Literature Cited

1. Bonner, D. M. and Yanofsky, C. The biosyntheses of tryptophan and niacine and their relationships. Jour. Nutrition 44: 603–616. 1951.
2. Dawson, R. F. Development of some recent concepts in the physiological chemistry of the tobacco alkaloids. Plant Physiol. 21: 115–130. 1946.
3. Dewey, L. J., Byerrum, R. U. and Ball, C. D. Biosynthesis of the pyrrolidine ring of nicotine. Biochim. Biophys. Acta 18: 141–142. 1955.
4. Fichter, F. and Beisswenger, A. Die Reduction des Glutärsaure anhydrids zum δ-Valerolactone. Ber. deut. chem. Ges. 36: 1200–1205. 1903.
5. Graebe, C. Ueber die Bildung aromatischer Methoxysäuren und von Anisol. Annalen Chem., Justus Liebigs 340: 205–212. 1905.
6. Grobbelaar, N. and Steward, F. C. Pipecolic acid in *Phaseolus vulgaris:* Evidence on its derivation from lysine. Jour. Amer. Chem. Soc. 75: 4341. 1953.
7. Hankes, L. V. and Urivetsky, M. Mammalian conversion of C^{14}-carboxyl-labeled 3-hydroxyanthranilic acid into N′-methylnicotinamide. Arch. Biochem. Biophys. 52: 484–485. 1954.
8. Heidelberger, C., Abraham, E. P. and Lepkovsky, S. Tryptophan metabolism. II. Concerning the mechanism of the mammalian conversion of tryptophan into nicotinic acid. Jour. Biol. Chem. 179: 151–155. 1949.
9. Leete, E. Biogenesis of nicotine. Chemistry & Industry 1955: 537. 1955.
10. Leete, E. Amer. Chem. Soc., Univ. of Minnesota meetings, September 1955.
11. Leete, E., Marion, L. and Spenser, I. D. The biogenesis of alkaloids. XIV. Biosynthesis of damascenine and trigonelline. Canadian Jour. Chem. 33: 404–410. 1955.
12. Lowy, P. H. The conversion of lysine to pipecolic acid by *Phaseolus vulgaris*. Arch. Biochem. Biophys. 47: 228–229. 1953.
13. Mitchell, H. K., Nyc, J. F. and Owen, R. D. Utilization by the rat of 3-hydroxyanthranilic acid as a substitute for nicotinamide. Jour. Biol. Chem. 175: 433–438. 1948.
14. Munier, R. Partition of microchromatography on paper of alkaloids and various biological nitrogenous bases. IV. Separation of some nicotinic acid derivatives and tryptophan. Bull. soc. chim. biol. 33: 857–861. 1951.
15. Nyc, J. F. and Mitchell, H. K. Synthesis of a biologically active nicotinic acid precursor: 2-amino-3-hydroxybenzoic acid. Jour. Amer. Chem. Soc. 70: 1847–1848. 1948.
16. Offerman, H. Zur Geschichte des Anthracens. Annalin d Chem., Justus Liebigs 280: 1–35. 1894.
17. Orechoff, A. and Menschikoff, G. Über die Alkaloide von *Anabasis aphylla* L. Chem. Ber. 64: 266–274. 1931.
18. Rothstein, M. and Miller, L. Loss of the α-amino group in lysine metabolism to form pipecolic acid. Jour. Amer. Chem. Soc. 76: 1459. 1954.
19. Spada, Di Alberto, and Gavioli, Ermanno Sull' acido 3-assiantranilico intermedio nella biosintesi dell' acido nicotinico. Bull. sci. facoltá chim. ind., Bologna 8: 101–103. 1950.
20. Tso, T. C. and Jeffrey, R. N. Paper chromatography of alkaloids and their transformation products in Maryland tobacco. Arch. Biochem. Biophys. 43: 269–285. 1953.
21. Zacharius, R. M., Pollard, J. K. and Steward, F. C. γ-methyleneglutamine and γ-methyleneglutamic acid in the tulip (*Tulipa gesneriana*). Jour. Amer. Chem. Soc. 76: 1961–1962. 1954.
22. Zeijlemaker, F. C. J. The metabolism of nicotinic acid in the green pea and its connection with trigonelline. Acta Bot. Neér. 2: 123–143. 1953.

THE INFLUENCE OF SALTS ON THE ACTIVITY OF PARTICULATE CYTOCHROME OXIDASE FROM ROOTS OF HIGHER PLANTS[1,2]

GENE W. MILLER and HAROLD J. EVANS

North Carolina State College, Raleigh, North Carolina

Recent investigations by Webster (21) have shown that cytochrome oxidase is widely distributed in higher plant species and it has been suggested (7) that this enzyme may play a major role in the terminal transfer of electrons in plant respiration. Lundegårdh (13) reported that the absorption spectrum of bundles of roots from wheat and other cereals revealed the existence of a complete cytochrome oxidase which was similar to that observed in animal tissues and many microorganisms. He calculated that this system was responsible for 50 to 75 % of the total aerobic respiration. Fritz and Beevers (6) observed that extracts from etiolated pea, wheat and barley seedlings contained cytochrome oxidase at all stages of development. When the amount of enzyme in the tissues was evaluated in terms of activity at an infinite concentration of cytochrome c, it was concluded that shoots or roots of peas, and shoots of either barley or wheat contained sufficient amount of the enzyme to account adequately for the respiration of these tissues. The roots of barley and wheat also contained the enzyme but the amounts extracted were not capable of mediating all the respiratory oxygen absorption. The results with wheat were in good agreement with those reported by Lundegårdh. It would appear that the importance of cytochrome oxidase in many higher plant species may approach that of this enzyme in the respiration of animals and aerobic microorganisms.

