# Effect of Temperature on Dissociation of Adult Mouse Liver with Sodium Tetraphenylboron (TPB).* (30953)

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The dissociation of adult mouse liver, and certain other tissue into a suspension of single cells with sodium tetraphenylboron (TPB) has been described(1,2). Other agents complexing $K^+$, *i.e.*, picrate and perchlorate, also dissociate the tissue. In the case of picrate and perchlorate, the concentration required for maximal dissociation at 4°C was related to the affinity of these anions for $K^+$. The concentration of TPB, however, required to dissociate the tissue was significantly higher than that at which salt formation with $K^+$ takes place. The possibility that diffusion of the bulky TPB molecule into the tissue was limiting the dissociation was investigated by studying dissociation with TPB at higher temperatures. It has been found that at 38°C, the concentration of TPB required to dissociate does correlate with its affinity for $K^+$.

It has also been found that the higher temperature *per se* facilitated dissociation. Efficiency of dissociation has been studied as a function of TPB concentration, temperature and pH. Under the optimal conditions, the tissue is dispersed rapidly and efficiently into a suspension of single cells.

*Results. Dissociation at 38°C.* Dissociation of adult mouse liver at 4°C and at 38°C is shown in Fig. 1. In these experiments, 0.3 g wet weight of cut tissue was incubated with 10 ml of the sucrose-salt solution (pH 7.8) at either 4° or 38°C for 2 hours and then 'reduced' by pipetting (see(1) for details). At 4°C, as already discussed(1) the concentration of TPB required to dissociate the tissue to any significant extent is nearly $10^{-3}$ M, or above that at which solubility data(3) show $K^+$ to be completely complexed by TPB. At 38°, however, concentrations as low at $5 \times 10^{-5}$ M significantly increase the number of cells released compared to that taking place in the sucrose-salt controls. The time required for complete dissociation of the tissue is also reduced.

Increasing the temperature was found to have quite an unexpected effect on dissociation. The quality of the cells released both in the presence of TPB and in the sucrose-salt control was greatly improved. The cells released were larger and less granulated than those obtained after treatment at 4°C. There is a considerable amount of acid produced during the treatment at the higher temperature. The higher yields obtained at 38°C may be the result, in part, of a stabilization of the cells afforded by metabolism.

*Preparation of liver cell suspension.* It has been found and will be reported later that cells released by TPB are viable and can attach to glass and grow *in vitro* under suitable conditions. It was, therefore, of interest to determine optimal conditions for treatment with TPB for the preperation of single cell suspensions.

The facilitating effect of increased pH on dissociation at 4°C has been presented(1). Addition of sodium glutamate was also found to have a qualitative effect on dissociation. The effect of these two variables, pH and concentration of glutamate on dissociation at 38°C was investigated. In these experiments, the tissue cut into pieces about 3-5 mm in size, was placed in an Erlenmeyer flask fitted with a magnetic stirrer and stirred vigorously from the moment it was covered with the solution. This was found easier and probably more effective than the pipetting which had been used previously for 'reducing' the fragments. The pH was adjusted to 8.6 by addition of $2 \times 10^{-2}$ M $NaHCO_3$ and further adjusted with .01 N NaOH if necessary. A volume of solution (25 ml per 0.3 g of tissue wet weight) was used sufficient to maintain the pH in spite of the acid produced by the

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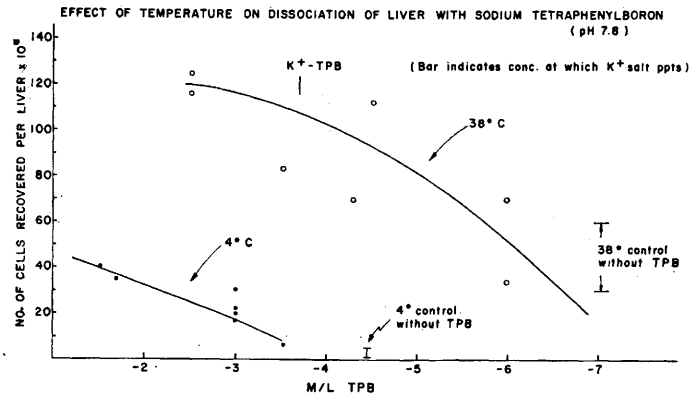


FIG. 1. Number of cells recovered on treatment of adult mouse liver with different concentrations of TPB at 4°C and at 38°C.

