Salt Inhibition and Enhancement of Cytochrome, Succinic, and Dopa Oxidase Systems of Mouse Melanomas. (18292)

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Sodium chloride by itself as physiological saline, or in conjunction with other salts in balanced isotonic solutions has often been used in the preparation of various tissue extracts for enzyme and other studies and has generally been considered to be an essential or desirable constituent of physiological media. While this is undoubtedly valid and important for many purposes where cellular integrity or morphological criteria are of importance, the use of these solutions has been extended to general applications in the study and manipulation of enzyme systems whose optimal activity requirements may be quite different.

Since the effects of various ions have been reported for certain systems(1-6) the present studies were undertaken to determine the quantitative influence of graded concentrations of salts frequently employed for preparing tissue extracts, and of those currently used in this laboratory for the control of adsorption and elution of cytoplasmic particulates in chromatography(7).

Experimental procedures and results. Unless otherwise indicated, all experiments were done under the following general conditions: Tumors of the Harding-Passey or Cloudman S91 mouse melanoma strains were ground with Celite and distilled water to yield a 20% suspension, and the enzyme-carrying cytoplasmic constituents were separated from the nuclei and tissue debris by the short Celite column procedure(8). The various enzyme determinations were carried out manometrically at 38°C employing 0.5 or 0.8 ml of tissue extract, 0.2 ml of substrate and cofactors, 0.1 ml of 2 N KOH in the well, and 0.5 or 1.0 ml of salt solution of proper concentration to establish the molarity in the vessel indicated by the tables and figures.‡ Sodium succinate concentration in most experiments was kept at 0.014 molarity to minimize its own salt effect. The small amount of residual salt contributed by the tissue is ignored in these experiments. Relative enzymic activities were calculated from the first 30 minutes of uptake following the tipping of substrates. This period is essentially linear and similar results can be obtained by calculating from the first 10 minutes of oxygen consumption.

Effect of sodium chloride. The influence of a wide range of salt concentration on the succinoxidase, cytochrome oxidase, and dopa oxidase systems was tested from 1.0 molar through 0.0001 molar and terminating with distilled water.§ Fig. 1 shows the inhibition and enhancement of the 3 enzyme systems at the concentrations studied. Inhibition of succinoxidase was obtained at all concentra-

* With the aid of Carson Brooks. N determinations made in the NIH micro-chemical laboratory under the supervision of William C. Alford.

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† Eight mg of dry cytochrome C (Nutritional Biochemical Corp.) dissolved in 4 ml of 0.5 M or 0.14 M sodium succinate to give vessel concentrations of 1.25×10^{-5} M cytochrome C, and 0.05 or 0.014 M sodium succinate. No buffer was added in the melanoma experiments where the effects of single salts were determined. The buffer capacity of the tissue and the relative neutrality of the salts employed kept the pH at approximately 7.

‡ No significant differences in response to salt have been observed between the two melanoma strains under the conditions studied.

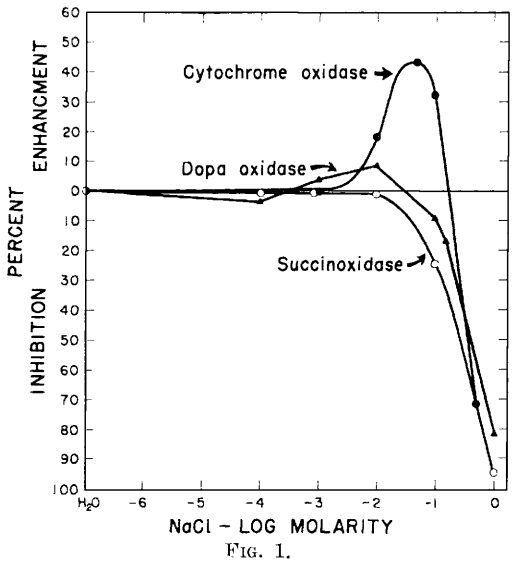

FIG. 1.