Several investigators have observed that salts

[1] Received April 3, 1956.

[2] Contribution from the Division of Biological Sciences, North Carolina Agricultural Experiment Station and published with the approval of the Director as paper No. 725. This research was supported in part by a grant from the Chilean Nitrate Educational Bureau.

stimulated the activity of cytochrome oxidase preparations from animal tissues. According to Smith (17) the activity of the enzyme in particulate fractions of kidney or heart was markedly increased by salt. The maximum effect was obtained with NaCl at a concentration of 0.11 M. A similar observation was made by Riley (16) who reported that a concentration of 0.1 M NaCl increased the activity of a cytochrome oxidase preparation from mouse melanomas by 40 %. Megumu (15) has reported that univalent cation salts at a concentration of 0.05 M maximally increased the activity of cytochrome oxidase from ox heart muscle. Divalent cation salts were stimulatory at lower concentrations.

There are relatively few experiments reported in the literature concerning the influence of salt on the activity of cytochrome oxidase from higher plant tissues. Lundegårdh (12) added macerated wheat roots to a solution containing reduced cytochrome c and observed that 41 % of it was oxidized in five minutes. In a comparable experiment where the solution contained KNO_3 or KCl at a concentration of 0.1 M, 73 % of the reduced cytochrome c was oxidized in a 5-minute period. Weinstein et al (22) observed the greatest cytochrome oxidase activity of a homogenate of sunflower leaves in a phosphate buffer (mixture of Na_2HPO_4 and KH_2PO_4) at pH 6.0 and at a concentration of approximately 0.13 M. The activity was markedly depressed with buffer concentrations greater or less than this value. No attempt was made to distinguish between the effect of anions and cations or to determine whether or not an osmotic phenomenon was involved.

It has been well established (14) that the addition of salts to plant tissues containing low salt contents markedly increased their respiratory rates. This increased respiration is sensitive to heavy metal inhibitors and carbon monoxide inhibition is reversed by light indicating the involvement of cytochrome oxidase. In view of the possible significance of these observations in an understanding of the mechanism of the salt accumulation and of the brief reports that the activity of plant cytochrome oxidase per se was influenced by salts, the experiments reported here were initiated in an effort to provide more detailed information on the influence of salts on cytochrome oxidase from plant sources. The experimental evidence also may have an important bearing on the role of certain cations in plant metabolism.

Materials and Methods

Preparations: Particulate preparations used in the various experiments were made from the roots of soybean (*Glycine Max* Merr.), tobacco (*Nicotiana Tabacum* L.), oat (*Avena sativa* L.) or spinach (*Spinacia oleracea* L.) plants which were grown in flats of sand in the greenhouse. The age of the seedlings ranged from 6 to 20 days. Extracts of root tissue were prepared as outlined by Webster (21) with the exception that the isolation medium contained 0.3 M sucrose and 0.05 M tris (hydroxymethyl) aminomethane (Tris) buffer instead of phosphate buffer. In the preparation of the extracts 1 gm of roots was ground in a cold mortar with 20 ml of a cold solution of buffer and sucrose, then homogenized for 1 minute with a Ten Broeck homogenizer and centrifuged at $1000 \times g$ for 15 minutes. The supernatant was decanted and centrifuged at $20{,}000 \times g$ for 15 minutes. The particles were collected and washed with 10 ml of the sucrose-buffer solution then suspended in a volume of sucrose-buffer equivalent to one fifth the original volume of the homogenate. Extracts prepared by this procedure were used in all experiments except that reported in figure 4. In this experiment (fig 4) the extract was prepared as described above, with the exception that buffer was omitted from the isolation medium. Also the suspension of particles used in this experiment was dialyzed over night in 2 liters of 0.3 M sucrose in an effort to remove endogenous salts.

A stock solution of 12 mg/ml of cytochrome c (Sigma Chemical Company of St. Louis, Missouri) in 0.05 M Tris buffer at pH 7.0 was chemically reduced with sodium hydrosulfite (3) and thoroughly aerated before use. The concentration of the stock solution was determined by use of the difference in molar extinction coefficients of oxidized and reduced cytochrome c at 550 $m\mu$ which is 1.96×10^4 (8). The stock solution of cytochrome c used in the experiment reported in figure 4 and the extract used in this experiment were dialyzed overnight against a 0.3 M sucrose solution before they were used. After dialysis the reduced cytochrome c concentration was determined by the spectrophotometric procedure. Solutions of reduced cytochrome c were relatively stable when stored at $-10°$ C.

Tris salts of Cl^- and phosphate (mixture of $H_2PO_4^-$ and $HPO_4^=$) used in the experiments reported in figure 3 were prepared by adding sufficient amounts of appropriate acids to Tris base to adjust the pH to 7.0. Tris buffer used in the various procedures was prepared by neutralization with HCl until the desired pH value was obtained. The solution of $NaHCO_3$ used in the experiments reported in figure 2 was adjusted to pH 7.5 by bubbling CO_2 into the solution.

In the experiment involving purification of the enzyme extract with ion resins, a mixture of hydroxyl saturated anion resin IRA-400 and hydrogen saturated cation resin IR-120 (both obtained from Rohm and Haas Company, Philadelphia) were mixed together in proportions that resulted in a pH of 6.5 in distilled water. The enzyme was dialyzed for 12 hours against a suspension containing 5 gm of mixed resins in 2 liters of cold distilled water.

