THE ROLE OF BASIC PROTEINS IN THE DECLINING RESPIRATION OF SENESCING CORN SCUTELLUM*

J. B. Hanson and H. R. Swanson

Department of Agronomy, University of Illinois, Urbana, Illinois

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The impairment of mitochondrial activity by ribonuclease or other basic proteins has been the subject of recent studies (Hanson, 1959; Person and Fine, 1961; Machinist, et al, 1962, Rivenbark and Hanson, 1962). We have tried to determine if this impairment is a factor in the declining respiration of senescing tissue. Beginning with the work of Akazawa and Beevers (1957) it has become evident that storage tissues of seedlings yield mitochondria with progressively lower P/O ratios and substrate oxidation rates as the tissues age and senesce. Akazawa and Beevers related the loss of mitochondrial activity to a decline in acid soluble phosphate (nucleotides). In our experiments with corn scutellum (Hanson, et al, 1959) the activity of the mitochondria declined while the nucleotide content was still high.

A preliminary inquiry showed that aqueous extracts of frozen scutellum mitochondria contained an inhibitor of oxidation and phosphorylation (Table I). The inhibitor proved to be non-dialyzable, was destroyed by boiling (Table II), increased with age of tissue, and was associated with ribonuclease activity. A short pretreatment of inactive scutellum mitochondria with chymotrypsin improved oxidation and phosphorylation (Table III).

These experiments suggested that ribonucleases and/or other basic proteins formed during senescence might be bound to the negatively charged scutellum mitochondria. If so, the use of a higher pH during isolation and in the Warburg vessel might dissociate some of the proteins and yield greater activities. Liebermann (1960) has suggested that extraneous proteins absorbed to apple

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fruit mitochondria at pH 6.0-6.9 might account for their inactivity. In our earlier work we had used pH 6.8 as an estimate of cytoplasmic pH. Investigation showed that a pH of 7.5-8.0 in the vessel gave greatly increased activity, and in subsequent experiments (Fig. 1) pH 7.5 was used.

TABLE I

IMPAIRMENT OF CORN SHOOT MITOCHONDRIA BY EXTRACTS OF SCUTELLUM MITOCHONDRIA

Age of scutellum	Extract protein/ vessel	RNase activity of extract protein	Uptake by shoot mitochondria O_2	PO_4
days	mg	µg RNA/mg prot./hr.	µ atoms	µ moles
-	none	--	4.4	7.6
1	.47	214	4.6	6.8
2	.58	509	4.3	6.4
3	.43	1851	3.8	5.0
4	.23	2610	3.2	3.8

Mitochondria from 100 scutella of indicated age were isolated and once washed in 0.5 M sucrose, 0.067 M potassium phosphate (pH 6.8), 0.005 M ethylene-diaminetetraacetate, suspended in 2 ml of H_2O and frozen overnight. After thawing, the flocculated mitochondria were centrifuged down and aliquots of the supernatant ("extract") added to Warburg vessels containing normal 3 day corn shoot mitochondria oxidizing α-ketoglutarate (Hanson, 1959). RNase was determined on extracts dialyzed overnight in cold water; the assay solution contained per ml 1 mg yeast RNA adjusted to pH 6.0, 20 µ moles KCl, and extract; incubation was at 30°C for 30 min. Decline in O_2 and PO_4 uptake are statistically significant at the 1% level (4 experiments).

TABLE II

EFFECT OF BOILING ON THE UNCOUPLING ACTIVITY OF DIALYZED SCUTELLUM MITOCHONDRIA EXTRACTS

Extract protein per vessel	Activity of corn shoot mitochondria Q_{O_2} (N)	P/O
none	374	1.63
.24 mg (dialyzed)	405	0.93
.30 mg (dialyzed & boiled)	363	1.71

Extracts of 3 day scutellum mitochondria (Table I) were dialyzed overnight in cold deionized H_2O. Portions were boiled until protein flocculated (about 5 min.), and assayed for effect on corn shoot mitochondria.

TABLE III

IMPROVEMENT OF OXIDATIVE PHOSPHORYLATION IN SCUTELLUM MITOCHONDRIA BY CHYMOTRYPSIN PRETREATMENT

Preincubation	mg mito. N recovered	Uptake μ atoms O_2	Uptake μ moles PO_4
29°C for 10 min.	13.9	6.7	1.3
+0.9 mg/ml chymotrypsin	10.8	8.5	5.3

Equal aliquots of washed 3 day scutellum mitochondria were preincubated as indicated, reisolated and oxidative phosphorylation determined.

The experiments cover the 2-5 day period of germination when the respiratory activity of the corn scutellum rises to a maximum and begins to decline (Fig. 1-A). The ribonuclease activity increases throughout the same period (Fig. 1-B). Preliminary column chromatography indicates that other basic proteins may also be increasing, but these have not yet been adequately separated from ribonucleases. Washed mitochondria bind considerable amounts of the ribonucleases, particularly if isolated at pH 6.8 (Fig. 1-C). Isolation at pH 7.6 reduces the amount of ribonuclease bound to the mitochondria, and permits increasingly rapid oxidation of α-ketoglutarate through the 5th day (Fig. 1-D). It is only with mitochondria isolated at the lower pH that there is a decline in oxidation rate with age roughly paralleling that of tissue respiration.