TABLE I. Effect of pH and Sodium Glutamate on TPB Dissociation of Mouse Liver at 38°C.

Tissue fragments stirred on magnetic stirrer in .05 M sucrose-.14 M NaCl solution pH 7.8 or pH 8.6 ($5 \times 10^{-3}$ M $NaHCO_3$). Number of single cells counted when tissue had completely dissociated.

| Supplements to .05 M sucrose-.14 M NaCl M/l | | No. of cells $\times 10^6$ recovered per liver | |
|---|---|---|---|
| TPB | Glutamate | pH 7.8 | pH 8.6 |
| $3 \times 10^{-3}$ | | 118, 125, 95 | 100, 91, 96, 106, 102 |
| | $5 \times 10^{-3}$ | 90 | 148, 133, 157, 130, 120, 124 |
| $3 \times 10^{-4}$ | | 94 | |
| | $5 \times 10^{-3}$ | | 123, 96 |
| $3 \times 10^{-5}$ | | 75, 110 | 155, 104, 109 |
| | $5 \times 10^{-3}$ | | |
| $3 \times 10^{-6}$ | | 60 | 74 |
| | | 52, 26 | 68, 58 |

cells to within 0.3 units during the treatment.

The results of these experiments are given in Table I. Each number represents the yield obtained by processing 0.3 g of tissue from different mice. The yield per liver has been derived by multiplying by a factor based on the actual wet weight of the livers, which in this strain of mice (2-4-month-old male C3H) was found to average 1.6 ± 0.3 g.

The data show the facilitating effect at 38°C of pH 8.6 and some effect of the presence of sodium glutamate. Glutamate did not always give a significant quantitative increase in cells. However, the quality of the cells usually appeared superior after dissociation in the presence of glutamate. The data indicate that about 130 million cells can be recovered from an adult male mouse liver by treatment at 38°C, pH 8.6 in the presence of $3 \times 10^{-3}$ M TPB and glutamate. The separation of tissue into a suspension of single cells is complete under these conditions in 30 to 45 minutes.

*Percentage of tissue recovered as single-cells by TPB.* Microscopic observation of tissue fragments during treatment with TPB as already reported indicated that this agent causes the separation of virtually every cell throughout the tissue from contiguous cells (1). Those observations were made under conditions in which a large number of the cells were lost, as indicated by the recovery of a large number of free nuclei in the final preparations. It was of interest to determine the percentage of cells recovered from tissue prepared under the improved conditions just described.

The efficiency of recovery was based on an estimate of the total number of parenchymal cells *in situ,* which was obtained by counting

TABLE II. Efficiency of TPB Dissociation of Mouse Liver as Determined by Nuclei Counts. Suspensions 1 and 2 prepared by treatment under "optimal conditions" *i.e.* $3 \times 10^{-3}$ M TPB in the sucrose-salt glutamate solution at pH 8.6 and 38°C. Suspensions 3 and 4 prepared with excess TPB.

| No. | g wet wt | Cells $\times 10^6$ recovered /liver | Free nuclei $\times 10^6$ in suspension | % cells binucleated | Cells equivalent to free nuclei | Total No. of cells $\times 10^6$ in liver | % recovery | No. of cells $\times 10^6$ per g wet wt |
|---|---|---|---|---|---|---|---|---|
| 1 | 1.8 | 144 | 1 | 56 | 1 | 145 | 99 | 80 |
| 2 | 1.6 | 147 | 2 | 50 | 1 | 148 | 99 | 92 |
| 3 | 1.5 | 101 | 31 | 43 | 27 | 128 | 72 | 86 |
| 4 | 1.5 | 112 | 60 | 57 | 33 | 157 | 72 | 105 |

the total number of nuclei, both free and in cells, recovered in the preparations. A large number of the cells recovered from the livers of the strain of C3H male mice used here are binucleated. The number of cells represented by the free nuclei in a suspension requires, therefore, a direct determination of the percentage of binucleated cells in the particular liver treated. This was determined by viewing samples of the suspensions at 600 × magnification and scoring the cells with respect to number of nuclei. At least 200 cells in each suspension were scored. It was found (Table II) that about 50% of the cells are binucleated. Less than 2.0% of cells were found which had more than two nuclei.

