in vitro into a suspension of single cells. 2. Evidence is presented that this is the result of the removal of K^+, which is the major cation involved in aggregation of cells in this tissue. 3. A coordination mechanism for aggregation is proposed in which the negatively charged surfaces in cell-cell aggregates or in cell-matrix-cell aggregates are neutralized by monovalent cations. 4. Two variables of coordination mechanisms, *i.e.*, cation coordinated and the coordination number are used in a model advanced to explain ordered movement and aggregation of cells in tissues.

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Further Studies on the Dissociation of Adult Mouse Tissue.* (30952)

CATHERINE RAPPAPORT AND GWENDOLYN B. HOWZE (Introduced by T. T. Puck)
(With the technical assistance of Mehda von Essen and Joan E. Marino)
Yale University School of Medicine, New Haven, Conn.

The dissociation of the adult mouse liver by sodium tetraphenylboron (TPB) has been reported(1). The selectivity of TPB for the K^+ ion(2) and the failure of EDTA(1,3), an agent complexing Na^+(3,4) and divalent cations suggested that the cells were held together in the tissue by coordination through K^+. This rather unexpected finding has been investigated further by studying the effect of other agents complexing cations on the dissociation of liver. Dissociation of other adult tissues, *i.e.*, kidney, brain and connective tissue, was also investigated in order to see if coordination through monovalent cations were a general mechanism for aggregation of cells in tissues.

The results indicate that K^+ is the major cation involved in aggregation in many adult mouse tissues. Certain differences have been found in the optimal agent for recovering particular types of cells from different tissues. The results indicate that coordination through monovalent cations is a general mechanism for aggregation, but that tissues may differ in the number of sites on the surface occupied by Na^+ or by K^+, and possibly also by the degree of hydration of the surface bound cations.

Materials and methods. Adult C3H male mice from 2-4 months old were used. The animals were killed by breaking the neck. The tissues were removed immediately and placed into the dissociation solution and allowed to stand at 4°C. The dissociation solution for liver contained 0.05 M sucrose, 0.14 M NaCl and .005 M sodium phosphate buffer pH 7.8. The complexing agents were diluted directly into this mixture from concentrated stocks and when necessary the pH was adjusted with NaOH.

In experiments with kidney, brain and connective tissue, the concentration of the sucrose was also varied.

All organic reagents were obtained from the K. and K. Special Chemical Laboratories. Sodium metaborate was obtained from Fisher Scientific Co.

Comparison of Na^+ and K^+ complexing agents. The agents which have been studied with respect to their capacity to complex Na^+ or K^+, as determined by a survey of the

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TABLE I. Effect of Sodium and Potassium Complexing Agents on Dissociation of Adult Mouse Liver *in vitro*.

Dissociation in .05 M sucrose-.14 M NaCl solution (pH 7.8) at 4°C. Yields are average of 3 or more experiments at the "optimal" concentration using tissue derived from different mice.

Anion (Na^+ salt)	Avg: Opt conc M/l	Avg: No. of cells × 10^6 recovered/liver	Complexing: Na^+	Complexing: K^+	Ref
Cl^-	.14	1	—		a
SO_4^{2-}	.10	1	—		a
Alanine	.10	1	—		a
Histidine	.10	1	—		a
Cysteine	.07	4			
Glycine	.10	1	—		a
Acetate	.10	3	—		a
Malate	.05	10	+		a
Malonate	.05	3	—		a
Pyruvate	.10	5	+		a
Citrate	.05	10	+		a
α-hydroxybutyrate	.05	15	+		a
EDTA	.05	10	+	—	a,b,c
Ethanol	2.0	4	+		a
Acetylacetone	.4	12	+		d
Metaborate	.0001	39	+	+	e,f
Violurate	.005	25	+	+	g
Picrate	.01	35	—	+	h
Tetraphenylboron	.005	45	—	+	h
Diphenyldithiocarbazone*	.0001	25			
Perchlorate	.10	35		+	f,h

a) NMR measurements(4).
b) Potentiometric titrations(5).
c) NMR measurements indicate no interaction with K^+(6).
d) Potentiometric titrations in H_2O-dioxane(7).
e) NMR(8).
f) Stability of K^+ salt in solutions(9).
g) Colored complex formation in Dimethyl formamide(10) and in H_2O (this laboratory in collaboration with Dr. R. Kozera).
h) Insoluble salt formation in H_2O(2).
* Concentration uncertain—has very low solubility in H_2O but absorbs at cell surfaces.