Standard Assay: Enzyme activities were assayed spectrophotometrically at 550 $m\mu$ with a Beckman DU spectrophotometer (3). The reaction mixture in a final volume of 1 ml contained the following constituents in micromoles: 25 Tris buffer at pH 7.0, 0.051 reduced cytochrome c, desired concentration and type of salt, and enzyme containing 0.1 to 0.2 mg protein. Enzyme activity is indicated as the decrease in optical density per mg protein between 15 and 75

seconds after the reaction was started by the addition of the enzyme. Reduced cytochrome c was not oxidized when boiled enzyme was used in the assay procedure with or without the various experimental additions to the mixture. Under the conditions of the experiments the oxidation rates remained linear for at least two minutes which is in agreement with the observations of Fritz and Beevers (6). The mixture was stirred prior to each reading in order to prevent errors due to the settling of particles in the cuvette. The various experiments were run three times and the results averaged. Maximum deviation from the mean was 8 % but in the majority of experiments the deviation was much less than this.

OTHER METHODS: Aliquots of purified assay mixtures were ashed in a muffle furnace at 600° C and analyzed for Na and K by use of a Perkin-Elmer flame photometer (23). Protein content of extracts was estimated by use of either Folin's phenol reagent (11) or by total N analyses (20). Results with the different methods were in good agreement.

EXPERIMENTAL RESULTS

EFFECTS OF VARIOUS CATIONS: Extracts of roots from four plant species were prepared and the effect on the activity of cytochrome oxidase of chloride salts at varying concentrations observed. In figure 1 A the influence of chloride salts on cytochrome oxidase activity of extracts from soybean roots is shown. Additions of univalent cation salts resulted in a striking increase in activity. A maximum stimulatory effect was exhibited at a concentration near 0.1 *N* and greater concentrations were inhibitory. It is of interest to note that chlorides of Na^+ and Li^+ at optimum concentrations activated less than chlorides of K^+ and NH_4^+. The degree of activation at optimum

TABLE I

EFFECT OF VARIOUS ANIONS AND CATIONS ON THE ACTIVITY OF CYTOCHROME OXIDASE FROM AN EXTRACT OF SOYBEAN ROOTS *

ADDITIONS	CONC OF ADDED SALT (*N*) OR SUCROSE (M)			
	0.00	0.05	0.10	0.20
	– O.D. change per min per mg protein			
Various anions				
NaCl	0.23	0.37	0.65	0.45
NaBr	0.23	0.35	0.70	0.40
NaI	0.23	0.38	0.65	0.43
Na_3 citrate	0.23	0.40	0.70	0.45
Various cations or sucrose				
$NaNO_3$	0.23	0.40	0.68	0.40
KNO_3	0.23	0.40	0.70	0.45
$Ca(NO_3)_2$	0.23	0.42	0.12	0.05
$Mg(NO_3)_2$	0.23	0.40	0.18	0.05
Sucrose	0.23	0.20	0.18	0.15

* The standard assay procedure was used with the addition of salts or sucrose as indicated. One tenth ml of soybean root extract containing 1.6 mg protein per ml was used as the enzyme.

TABLE II

EFFECT OF COMBINATIONS OF SALTS ON CYTOCHROME OXIDASE ACTIVITY FROM AN EXTRACT OF SOYBEAN ROOTS *

SALTS	TOTAL SALT CONC (*N*)					
	0.00	0.05	0.075	0.10	0.15	0.20
	– O.D. change per min per mg protein					
NaCl	0.33	0.52	0.73	0.77	0.67	0.37
KCl	0.33	0.55	0.70	0.79	0.67	0.40
½ KCl + ½ NaCl	0.33	0.47	0.70	0.80	0.67	0.37
$CaCl_2$	0.33	0.50	0.30	0.20	0.10	0.08
½ CaCl + ½ KCl	0.33	0.50	0.45	0.35	0.15	0.13

* The standard assay procedure was used with the addition of salts as indicated. The assay included 0.10 ml soybean extract containing 1.4 mg protein per ml.

univalent salt concentration in this particular experiment was greatest with the least hydrated cations possessing greatest relative mobilities (9). Divalent cation chlorides activated slightly up to 0.05 *N*, but increased concentrations caused a sharp decline in activity resulting in almost complete inhibition at 0.2 *N*. $CaCl_2$ at concentrations below 0.05 *N* stimulated enzyme activity more than $MgCl_2$ at comparable concentrations. At all except low concentrations, univalent cation salts were more effective than the divalent ones. The influence of nitrate salts of Mg^{++}, Ca^{++}, K^+ and Na^+ on cytochrome oxidase activity of soybean extract was similar to that observed with the respective chloride salts (table I). These results were not included in figure 1 because the effect of the two anions were so similar that an overlapping of many curves would have resulted. The data in this table also show that various concentrations of sucrose had little effect on the activity of the enzyme.

The effect of chloride salts of various cations on the activity of cytochrome oxidase from roots of tobacco, spinach and oat is shown in figures 1 B, 1 C and 1 D and the results are generally comparable with those obtained with soybean. In all experiments with the exception of those where extracts of oat roots were used, KCl at optimum concentrations stimulated enzyme activity slightly more than NaCl. The effect of divalent cation salts on cytochrome oxidase activity from oat and spinach extracts was similar to that observed with soybean, but with extracts from tobacco, $MgCl_2$ was considerably more effective than $CaCl_2$ at low as well as at high concentrations. This is particularly noticeable at the concentration of 0.05 *N*.