The efficiency of recovery from treatment with TPB has been presented for 4 livers in Table II. The first 2 suspensions were obtained by treatment under 'optimal' conditions, *i.e.*, at 38°C in the presence of $3 \times 10^{-3}$ M TPB in the sucrose-salt glutamate solution at pH 8.6. The third and fourth suspensions were obtained by treatment under sub-optimal conditions, *i.e.*, higher concentrations of TPB solution. These suspensions contained a smaller number of single intact cells. If the nuclei are stable to TPB under these conditions, the total population based on nuclei counts in these suspensions should be the same as that derived by counts on the good suspensions. This was found to be the case. Counts on both types of suspension indicate that the liver of an average 2-4-month-old male C3H mouse contains about 150 million parenchymal cells. The number of single intact cells recovered in the suspensions prepared by treatment under the optimal conditions was more than 80%.

*Discussion.* It has been found that at 38°C, the concentration of TPB required for dissociation of adult mouse liver correlates well with its affinity for $K^+$. The anomalously high concentrations which were found to be required at 4°C would seem to be explained by the impeded diffusion of this molecule into the tissue mass. Thus, the findings here and those reported previously with other $K^+$ complexing anions, are consistent with the hypothesis that aggregation of cells in liver takes place through coordination about $K^+$.

It has also been found that dissociation is facilitated by increased temperatures *per se*. Most work on dissociation has adhered to the precaution of keeping the tissue cold, presumably to avoid autolysis. It was found, however, that the number of liver cells recovered was greater after treatment at 38°C than at 4°C. More importantly, the quality of the cells was superior after dissociation at the higher temperature. It would appear that the metabolism which can take place at 38°C actually helps to stabilize the cells. Similar beneficial effect of temperature on dissociation of brain and spleen has also been observed.

Apart from 'stabilization' of cells during dissociation, increased temperature may have direct effects on the aggregation mechanism. In a mechanism in which cells are aggregated about $K^+$, the simplest configuration would be one in which all of the available coordination positions are occupied by 'fixed' sites on the surface of the adjacent cells. This configuration would be the most stable but it would also mean that, at these sites of aggregation, there would be a 'shut-down' in electrolyte exchange. The model has proposed, however, that metabolism does take place at the aggregation sites through a process in

which some of the 'fixed' sites are displaced by small molecular 'mobile' ligands, *e.g.*, $H_2O$, $Cl^-$, etc., and perhaps even metabolites such as pyruvate and citrate(4) passing in and out of the cell. Thus, during metabolism, the aggregate would be 'opened up' and the tissue should dissociate more readily. The facilitating effect of high pH on dissociation observed not only in liver, but in other tissues (5,6) may be explained in part similarly. Hydroxyl ion coordinates with cations and would diffuse in and open up the tissue by displacing some of the 'fixed' surface sites.

According to the coordination hypothesis of aggregation which we are considering, the primary event is the completion of the coordination requirements of the intercellularly held cation through the formation of one or another of the configurations 'permitted' for that cation. It is not necessary to distinguish between the cases in which cells are aggregated directly to other cell surfaces, and those in which there is intercellular matrix material. In both cases, the stability of the complex may be expected to be determined, and amplified, by secondary cooperative effects taking place along the membrane. The sensitivity of aggregation to pH and to temperature observed here and with other systems(7) may be the result not only of changes in metabolism but to conformational changes of the macromolecules involved.

*Summary.* 1. Dissociation of adult mouse liver *in vitro* was found to proceed more rapidly at 38° than at 4°C. The number of cells recovered either in the presence or the absence of TPB was increased. The physiological condition of the cells recovered by dissociation at 38°C was superior. 2. At 38°C, the concentration of TPB required for dissociation correlates well with the concentrations where it complexes $K^+$. 3. A procedure has been developed which results in the recovery of about 80% of the total population of parenchymal cells as a suspension of single intact cells.

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## Role of 'Intercellular' Matrix in Aggregation of Mammalian Cells. (30954)

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Previous work(1) has shown that $K^+$ complexing agents can dissociate adult mouse liver into a suspension of single cells. On the basis of this finding, we have proposed a coordination model for aggregation of cells in tissues. In the course of investigating various aspects of this model, we have reinvestigated the dissociation of liver by acid phosphate, first reported by Langmuir and ap Rees(2). It has been found that dissociation in acid phosphate is inhibited by hydrocortisone. In the presence of low concentrations of hydrocortisone, long strands of an intercellular material not apparent under other conditions of dissociation, aggregate and trap the cells in an entangled mesh.

This finding helps to clarify some specific physical questions presented by the coordina-

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