literature, were tested. The number of studies on complexing of these monovalent cations is rather limited. The most extensive study appears to be the nuclear magnetic resonance (NMR) study of Jardetsky and Wertz(4), in which a series of compounds, some of which were naturally occurring metabolites, were investigated for weak complex formation with Na^+. A few other compounds have been studied for complex formation with Na^+ by spectrophotometric or potentiometric titration methods. However, none of these compounds were also studied for their capacity to complex K^+.

The effect of these agents on the dissociation of adult mouse liver was determined as previously described(1), by allowing fragments of liver to stand in a sucrose-salt solution at pH 7.8 at 4°C for 2 hours. This was followed by 'reducing' the fragments by gently forcing them through a series of pipettes with decreasing bore sizes. The number of single cells recovered was determined by haemocytometer counts. Each agent was tested over a range of the concentrations from about 10^{-4} M to the limit of its solubility in the sucrose-salt solution, usually about 0.5 M. After the optimal concentration for a given compound had been determined, dissociation at this concentration was repeated using portions of livers derived from several different mice.

The number of single cells recovered by treatment with the different agents at their optimal concentration is given in Table I. The degree to which they complex Na^+ or K^+ at these concentrations is indicated by plus or minus. This scoring was based on an evaluation of the data in the literature, and the pertinent information for the scoring has been

given in the footnote to the Table.

The most general finding from this survey is that none of the compounds known to complex Na^+ were as effective in dissociation as the agents complexing K^+. Thus, pyruvate, malate, citrate, α-hydroxybutyrate, EDTA and acetyl acetone, compounds for which there is evidence of weak complex formation, release at the 0.1 M level only 10-25% the number of cells as .003 M TPB. The other anions complexing K^+, *i.e.,* violurate, picrate, and perchlorate, were also more effective than the sodium complexing agents, yielding a larger number of cells at lower concentrations. More importantly, the quality of the cells released by the K^+ complexing anions is superior to those recovered after treatment with the Na^+ complexing anions. Dissociation with TPB has been shown microscopically(1) to be the result of a process, limited to the cell surface, in which the cells simply separate from each other without any change in gross morphology of the cell *in situ*. The process starts by the formation of gaps between all the cells in the tissue. Formation of gaps has been found to be characteristic of the K^+ complexing agents and is never seen to occur during treatment with the other types of anions. The cells which are recovered by treatment with citrate, EDTA, etc. show gross morphological changes compared to the cells *in situ*.

The data show, however, that there is some correlation between the capacity to complex Na^+ and to dissociate the tissue. Although dissociation with the anions complexing Na^+ was poor compared to that obtained with TPB, it is significantly greater than with agents which do not complex Na^+. Thus, the inorganic agents NaCl, Na_2SO_4, which were not found by NMR measurements to complex Na^+ were without effect on dissociation, whereas $Na_2B_2O_4$, which complexes Na^+ was effective. The organic reagents, alanine, glycine, histidine and acetate have been shown not to complex Na^+ and did not dissociate the tissue. Malate complexes Na^+, whereas malonate with the hydroxyl one carbon removed, does not. Malate increases the yield of cells some 10 times over that obtained in the controls, whereas malonate was without significant effect.

