and 5×10^{-3} *M* sodium glutamate (adjusted to pH 7.4); (b) buffered sucrose–salt solution with 5×10^{-3} *M* KCl; (c) buffered sucrose–salt solution with TPB (3×10^{-5} *M*); and (d) buffered sucrose–salt solution with 5×10^{-3} *M* KCl (adjusted to 400 milliosmolarity with sucrose).

The enzymatic dissociation procedure was that of Howard and Pesch (3), which employed 0.05% collagenase and 0.10% hyaluronidase in calcium-free and glucose-free Hanks' solution. The cells obtained by this procedure were suspended in KCl-free Ringer's phosphate buffer (pH 7.4) to form a stock solution.

The oxygen uptake of cells or slices was measured in a Yellow Springs oxygen monitor, using a Clark oxygen electrode. Measurements were made in Hanks' MEM at 37°.

The potassium content of the freshly prepared, leached, or incubated cells or slices from which K^+-uptake could be calculated was determined by flame photometry. Protein was determined by the biuret method of Weichselbaum (8).

Results. The effect of TPB at varying concentrations on the O_2-uptake, K^+-content, and K^+-accumulation of rat liver slices is presented in Table I. The freshly cut slices behaved in the expected manner (7, 9). Their respiration in the presence of succinate was initially 15.4 μl of O_2/mg of protein/hr, which did not change appreciably after the leaching period (17.7 μl of O_2/mg of protein/hr) nor after the incubation period (19.6 μl of O_2/mg of protein/hr). Similarly, the initial value of 149 μeq of K^+/g of protein was in the expected range (7). This diminished to a value of 49, which is approximately 30% of the initial value and represents the unleachable potassium (7). Following incubation, this value returned to 128, which is considered to be essentially a return to the normal value, indicating that the K^+-pump was operating in the physiologic range.

When TPB was present in the leaching solution, it was evident that an impairment of both respiration and K^+ metabolism had occurred which was dose related. At 3×10^{-5} *M* TPB there was little or no change, and the values were all similar to those described above for the normal liver slices. However, when TPB was present in 10-fold and 100-fold greater concentrations, there was dose-related decreases in O_2-uptake of the slices both after leaching and after incubation (cf. Table I). These higher concentrations of TPB also caused a decrease of the

TABLE I. Oxygen Uptake and K^+ Content of Freshly Cut, Leached, and Incubated Liver Slices at 37°.

Treatment	No. of expts.[a]	Control		Incubated	
		Oxygen (μl/mg of protein/hr)[b]	Potassium (μeq /g of protein)[c]	Oxygen (μl/mg of protein/hr)[b]	Potassium (μeq /g of protein)[c]
Freshly cut slices	4	15.4 (13.4–17.6)	149 (104–205)		
Slices leached in cold					
Saline	4	17.7 (14.2–20.1)	46 (34–59)	19.6 (14.4–21.1)	128 (81–192)
" + TPB 3×10^{-5} *M*	3	21.6 (14.9–28.8)	52 (38–61)	19.2 (17.4–20.1)	144 (105–179)
" + " 3×10^{-4} *M*	2	11.4 (10.8–18.0)	33 (23–42)	9.2 (7.7–18.0)	62 (48–75)
" + " 3×10^{-3} *M*	2	1.2 (1.1–1.3)	18 (15–21)	1.6 (0–1.6)	42 (36–48)

[a] Each value is the mean of 3 or more independent measurements with range given in parentheses.

[b] The reaction mixture contained 4.9 ml of MEM and appropriate weight of slices +0.1 ml of 110 m*M* succinate. Protein was estimated in each group separately.

[c] The incubation media for K^+ uptake contained NaCl (125 m*M*), KCl (6 m*M*), $MgSO_4$ (1.2 m*M*), NaH_2PO_4 (1 m*M*), $CaCl_2$ (1.2 m*M*), glucose (200 mg/100 ml), NaH_2PO_4 buffer, pH = 7.4 (15 m*M*), and 5% newborn calf serum.

unleachable K^+ below the expected 30% value, and incubation only raised these values approximately twofold. Thus there was definite evidence of impairment of the K^+-pump.