It is apparent from figure 1 that univalent cation salts have similar effects on enzyme activity. The activating effect of combinations of salts as compared with single salts is shown in table II. A mixture containing equal concentrations of NaCl and KCl behaved in a manner similar to the same total concentration of either of the single salts indicating an additive effect of univalent cation salts. The activation of cytochrome oxidase by an equal mixture (N) of $CaCl_2$ and KCl was similar to that noted with the single

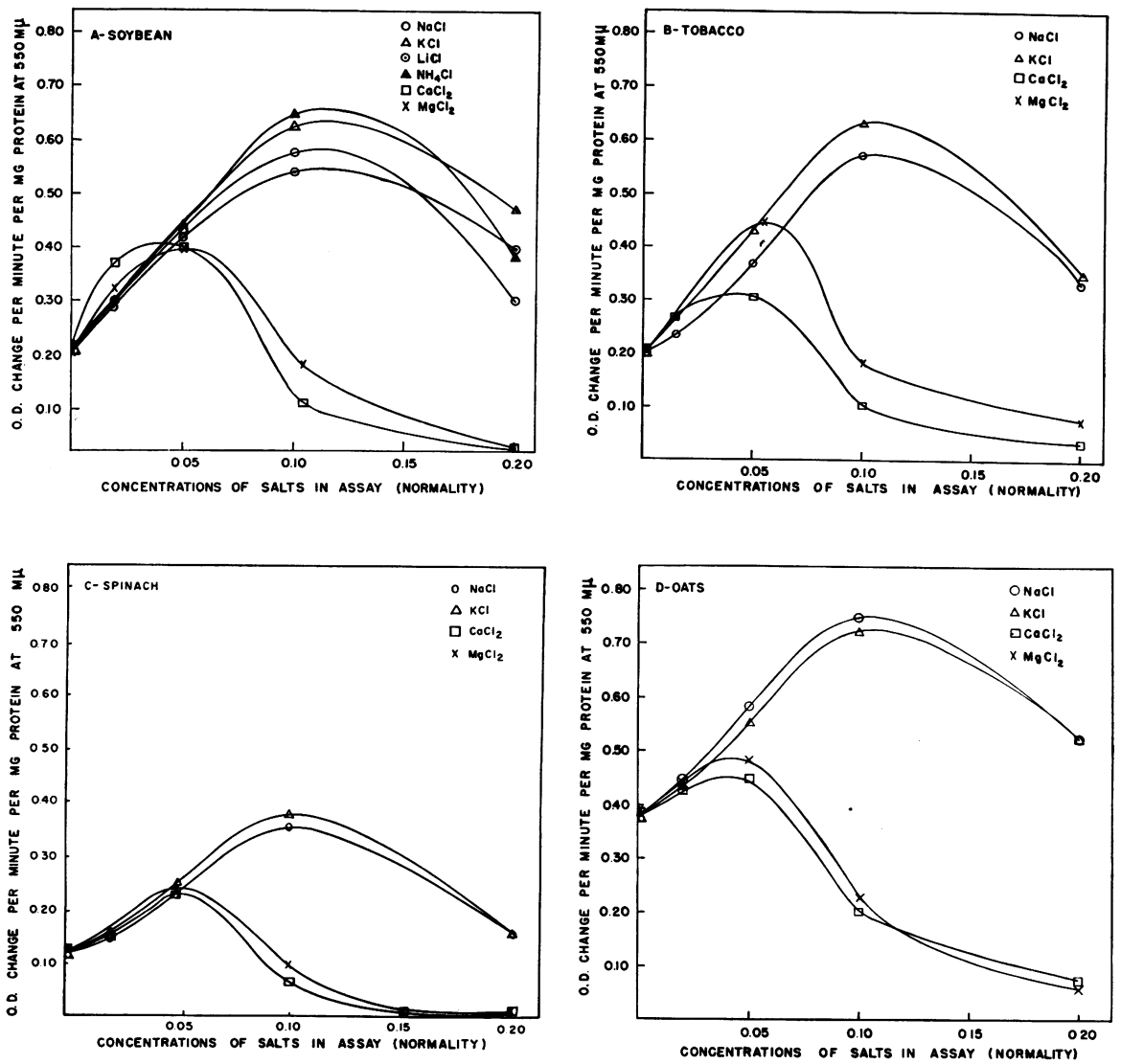


FIG. 1. Effect of various concentrations of salts on the cytochrome oxidase activity of extracts from roots of several plant species. Procedure was that of standard assay with variations in concentration and type of salt as indicated. The enzyme preparations were from the following species: A—0.10 ml soybean extract containing 1.2 mg protein per ml; B—0.10 ml tobacco etxract containing 1.3 mg protein per ml; C—0.10 ml spinach extract containing 1.7 mg protein per ml; D—0.10 ml oat extract containing 1.4 mg protein per ml.

In this figure and the subsequent figures the O.D. changes are negative.

salts up to a total concentration of 0.05 *N*. At greater concentrations an interaction between Ca^{++} and K^{+} salts was apparent as indicated by the difference in enzyme activities obtained from combinations of salts as compared with single salts. Where Ca^{++} was at maximum stimulatory level, the addition of univalent cation salts suppressed activity.