In analyzing the correlation between association with Na^+ and dissociation, it is necessary to consider the fact that most of these compounds have not been studied for their capacity to form weak complexes with K^+. Thus, effectiveness of anions such as citrate, malate, etc. may only indicate that they have some affinity for K^+. The activity of EDTA, however, cannot be attributed to the complexing of K^+. The potentiometric titration studies of Schwarzenbach *et al*(5) showed that EDTA forms weak complexes with Na^+ at concentrations as low as 10^{-3} M. On the other hand, the NMR studies of Nikol'skii *et al*(6) indicated no interaction of EDTA with K^+ even at high concentrations and over a wide pH range. These data indicate a high selectivity for Na^+ over K^+. It seems unlikely that even given some unusual physical environment at the cell-cell interphase, EDTA would complex K^+. In these studies, and also those of Easty and Mutolo on rat liver(3) EDTA was found to have no significant effect at concentrations where it complexes Na^+, *i.e.,* the .001 M range. Concentrations of 0.1 M were required for significant effect on dissociation. In the case of the other Na^+ complexing agents, citrate, malate, α-hydroxybutyrate and acetyl acetone, the concentration required for dissociation was found to correlate well with the concentrations where these anions complex Na^+. The relatively high concentrations required with EDTA may only indicate that its diffusion into the tissue mass is restricted. It is possible, however, that dissociation is not the result of removal of Na^+, but due to some other effect, such as ionic strength, on the cell aggregate.

Diphenyldithiocarbazone was found to be an effective dissociator of liver tissue. The possible mechanism cannot be evaluated at this time. Diphenyldithiocarbazone is a lipid soluble compound and can be seen, since it is bright yellow, to adsorb on or within the surface membrane. In aqueous solutions, it is known to complex Zn^{2+} specifically, but certain effects on removing materials coordinated to glass surfaces may indicate that it has a high affinity for the monovalent cations in aqueous phase.

The results of this survey of cation-complexing agents indicate that the most effective agents for dissociation of liver tissue are those which complex K^+. This indicates that the major cation of aggregation is K^+. In an effort to determine whether other cations played a secondary role in aggregation, experiments were set up to determine a synergistic effect of other cation-complexing agents on TPB. The tissue was incubated at 4°C in the sucrose-salt solution containing 10^{-3} M TPB, supplemented with another complexing agent. The addition of 0.1 M EDTA, 0.1 M acetylacetone and of .0001 M diphenyldithiocarbazone to the TPB did not result in an increase in rate of dissociation or in the number of cells recovered compared to the TPB control. The addition of glutamate sometimes seemed to improve the quality of the cells released. The findings suggest that K^+ is the predominant, if not exclusive cation of aggregation in the liver.

Dissociation of other tissues. The dissociation of brain, kidney and gastric mesentery of the adult mouse has been investigated. The various compounds found to be effective for dissociation of liver were tested using the same procedure as that used with the liver, *i.e.,* pre-incubation of cut tissue at 4°C with the agent in the sucrose-salt solution at pH 7.8 for one hour. The fragments were then pipetted and when the turbidity of the fluid indicated that a large number of cells had dissociated off, the fluid was removed from the residual tissue, and replaced with 3-5 ml of fresh solution. The procedure was continued in some cases for many hours. The different agents were evaluated by the quality of the cells released and by the relative number recovered.

TPB was found to be an effective agent for the dissociation of mouse brain. Other agents, *i.e.,* EDTA, citrate, malate, glutamate, and diphenyldithiocarbazone, were not so effective. The dissociation in TPB appeared to be facilitated with sodium glutamate. In the case of brains from very young mice (5 days old), where development of the long and entangled axons has not yet taken place, dissociation proceeded rather rapidly and was about 50% complete after 2-3 hours at 4°C. A large number of the various cellular elements characteristic of the brain were recovered as intact single cells (Fig. 1). Brains from adult (4 months old) mice dissociated much more slowly. It is considered that this is due in large part to the limited rate of diffusion of TPB into the dense and more involved tissue mass. Breakdown of the long axons during the pipetting used to 'reduce' the tissue fragments was also found to be a problem with the adult tissue. This was prevented to some extent by increasing the concentration of sucrose to 0.5 M. Increasing the pH, by addition of $NaHCO_3$, which had been found in liver to increase dissociation, was also found to facilitate the separation of brain cells (Fig. 2, 3).

Tetraphenylboron was also found to dissociate adult kidney tissue. However, these preliminary studies indicated that the best dissociation of the adult kidney was in a mixture of 0.05 M citrate and 0.05 M L-malate, which caused the cells to separate off fragments without change in morphology (Fig. 4). A large number of cells with brush borders were recovered. The physiological conditions of these cells are indicated by the drop in pH during dissociation and by the fact that the recovered cells stain supravitally with Janus Green B (Fig. 5).