These data show a definite log-dose response by the slices to TPB with respect to both the respiratory action of the slices and the operation of the K^+-pump which is similar to other responses by pharmacologic and toxic agents. Furthermore, it is evident that TPB in the higher concentrations (3×10^{-3} *M*), as recommended by Rappaport and Howze (4), definitely reduced the unleachable or cell-bound potassium much below that normally found (8). On the other hand, 3×10^{-5} *M* TPB did not alter either of the normal biochemical parameters studied, and this level therefore was used for additional work on the biochemical status of dissociated liver cells.

Our results on the biochemical integrity of rat liver cells dissociated from tissue using five different procedures or media, including one using 3×10^{-5} *M* TPB, are given in Table II. These data show elevated O_2-uptake of the freshly prepared cells in comparison with the data for slices of Table I whether the medium was sucrose–salt solution (method 1), sucrose–salt solution with K^+ (method 2), or sucrose–salt with 3×10^{-5} *M* TPB (method 4). Likewise, the unleachable K^+ values are all very much lower than those which were found for freshly prepared liver slices even when 3×10^{-5} *M* TPB was in the leaching medium of the slices. Thus all of the methods which we used for dissociating cells drastically altered these basic biochemical parameters from those which might be expected on the basis of the values found with freshly prepared liver slices.

In addition, it was found that the O_2-uptake of cells *after* incubation with K^+ medium had dropped in the three methods studied; namely, methods 1, 2, and 4, a situation which did not occur with normally functioning slices. The most drastic change, however, was again found to be the inability of these dispersed cells to accumulate K^+ against a gradient; and a most interesting finding was that the amount of K^+ accumulated was less than that which would normally have been expected to remain as bound K^+ in the leached cells or tissue (7).

Our results with cells dissociated in the

TABLE II. Oxygen Uptake and K^+ Content of Cells Prepared by Using Different Dissociating Solutions and Enzyme Digestion at 37°.

Liver tissue	No. of expts.[b]	Fresh cells		Incubated cells	
		Oxygen (μl/mg of protein/hr)[c]	Potassium (μeq /g of protein)[d]	Oxygen (μl/mg of protein/hr)[c]	Potassium (μeq /g of protein)[d]
Dissociated in[a]					
1. Sucrose–salt solution	3	32 (29–47)	5 (2–9)	18 (13–27)	28 (26–30)
2. 5 m*M* KCl sucrose–salt solution	5	23 (12–29)	6 (4–7)	16 (12–19)	36 (30–42)
3. 400 milliosmolar sucrose–salt solution with 5 m*M* KCl	2	—	14 (11–16)	—	32 (32–33)
4. Sucrose–salt TPB solution (3×10^{-5} *M*)	2	41 (41–42)	8 (7–9)	27 (21–33)	28 (27–29)
5. Digested by enzyme	3	—	29 (24–36)	—	40 (33–48)

[a] The composition of solutions is described under Materials and Methods.

[b] Each value is the mean of 4 or more independent measurements with range given in parentheses.

[c] The reaction mixture, total of 5.0 ml contained 4.5–4.7 ml of MEM solution (pH = 7.2–7.4), 0.2–0.4 ml of cell suspension (25–50 mg of protein/ml), 0.1 ml of 110 m*M* succinate.

[d] The incubation media for K^+ uptake contained NaCl (125 m*M*), KCl (5–7 m*M*), $MgSO_4$ (1.2 m*M*), NaH_2PO_4 (1 m*M*), $CaCl_2$ (1.2 m*M*), glucose (200 mg/100 ml), NaH_2PO_4 buffer, pH = 7.4 (15 m*M*), and 5% newborn calf serum.

five different ways have a consistency which would indicate that the severe alteration in respiratory and K^+-pump activity was somehow related to the process of dissociation rather than to any specific method. Furthermore, the data cannot implicate 3×10^{-5} *M* TPB as being in any way specifically involved in these changes.

This conclusion that the dissociation of liver tissue into cells causes drastic disturbances in several normal metabolic functions is further emphasized in a preliminary investigation of the integrity of the mitochondria isolated either from fresh liver tissue (method 1) or from liver cells dissociated in sucrose–salt solution containing 3×10^{-5} *M* TPB (method 4). When mitochondria in the tightly coupled state of oxidative phosphorylation were studied (10), it was found that those obtained from liver tissue directly responded to added ADP in the presence of succinate with a sharp rise in O_2-uptake, whereas those derived from the dissociated liver cells gave no response to added ADP. This indicates that dissociation of tissue into cells may drastically alter the energy-converting mechanism and thus could cause most of the aberrations mentioned above involving O_2-uptake and K^+-accumulation.