EFFECT OF VARIOUS ANIONS: Univalent and divalent cation salts differ in their influence on cytochrome oxidase activity but the effect is not independent of the anion present. It can be seen from figure 2 that sodium salts of Cl^{-}, NO_3^{-}, and $SO_4^{=}$ were equally effective in activation of the enzyme. The sodium salts of Br^{-}, I^{-} and citrate (tri-sodium) influenced the activity in a manner similar to the chlorides as shown in table I. From figure 2 it is apparent that the addition of phosphate ($HPO_4^{=} + H_2PO_4^{-}$) increased the activity of the enzyme from soybean roots to a greater degree than other anions. The increased stimulatory effect of phosphate also was observed with extracts of spinach, oat and tobacco roots. The stimulation from phosphate salt at most concentrations was approximately 20 % higher than that of other salts. The effect of phosphate was manifested at con-

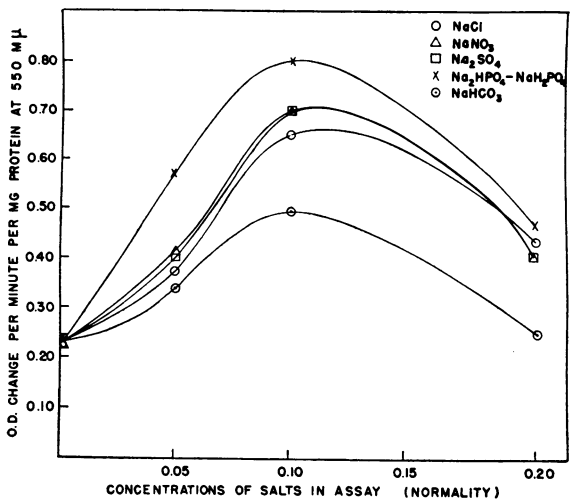
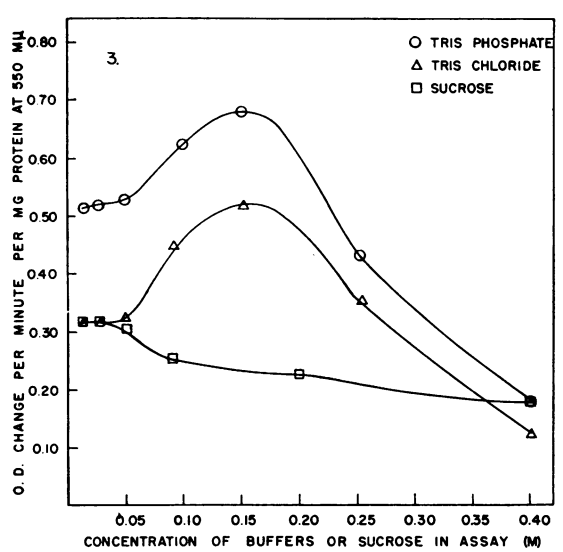


FIG. 2. The effect of various concentrations of sodium salts on the activity of cytochrome oxidase from soybean roots. The procedure of standard assay was followed with the exception that all determinations were made in 0.025 M Tris buffer at pH 7.5. The assay mixtures contained variable concentration and kinds of salts as indicated. The preparation of the bicarbonate solution is described in Materials and Methods. Assays included 0.1 ml soybean extract containing 1.7 mg protein per ml.

centrations as low as 0.0005 M. Bicarbonate salts at the same pH and cation concentration as the above mentioned salts resulted in little activation at concentrations that were optimum for other salts and pronounced inhibition at high concentrations. This inhibition was noted with extracts of various plant species.

REQUIREMENT OF SALT FOR ACTIVITY: In the experiments where the effect of various cations and anions on the activity of cytochrome oxidase was studied, marked stimulation was noted from added salts. Since all these assay mixtures contained 0.025 M Tris chloride as a buffer, it was deemed necessary to test the influence of various concentrations of buffer on the enzyme activity. The results of such an exeriment are reported in figure 3. Tris salts of Cl^- and phosphate (mixture of $H_2PO_4^-$ and $HPO_4^=$) activated the enzyme in a manner similar to other univalent cation salts. The difference in stimulation between chloride and phosphate salts was observed again and indicated a specific action of the phosphate ion. Tris phosphate at concentrations of 0.0125 M resulted in marked increases in activity when compared with Tris chloride at the same concentrations.

An experiment was set up to determine enzyme activity in an assay mixture containing no added Tris or inorganic salts. The preparation of materials is described in Materials and Methods. Enzyme activ-



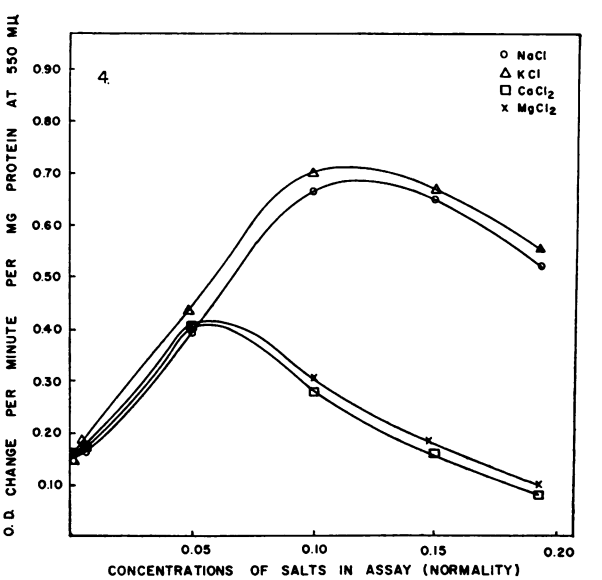


FIG. 3. Effect of various concentrations of Tris salts on the activity of cytochrome oxidase from soybean roots. The standard assay procedure was used with variation in buffers (preparation of Tris phosphate and Tris chloride is described in Materials and Methods) as indicated. Extracts were prepared in either Tris chloride or Tris phosphate depending on which buffer was used in the assays. Assays included 0.1 ml soybean extract containing 1.1 mg protein per ml.