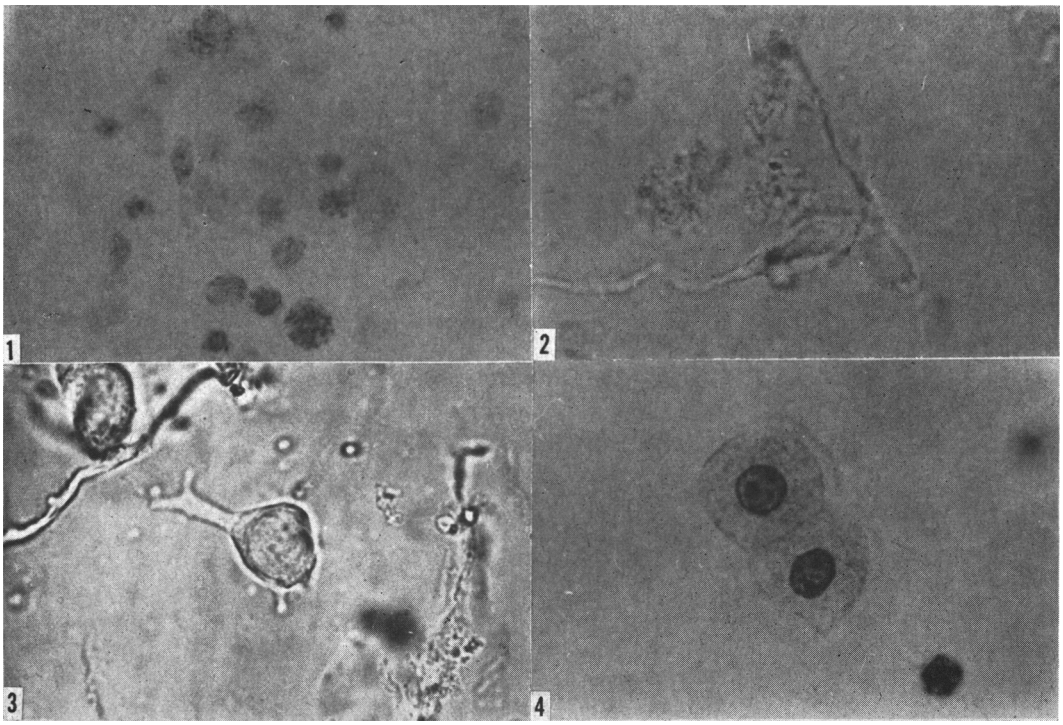
Connective tissues from the adult mouse were found to behave quite differently from liver, brain or kidney tissues. Gastric mesentery was found to be refractory to TPB, citrate, malate, EDTA or glutamate. However, a significant dissociation was obtained using 4 M ethanol in the sucrose-salt solution at pH 8.3.

Discussion. A large number of compounds has been investigated with respect to their capacity to dissociate adult mouse liver. Agents which are known to complex K^+ were strikingly more effective than agents which complex Na^+ or divalent cations. Solutions containing both the K^+ complexing agent tetraphenylboron (TPB) and also an agent complexing Na^+ did not increase either the rate of dissociation of the tissue or the quality of the cells obtained over that obtained in the presence of TPB alone. Thus, in liver it would appear that the sites on the cell sur-

face involved in aggregation are predominantly, if not exclusively, coordinated with K^+. This finding does not exclude the possibility that Na^+ and perhaps also the divalent cations may be involved secondarily in aggregation by stabilizing the surface membrane or intracellular matrix material.

TPB was also found to dissociate adult mouse brain and kidney, liberating cells with the morphology of the various elements of these tissues. Some of the agents known to complex Na^+ were effective in these tissues. The only tissue of those studied which was found to be refractory to TPB was adult gastric mesentery. Dissociation could be effected with 4 M ethanol; a solution in which there is some evidence that Na^+ may be complexed (4).