Relative enzymic inhibition and enhancement of mouse melanoma extracts at various NaCl concentrations.

tions above 0.01 molar. In no case was enhancement of succinoxidase activity noted at any concentration tested. When PPDA was substituted for succinate, as a measure of cytochrome oxidase activity, a distinct enhancement peak was obtained in the range between 0.01 and 0.1 molarity (Fig. 1). The dopa oxidase activity curve (Fig. 1) is similar to that obtained for succinoxidase. The slight enhancement obtained in the vicinity of 0.01 molar is not statistically significant, although a small enhancement effect at this concentration is not ruled out. Fig. 2 illustrates the degree of inhibition of the succinoxidase system by NaCl as compared with KCl from 0 to 0.7 molarity.‖

Inhibition by potassium chloride. Since sodium and potassium are antagonistic under some conditions(9) it was thought that the addition of the potassium ion might possibly counteract the toxicity of the sodium if the phenomena were due to cation inhibition. Table I indicates that no antagonistic effects were obtained with the concentrations used and that KCl acted in an additive way to increase the inhibition as though the Cl ion were the toxic agent; or alternately, that Na and K were behaving as a single toxic ion. An additional test was made, comparing distilled water with an isotonic solution containing a potassium-sodium ratio of 9 to 1. Similar inhibition effects were obtained with this solution. Fig. 2 illustrates the inhibition curve for KCl on the succinoxidase system.

Inhibition by potassium nitrate. Identification of chloride as an inhibitor for succinic dehydrogenase has been reported but with no quantitative data given(3). Since chloride was the common ion in the preceding experiments it appeared that it may have been responsible for the inhibition observed. In an attempt to check this, a similar experiment was done but with the substitution of NO_3 for the Cl anion. Fig. 3 indicates that es-

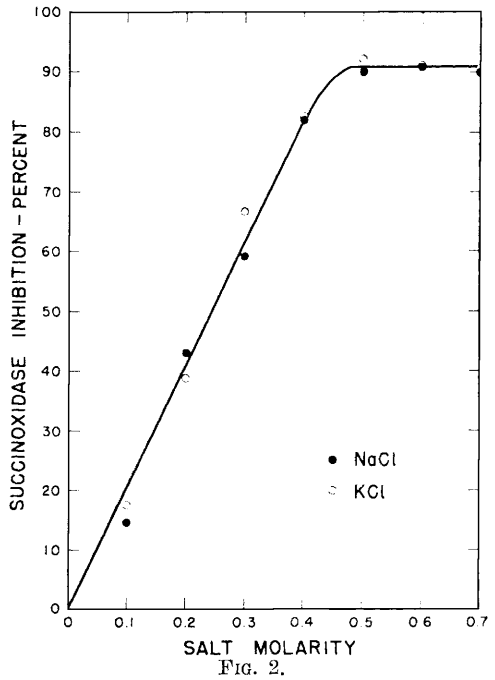

FIG. 2.

Inhibition of the succinoxidase system with sodium and potassium chloride.

§ For purposes of orientation in estimating the absolute activity of the preparations in Fig. 1 the mean oxygen consumption of the various distilled water reference points were as follows: The succinoxidase mean of 10 vessels was 126, the cytochrome oxidase mean of 3 vessels was 190, and the dopa oxidase mean of 3 vessels was 59 μl O_2/hr.

‖ Oxygen consumption at the distilled water reference point was 126 μl O_2/hr. Each point represents the mean of 5 exps.

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TABLE I. Additive Inhibitory Effect of KCl When Superimposed on a Physiological NaCl Melanoma (Harding-Passey) Tissue Extract.

Salt combinations	Succinoxidase inhibition,† %
Distilled water suspension	0
0.9% NaCl suspension	47
0.9% NaCl suspension + .0001 M KCl	49
0.9% NaCl suspension + .001 M KCl	52
0.9% NaCl suspension + .01 M KCl	54
0.9% NaCl suspension + .1 M KCl	73
0.9% NaCl suspension + 1.0 M KCl	97
Distilled water extract	0
9 to 1 K-Na solution*	44
0.9% NaCl solution	49

* KCl, 8.946 g; NaCl, 0.76 g per l.

† The μl O_2/hr at 0 inhibition was 157 and 176 in the distilled water fractions.

sentially the same result was obtained with KNO_3 for succinoxidase as with KCl, but with the interesting difference that the dopa oxidase system was but slightly, if at all, inhibited.[¶] These results demonstrate an interesting difference in enzyme-ion response between the two systems, although it did not establish the specific ion responsible for the inhibition of succinoxidase.

Inhibition and purification. Since this work was done with partially purified preparations it was thought that the inhibition might not be demonstrable in the cruder tissue extracts where there was a higher concentration of protective tissue colloids and tissue ions. To test this, an experiment was run in which the inhibition effect was tested at different stages of purification. As shown in Table II, there tends to be a slight contrary effect, but with no significant difference in the inhibition of the crude preparations as compared with more purified fractions.