FIG. 4. The influence of salts on activity of cytochrome oxidase from soybean roots in a purified medium (see Materials and Methods). The assay mixture in a final volume of 1 ml contained 0.051 micromoles of dialyzed reduced cytochrome c; 0.1 ml dialyzed extract containing 1.6 mg protein per ml, and the concentration and types of salts indicated. No buffer was used in the assay mixtures and the pH varied between 5.2 and 5.3 during the determinations. All salt solutions were adjusted to pH 5.2 before use.

ity was present in this purified medium as shown in figure 4, and the salt stimulation was similar to previous experiments where buffers were used. An analysis of a sample of the entire purified reaction medium showed that concentrations of 1.5×10^{-4} M potassium and 7.5×10^{-5} M sodium were present in the mixture.

In another experiment, dialysis of the enzyme against a mixed resin, as described in Materials and Methods, failed to decrease the cytochrome oxidase activity in the reaction mixture containing no added salts. An analysis of the entire reaction mixture under these conditions showed 1.25×10^{-4} M potassium and 6.3×10^{-5} M sodium were present. In another attempt to remove salts, the enzyme preparation was placed for 30 minutes in a cold Gooch crucible in contact with a mixture of the anion and cation resins described in Materials and Methods. The enzyme was recovered by suction and assayed for activity but no absolute dependence on salt was observed.

Discussion

The results of these experiments clearly show that both type and concentration of salts in contact with suspensions of particulate matter from roots of various plant species profoundly influenced the cytochrome oxidase activity of the particles. When the anions were held constant as the chlorides (fig 1 and table I) the various cations up to a concentration of 0.05 *N* generally resulted in similar effects on the activity of cytochrome oxidase. When concentrations of cations greater than 0.05 M were added to assays then univalent cations continued to stimulate activity up to a concentration of 0.12 M whereas concentrations of divalent cations greater than 0.06 M were inhibitory. When the results were plotted on an ionic strength basis different effects of univalent and divalent cations were observed at concentrations greater than 0.07 *N*. The influence of salts on the activity of cytochrome oxidase from soybean roots is not independent of the anion associated with salt as indicated by the results in figure 2 and table I. The sodium salts of NO_3^-, $SO_4^=$, Cl^-, I^- and citrate$^{\equiv}$ at comparable pH values were strikingly similar in their effect on the activity. Sodium phosphate (Na_2HPO_4-NaH_2PO_4) on the other hand consistently increased activity to a greater degree than the other sodium salts. The specific inhibitory action of bicarbonate ions on cytochrome oxidase has been observed consistently with enzyme preparations from various species and is the subject of another communication.

In view of the relatively high concentrations of salts required for maximum cytochrome oxidase activity in isolated particulate matter from soybean root and the relatively low concentration of salts needed for maximum salt respiration of intact tissues (4) one might conclude that entirely different phenomena were involved in each case. These differences may be accounted for by assuming that intact tissues possess a greater accumulatory capacity for salts than the particulate preparations containing cytochrome oxidase.

It seems possible that different concentrations and types of salts may influence cytochrome oxidase activity by their effects on the physical structure of the particles. Stafford (18) has established that cytochrome oxidase in higher plant tissue occurs in intracellular particles of the size of mitochondria. Farrant et al (5) have shown with the electron microscope that mitochondria from red beet prepared in 0.45 % KCl appeared as large diffuse discs whereas those prepared in 30 % sucrose appeared as compact spheres. It has been demonstrated that the cytochrome oxidase activity of particles isolated from animal livers was strongly affected by the physical state of the particles and that salts markedly influenced the physical appearance of them (2). One might speculate that the type and concentration of the various salts used in the experiments reported in this manuscript affected the physical properties of the particles and therefore possibly altered the accessibility of the enzyme to either oxygen or reduced cytochrome c. A microscopic examination of the particles from soybean seedlings in both water and 0.1 *N* KCl indicated dimensions of mitochondrial size, but no gross differences in physical structure were observed. It is necessary to conduct more detailed studies with these particles before a definite conclusion concerning the effect of salt on gross structure can be made.

Since it has been clearly established (5) that beet mitochondria possess a membrane and that cytochrome oxidase is associated with mitochondria (18), it is necessary to consider the possibility that the various reactants in the cytochrome oxidase reaction have to penetrate a membrane during the course of the enzymatic reaction. In this event various species and concentrations of ions would very likely have differential effects on membrane permeability (22) and therefore the rates of the enzymatic reaction. Lehninger (10) has pictured an "internal" and "external" pathway of electron transport from the substrate to molecular oxygen in mitochondrial preparations. Externally added reduced cytochrome c apparently does not penetrate to the internal site of electron transport within the mitochondrial body unless some alterations are made in mitochondrial permeability or structure. Apparently it is necessary for electrons to be transported through the "internal" pathway in order to obtain oxidative phosphorylation. Information concerning the relationship of salts to the pathway of electron transport and to oxidative phosphorylation in plant mitochondria remains for future study.