The findings suggest that the monovalent cations play a major role in the aggregation of most adult mouse tissues. The differences in behavior among the tissues with respect to the agent optimal for dissociation might at first suggest that the cells differ in the proportion of the binding sites coordinated with K^+ or with Na^+. Thus, whereas liver is coordinated predominantly with K^+ according to this interpretation, kidney tissue would be



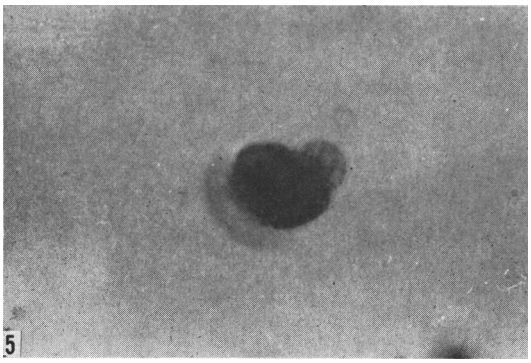


FIG. 1. Brain tissue from 5-day-old mouse dissociated with 0.3% TPB in sucrose-salt solution. Stained with Giemsa. Magnification, 260×.

FIG. 2. Neurons with long axons dissociating from adult mouse brain during treatment with 0.3% TPB in 0.5 M sucrose-salt solution at pH 8.3. Magnification, 260×.

FIG. 3. Bifurcated neurons recovered from TPB dissociation of adult mouse brain. Magnification, 260×.

FIG. 4. Brush border cells recovered from adult kidney on treatment with 0.05 M citrate-0.05 M l-malate in sucrose-salt solution. Stained with Toluidine blue. Magnification, 260×.

FIG. 5. Brush border cells stained supra-vitally with Janus Green B. Magnification, 260×.

coordinated with both cations, and gastric mesentery would be coordinated predominantly with Na^+. We have presented elsewhere (1) a theory based on coordination mechanisms of cellular aggregation and movements. One of the important aspects of this theory is that it can account for stability of cell-cell adherence, even under conditions where there is a continuous electrolyte exchange taking place at the surfaces. Since cells differ in the characteristic rates of exchange of Na^+ and K^+, evidence indicating actually a difference in the amount of the 2 cations coordinated at the surfaces of the different cell types would suggest an important aspect of the mechanism which couples metabolism with cellular aggregation and movements in tissues.

The data, however, do not actually permit an evaluation of the role of coordination through Na^+, in the aggregation of these tissues. The effectiveness of the agents, determined by the number of cells recovered, is the result of 3 factors which cannot be evaluated at this time: 1) The diffusion of TPB into a tissue may be virtually prevented in certain tissues. Data to be presented later indicate that even in liver it may be rate-limiting. It may also be added that in preliminary studies with hamster embryos where the tissue is more hydrated, both kidney and connective tissue were easily dissociated with TPB. 2) The degree to which the agents complexing Na^+ also complex K^+ is not known. Thus, the effectiveness of malate and citrate with certain tissues may be due to the fact that they are actually complexing K^+. 3) Finally, the effectiveness of an agent is the result of the stability of the cells released in its presence. The apparent superiority of certain agents in some adult tissues over TPB may be due to the fact that the cells have a lower tolerance for TPB under the conditions used here than for the other agents.

Regardless of the uncertainties about the relative amount of Na^+ or K^+ coordinating the cells in different tissues, the findings suggest a new approach to separation of mammalian tissues. Cells released from tissues by these complexing agents retain the morphology of the various cellular elements in the tissue. This is in marked contrast to the results obtained by digestion of tissues with various enzymes. The grossly distorted morphology of the cells and the low yields obtained by enzymes may indicate that the cells are freed to a large degree simply because the cells adjacent to them in the tissue have been completely broken down. Although only a few compounds were studied here, *i.e.*, those already reported in the literature to complex Na^+ of K^+, 4 effective agents for dissociation were discovered, *i.e.*, TPB, metaborate, citrate and malate. It may be expected that a wider survey may indicate even better agents, and that with the proper attention to factors affecting the stability of the cells released, virtually any cell type in a tissue may be recovered separate and intact.

Summary. 1. A series of compounds known to complex Na^+ or K^+ has been studied with respect to their capacity to dissociate various tissues of the adult mouse. 2. Single cell suspensions of kidney, brain and connective tissue retaining the morphology of the various cellular types *in situ* may be obtained with the appropriate complexing agent. 3. The findings suggest that cells in most tissues are aggregated predominantly by coordination about K^+. The extent to which cells may be aggregated by coordination through Na^+ in some tissues cannot now be evaluated.

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