Sodium chloride inhibition of tissue slices. The preceding results were obtained with cytoplasmic extracts or isolated intracellular granules. This posed the question as to whether the effect would be found when measuring the respiration of intact cells, since it has been reported that some cell membranes are impermeable to Na or Cl ions. Thin slices from a young, rapidly growing, mouse melanoma (Harding-Passey) were weighed and nearly equal amounts were transferred to chilled Warburg vessels. Two manometric series were run at 38°C employing succinate or dopa.** Table III indicates that similar inhibition effects were obtained with tissue slices as with the cytoplasmic extract or the isolated granules. The inhibiting effect of salt on the respiration of tissue slices suggests several possibilities. If the inhibition is due to the Cl ion it means that this anion can gain entrance to the cell interior rapidly and in substantial quantities, contrary to some reports(10), or acts indirectly in some manner, possibly affecting cell permeability. The similarity of the inhibition curve of granules and slices however, suggests the more direct inhibition of the intracellular succinoxidase system.

Reversibility of salt inhibition. A 20% Harding-Passey extract was prepared(8) in distilled water and the enzyme-carrying mel-

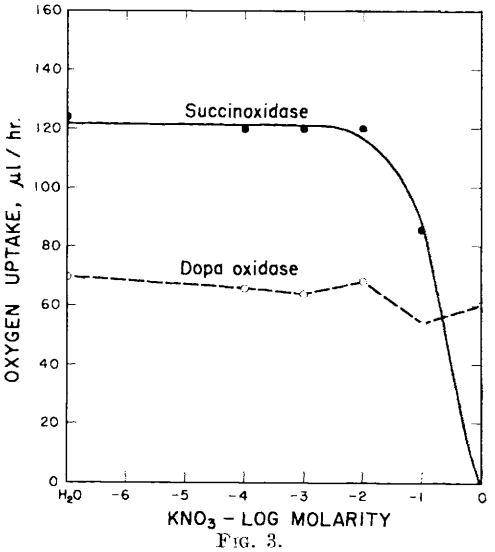

FIG. 3.

Differential inhibition of succinoxidase and dopa oxidase with KNO_3. (Cloudman S91 melanoma).

¶ Barring the possibility of an antagonistic effect, it would appear that the chloride anion is inhibitory for dopa oxidase while potassium and NO_3 are relatively non-toxic.

** Dopa oxidase was determined as described for succinoxidase except that 0.2 ml of dopa (1 mg) was employed as substrate.

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TABLE II. Effect of Purification on Salt Inhibition of the Succinoxidase System.

Fraction	Succinoxidase activity§				Salt inhibition, %
	Dist. water		0.9% NaCl		
	μl O_2/hr	$Q_{O_2}(N)$	μl O_2/hr	$Q_{O_2}(N)$	
Crude tumor homogenate*	426	122	261	75	39
Centrifugally cleared homogenate†	365	181	230	114	37
Granules centrifugally purified‡	134	244	89	162	34

* Harding-Passey mouse melanoma ground in mortar with distilled water to yield a 20% homogenate. Suspension contains intact cells, nuclei, and all cytoplasmic constituents.

† Crude homogenate centrifugally cleared to remove the bulk of the tissue debris, intact cells, and nuclei. The resulting supernatant contains most of the cytoplasmic elements.

‡ The cytoplasmic melanized granules partially purified by 2 cycle centrifugation. All the granules in the starting material were not recovered.

§ Determined manometrically with the addition of sodium succinate and cytochrome C. The tissue fraction volume was 1.0 ml. $Q_{O_2}(N) = \mu l\ O_2/hr/mg\ N$.

TABLE III. Inhibition of Succinoxidase in Harding-Passey Tumor Tissue Slices with Various Concentrations of Sodium Chloride.

NaCl conc. in vessel (molarity)	Inhibition, %
.0*	0†
.1	37
.2	49
.3	66
.4	83
.5	86
.6	89

* Distilled water.

† Oxygen consumption for the distilled water fraction was 70 μl O_2/hr for 20 mg of tissue wet weight.

TABLE IV. Reversibility of Inhibition of the Dopa and Succinoxidase Systems Following Exposure to NaCl in the Cold or at 38°C.

Fraction	Succinoxidase activity*		Dopa oxidase activity, 4°C, %
	4°C, %	38°C, %	
Granules maintained in dist. water	100	100	100
Granules maintained in 0.5 M NaCl	31	46	41
Salt replaced with dist. water	90	194	114

* The different temperature levels were run in separate experiments. The 4°C experiment employed Harding-Passey tissue, and the 38°C experiment was with the Cloudman S91 melanoma tissue. The μl O_2/hr/ml for 4°C and 38°C with succinate was 67 and 63, and for dopa oxidase at 4°C was 108.

anized granules were centrifugally sedimented.†† The resulting pellets were resuspended in 2 groups employing distilled water or 0.5 M NaCl as suspending fluids. After approximately 30 minutes at 4°C these suspensions were sedimented as before and again resuspended in fresh fluid but with one of the saline pellets taken up in distilled water rather than 0.5 M salt. A similar experiment was run but with the salt exposure carried out at 38°C. The reversibility of the effect of temporary exposure to strong salt, under the given conditions, is demonstrated by Table IV. The apparent enhancement in the second experiment of the fraction with the salt replaced by distilled water is probably due to the washing out of co-factors in the distilled water fraction at the higher temperature.