In an effort to explain the influence of salts on the oxidation-reduction balance of cytochromes in living wheat roots, Lundegårdh (14) proposed that if two molecules capable of undergoing oxidation-reduction were separated from each other, apparently in some structural unit, they may fail to react unless salts are present in the surrounding medium. In this proposal the movement of ions may compensate for the electrostatic potential between the reactants involved in the oxidation-reduction. In order to apply this generalized explanation to the cytochrome oxidase reaction it

seems that particulate cytochrome oxidase would be necessary to meet the structural requirements of a salt dependent system. If cytochrome oxidase were in true solution and in intimate contact with a solution of reduced cytochrome c it seems logical to expect that no salt influence would be manifested if the general mechanism proposed by Lundegårdh is operative.

The striking stimulatory action of univalent cation salts on the activity of cytochrome oxidase may have a bearing on the role of univalent cations in plant metabolism. It is well established that K^+ is the major cation in plant tissues and that Na^+ will partially replace K^+ in its role as a univalent cation in certain species. Since Na^+ and K^+ are approximately equally effective in the activation of cytochrome oxidase it seems that this enzyme may represent a metabolic site where replacement could occur. It is of interest to note that dry plant tissues containing 2 to 4 % K (1) would contain a concentration calculated on a fresh tissue basis of approximately 0.05 to 0.1 *N*. As already indicated this range in concentration of univalent cation salts markedly stimulated the activity of cytochrome oxidase in vitro. A significant physiological role of univalent cations other than K^+ and Na^+ in the activation of cytochrome oxidase in vivo seems unlikely in view of either the low concentrations or relative toxicities of other such cations that are normally present.

Summary

A study has been made of the influence of various concentrations and kinds of salts on the activity of cytochrome oxidase associated with particulate preparations showing characteristics of mitochondria from the roots of certain higher plant species.

The activity of the enzyme from all the species studied including soybean, tobacco, oat and spinach was markedly influenced by salts. When the anions were held constant as chlorides and the effect of cations compared on an equivalent basis, increasing concentrations of univalent cation salts stimulated activity until a maximum effect was obtained at a concentration of approximately 0.12 *N*. Concentrations greater than this resulted in a progressive decline in activity. The addition of chloride salts of divalent cations was less stimulatory than salts of univalent cations. The maximum increase in activity was obtained at a concentration of near 0.05 *N* and greater concentrations were inhibitory. The concentration of Tris chloride used as a buffer also influenced the activity of the enzyme.

Experiments were conducted with extracts from soybean roots where the cation of salts was kept constant as Na^+ and the influence of equivalent concentrations of anions studied. Cl^-, NO_3^-, $SO_4^=$, Br^-, I^- and citrate influenced the activity in a similar manner but phosphate ($H_2PO_4^- + HPO_4^=$) resulted in approximately 20 % greater activity than other anion salts. The effect of $NaHCO_3$ on the activity when compared with other anion salts at the same pH values was inhibitory.

An absolute salt requirement for cytochrome oxidase activity was not demonstrated; however, analyses of purified assay mixtures indicated the presence of small concentrations of both Na^+ and K^+.

The influence of salts on the activity of cytochrome oxidase was not an osmotic manifestation since various concentrations of sucrose in the assay medium had no appreciable effect.

The experimental results are discussed in relation to the possible mechanism of the differential action of various salts and also in relation to the possible role of certain cations in plant metabolism.

The authors gratefully acknowledge the technical assistance of Mrs. Thea Schostag.

Literature Cited

1. Beeson, K. C. The mineral composition of crops with particular reference to the soils in which they were grown. U. S. Dept. Agr., Misc. Publ. 369. 1941.
2. Claude, A. J. Fractionation of mammalian liver cells by differential centrifugation. Jour. Exptl. Med. 84: 51–61. 1946.
3. Cooperstein, S. J. and Lazarow, A. A microspectrophotometric method for the determination of cytochrome oxidase. Jour. Biol. Chem. 189: 665–670. 1952.
4. Epstein, E. Cation-induced respiration in barley roots. Science 120: 987–988. 1954.
5. Farrant, J. L., Robertson, R. N. and Wilkins, Marjorie J. The mitochondrial membrane. Nature 171: 401–402. 1953.
6. Fritz, G. and Beevers, H. Cytochrome oxidase content and respiratory rates of etiolated wheat and barley seedlings. Plant Physiol. 30: 309–317. 1955.
7. Hill, R. and Hartree, E. F. Hematin compounds in plants. Ann. Rev. Plant Physiol. 4: 115–150. 1953.
8. Horecker, B. L. and Heppel, L. A. The reduction of cytochrome c by xanthine oxidase. Jour. Biol. Chem. 178: 683–690. 1949.
9. Kachmar, J. F. and Boyer, P. D. The potassium activation and calcium inhibition of pyruvic phosphoferase. Jour. Biol. Chem. 200: 669–682. 1953.
10. Lehninger, A. L. The Harvey Lectures. Pp. 176–215. Academic Press Inc., New York 1955.
11. Lowry, D. H., Rosebrough, Nira J., Farr, A. L. and Randall, Rose J. Protein measurement with the Folin phenol reagent. Jour. Biol. Chem. 193: 265–275. 1951.
12. Lundegårdh, H. Controlling effects of salt on the activity of cytochrome oxidase. Nature 171: 477–478. 1953.
13. Lundegårdh, H. A new cytochrome in living roots. Nature 173: 939–941. 1954.
14. Lundegårdh, H. Mechanism of absorption, transport, accumulation and secretion of ions. Ann. Rev. Plant Physiol. 6: 1–23. 1955.
15. Megumu, I. Studies on the cytochrome oxidase. Sci. Bull. Fac. Agr., Kyushu Univ. 13: 167–170. 1951.
16. Riley, V. Salt inhibition and enhancement of cytochrome, succinic and dopa oxidase systems of mouse melanomas. Proc. Soc. Exptl. Biol. Med. 75: 644–648. 1950.