Discussion. Recently several reports have appeared which confirm our conclusion that TPB in the concentrations originally recommended by Rappaport and Howze (4) are toxic. These reports also confirm our findings that the dissociation of liver cells, regardless of the method used, causes biochemical aberrations.

Thus, Utsumi and Packer (11) reported that a concentration of 8×10^{-5} to 2×10^{-4} *M* TPB caused marked alteration of the parameters used by them to evaluate the state of oxidative phosphorylation in rat liver mitochondria. Since the oxygen uptake of cells is in the same order of magnitude as that shown by mitochondria, their results and those reported by us above are in harmony.

Although very little is known about the membrane function in dissociated cells, our findings with K^+-accumulation appear to

bear on this aspect. The large reduction in K^+-accumulation found with slices when TPB was present in the higher ranges of concentration, and when cells were obtained by the mechanical dissociation procedure which we used, can be explained by a drastic inhibition or uncoupling of oxidative phosphorylation, as shown by Utsumi and Packer (11).

Friedmann and Epstein (2) reported the failure of cells dissociated with either TPB or citrate to carry on normal protein synthesis. Recently Lipson *et al.* (5) indicated that TPB-dissociated cells incorporate ^{14}C-amino acids into protein at about one-fifth the rate of cells isolated by mechanical means or by enzymatic digestion of the tissue. In neither report, however, was a clear delineation of the concentration of the dispersing agents given. In the latter report there was no indication whether or not a normal level of incorporation was achieved. Harris and Leone (12) reported that 0.1% EDTA and TPB (3×10^{-6} to 3×10^{-3} *M*) cause an alteration of the ultrastructure of mouse liver cells when used in the dispersing media.

Our finding that the "unleachable" K^+ is greatly reduced in dissociated cells and in slices treated with 3×10^{-3} *M* TPB indicates a further definite effect on the utilization of energy by the cells. The fact that the effect of TPB on slices is log–dose related is of importance, since it indicates a drug-like action which may be further studied by classical pharmacologic methods.

From our data and those of the literature cited here, it would appear that liver cells prepared by dispersing agents presently available, by mechanical means alone, or by enzymatic digestion, have limited value for the study of normal metabolic processes.

Summary. Rat liver cells prepared by using various dissociating solutions were unable to accumulate K^+ from the media. The respiration of dissociated liver cells in the presence of succinate was not altered by the presence of 3×10^{-5} sodium tetraphenylborate in the dissociating media. Accumulation of K^+ and respiration in the presence of succinate of rat liver slices were shown to be subject to inhibitory action of TPB on a log–dose basis in the concentration range of 3×10^{-5} to 3×10^{-3} *M*. Liver slices exposed to 3×10^{-3} *M* TPB showed the same loss of ability to accumulate K^+ which was shown by liver cells dissociated by any of the methods tried, whether TPB was present or not. From these studies it is concluded that freshly isolated cells which exhibit oxygen uptake in the presence of succinate are unable to perform other metabolic functions, such as K^+ uptake.

1. Noda, K., Karome Med. J. 14, 1 (1967).
2. Friedmann, T. and Epstein, C., Biochem. Biophys. Acta 138, 622 (1967).
3. Howard, R. B. and Pesch, L. A., J. Biol. Chem. 243, 3105 (1968).
4. Rappaport, C. and Howze, G. B., Proc. Soc. Exptl. Biol. Med. 121, 1010, 1016 (1966).
5. Lipson, L. G., Margolis, S., and Capuzzi, D. M., Federation Proc. 28, 366 (Abstract No. 607) (1969)
6. McIlwain, H., Biochem. J. 78, 213 (1961).
7. McLean, A. E. M., Biochem. J. 87, 161 (1963).
8. Weichselbaum, T. E., Am. J. Clin. Pathol. 7, 40 (1946).
9. Mudge, G. H., Am. J. Physiol. 165, 113 (1951).
10. Lehninger, A. L., "The Mitochondrion," p. 135. Benjamin, New York (1965).
11. Utsumi, K. and Packer, L., Arch. Biochem. Biophys. 122, 509 (1967).
12. Harris, C. C. and Leone, A. C., J. Cell Biol. 28, 405 (1966).

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