NaCl inhibition of liver mitochondria. While the salt effects reported here have been largely carried out on tumor tissue it is reasonably expected that similar findings for these enzyme systems will be found in other tissues. The following data show the results obtained with isolated liver mitochondria purified by differential centrifugation, as well as the response of relatively crude extract (Table V).

Discussion. The data of this report indicate that optimum activity of some of the oxidative enzymes carried on intracellular particles‡‡ is not obtained with the conventional "physiological" concentrations of salts originally designed to meet the osmotic or other requirements of intact cells or mitochondrial elements. Thus, what may be the

†† Servall angle centrifuge, type SS-1, 9″ rotor, sedimented at approximately 10,000 times gravity for 15 min. plus a run up to 18,000 x g to pack the pellets.

TABLE V. Inhibition of Normal Mouse Liver Preparations with Sodium Chloride at Various Levels of Activity and Purification.

Fractions	Succinoxidase activity: $\mu l\ O_2$/hr/ml	Succinoxidase activity: Q_{O_2} (N)	% inhibition
Crude extract*			
Distilled water	1092	284	4
Distilled water + .0001 M NaCl	1140	297	0
Distilled water + .001 M NaCl	1104	288	3
Distilled water + .01 M NaCl	1128	294	1
Distilled water + .1 M NaCl	768	200	33
Distilled water + 1.0 M NaCl	96	25	92
Isolated mitochondria†			
Distilled water	1880	3123	0
0.1 M NaCl	1048	1741	44

* Prepared by the short column procedure(8) in 10% sucrose and run manometrically under conditions of ionic substrates and co-factors for submaximal succinoxidase activity.

† Purified by differential centrifugation and run manometrically under conditions(6) for maximum activity.

best ionic conditions for maintaining morphology are not optimum for demonstrating maximum enzyme activity.

In determining enzyme rates, the highest activities obtained in the manometric vessel may not be the normal biological rates at the site of function in the cell(13). Thus, if in nature the succinoxidase system is exposed to "physiological" concentrations of sodium and potassium salts the present data indicate that its normal operative rate may be materially lower than the optimum potential rate. Some degree of normal enzyme inhibition would be advantageous considering the operative flexibility it would provide the system for acceleration and depression of the metabolism of the cell. These data thus emphasize the possible role that ions may play in the automatic regulation of enzymic rates as a result of ion movements or shifts across cell membranes.

The data establish that sodium and potassium chloride are distinctly inhibitory for certain enzyme systems. These effects, however, may be outweighed in some studies where salts are used to preserve the integrity of labile sub-cellular elements during isolation and purification procedures. The data, however, also indicate that salts may be employed in the preparation of material if they are removed or carefully corrected for in the measurement of enzyme activity. It should be noted that many of the values reported in the literature for these enzymes have been measured under various degrees of ionic inhibition and a correction factor should be applied when comparing them with optimum values.

It may be emphasized, that the inhibiting and enhancing results reported here have been primarily obtained with single salts under *in vitro* conditions. The synergistic and antagonistic effects of salt combinations on the various enzyme systems are yet to be systematically explored.

Summary. The quantitative inhibiting effects of NaCl, KCl, and KNO_3 on the succinoxidase, cytochrome oxidase, and dopa oxidase systems are reported. Enhancement, exceeding 40%, of the cytochrome system at certain concentrations of NaCl was observed. No significant enhancement of the succinoxidase or the dopa oxidase systems with the above salts was found at any of the concentrations tested. Inhibition of the succinoxidase system with NaCl and KCl was a rapidly increasing function from about 0.01 through 1.0 molarity, with physiological concentrations (0.9%) inhibiting up to about 50%. Reversibility of this effect was shown. No significant difference in degree of inhibition was found between crude and purified preparations. A differential inhibition response between the succinoxidase and dopa oxidase systems was observed for KNO_3. The former was inhibited as with KCl while dopa oxidase was inhibited but slightly if at all.

‡‡ Evidence has been presented that these melanized granules are mitochondrial elements(11,12).

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