17. SMITH, F. G. and STOTZ, E. A colorimetric method for the determination of cytochrome oxidase. Jour. Biol. Chem. 179: 891–902. 1947.
18. STAFFORD, HELEN A. Localization of enzymes in pea seedlings. Physiol. Plantarum 4: 696–741. 1951.
19. STEINBACH, H. B. Permeability. Ann. Rev. Plant Physiol. 2: 323–337. 1951.
20. UMBREIT, W. W., BURRIS, R. H. and STAUFFER, J. F. Manometric techniques and tissue metabolism. Pp. 161. Burgess Publishing Co., Minneapolis, Minnesota 1951.
21. WEBSTER, G. C. The occurrence of a cytochrome oxidase in the tissues of higher plants. Amer. Jour. Bot. 39: 729–745. 1952.
22. WEINSTEIN, L. H., ROBBINS, W. R. and WAINIO, W. W. Assay of cytochrome oxidase activity of sunflower leaf tissue in relation to pH value and cation concentration of the buffer. Plant Physiol. 29: 398–399. 1954.
23. WILLARD, H. H., MERRITT, L. L. and DEAN, J. A. Instrumental Methods of Analysis. Pp. 49–84. D. Van Nostrand Company, Inc., New York 1951.

OVERWINTERING TRENDS OF COLD RESISTANCE AND CARBOHYDRATES IN MEDIUM RED, LADINO, AND COMMON WHITE CLOVER[1,2]

O. CHARLES RUELKE[3] AND DALE SMITH
DEPARTMENT OF AGRONOMY, WISCONSIN AGRICULTURAL EXPERIMENT STATION, MADISON, WISCONSIN

Medium red clover, *Trifolium pratense* L., and ladino clover, *Trifolium repens* L., are used extentively in the northern areas of the United States in hay and pasture mixtures while common white clover, *Trifolium repens* L., occurs naturally in most of the closely grazed pastures. Unfortunately, these true clovers are often killed or weakened during the winter months, and maintaining stands over winter is one of the important problems related to their culture in the north. A better understanding of the overwintering physiology of these legumes is needed in order to provide information that will aid in the maintenance of stands during the winter period.

Several workers have studied the trend and development of cold resistance in alfalfa during the fall and winter months (2, 3, 5, 8, 14). Similar work with red, ladino, and common white clover is limited. Bula and Smith (2) compared the cold resistance trend in medium red clover with that in Ranger alfalfa and biennial sweetclover under Wisconsin conditions. The trends in field-grown seedling plants of alfalfa and sweetclover were similar although sweetclover developed resistance more rapidly and reached a higher level than alfalfa. Red clover developed cold resistance later, more slowly, and did not reach as high a level as alfalfa or sweetclover. Most of the resistance in these legumes was developed by the time of the first sampling following permanent freezing of the soil surface in late November to mid-December. Cold resistance in red clover and alfalfa began to decrease after mid-February while it was retained longer in sweetclover. Cold resistance was not lost rapidly until after the snow was gone and the soil surface thawed in late March. Bula et al (3) have shown further with alfalfa that certain responses differed when the same varieties were grown at diverse latitudes. Smith (15) has compared the survival of field-hardened stolons of common white and ladino clover when encased in solid ice at non-injurious temperatures during winter dormancy. The more severe injury sustained by ladino appeared to be associated with the larger stolons and the higher level of metabolic activity during winter dormancy.

The overwintering of such legumes as alfalfa, sweetclover, and red clover has been shown to be associated with a high level of carbohydrate storage and with subsequent changes in certain carbohydrate components (2, 3, 7, 8, 9, 10, 16, 17, 19). Wood and Sprague (21) at New Jersey have reported that the more cold-hardy clones of ladino clover were higher in most carbohydrate fractions during the winter than the less hardy ones. Changes in the levels of sucrose, total sugars, and total polysaccharides in the hardy clones were made at a more gradual rate during winter. Fall cutting decreased some carbohydrate fractions and increased others but had no measurable influence on low temperature survival. Tesar and Ahlgren (18) at Wisconsin have shown that the percentages of total available carbohydrates in ladino clover stolons in November were not significantly different even though various heights and frequencies of cutting were applied during the growing season.

The investigations reported here deal with the trends of cold resistance and the trends of certain carbohydrate fractions from fall to spring in the overwintering parts of medium red, ladino, and common white clover, and the relationship of these trends to the prevailing weather. The studies were made at Madison, Wisconsin, during the winters of 1952–53 and 1953–54.

MATERIALS AND METHODS

Wisconsin-grown seed of medium red clover, *Trifolium pratense* L., and of ladino and common white clover, *Trifolium repens* L., was seeded in rows and in broadcast plots during June of 1952 and of 1953. Plants grown in rows were used in the cold tests initi-

[1] Received April 6, 1956.
[2] Published with approval of the Director, Wisconsin Agricultural Experiment Station.
[3] Present address: Department of Agronomy, University of Florida, Gainesville, Florida.