Oxidative phosphorylation is somewhat improved throughout germination by isolation at the higher pH (Fig. 1-E). However, there is a decline in P/O ratio with age independent of isolation pH, indicating a continuous endogenous attrition of the phosphorylating system. Calculations of P/N ratio show impaired net efficiency in energy production by the 4th or 5th day (Fig. 1-F).

These experiments show that ribonucleases (and probably other basic proteins) increase in senescing scutella, and can bind to the mitochondria; in consequence oxidative and phosphorylative efficiency is impaired. The decline in tissue respiration is probably due to *in vivo* binding of proteins, for if

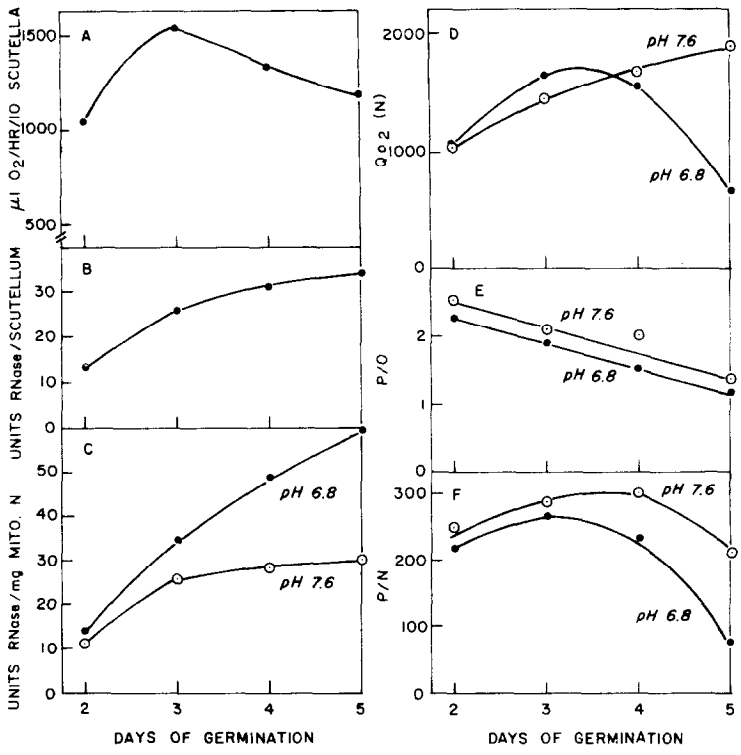

Figure 1. Corn (Zea mays, WF9 X M14) germinated at 28°C in 10^{-4}M $CaCl_2$ for days indicated, and scutella removed for analysis. Mean values from 3 experiments.

A - Respiration rate of 10 intact scutella at 30°C. No ambient buffer was used as H_2O greatly restricts respiration rate (Ohmura and Howell, 1960).

B - Ribonuclease activity in sucrose-phosphate-EDTA homogenates. Assay solution contained 4 mg yeast RNA, 200 μ moles cacodylic acid (to pH 5.8 with imidazole), 400 μ moles KCl, and homogenate in 2.5 ml. After incubation for 30 minutes at 37°C, proteins and RNA were ppt. with 0.5 ml of 25% $HClO_4$ containing 0.75% uranyl acetate. Unit of activity = increase of 0.1 in optical density of supernatant at 260 mμ over non-incubated initials.

C - Ribonuclease activity of scutellum mitochondria isolated and washed in 0.4 M sucrose, 0.05 M potassium phosphate, 0.005 M EDTA of indicated pH.

D - Oxidation of α - ketoglutarate by the mitochondria of Fig. 1-C. $Q_{O_2(N)}$ = μl O_2/hr/mg mitochondrial N. Procedure that of Hanson, et al (1959) except that vessel pH was 7.5 and cytochrome c omitted.

E - P/O ratios of mitochondria of Fig. 1-C.

F - P/N ratios (μ moles phosphate/mg mito. N/hr) of mitochondria of Fig. 1-C.

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the in vivo mitochondria were as "clean" as those isolated at pH 7.6 tissue respiration should rise (substrates are not limiting). In addition, there is a true endogenous uncoupling with age, in that with "clean" mitochondria

oxidation rates rise as phosphorylation declines. The uncoupling might be due to ribonuclease acting as an enzyme, degrading an enzyme-binding polynucleotide such as that described by Shibko and Pinchot (1961). We have noticed a number of times that a mild or short-term treatment of mitochondria with ribonuclease will produce true uncoupling (Hanson, 1959); so will the ribonuclease containing extracts (Table II). Scutellum mitochondria lose up to 20% of their RNA in 4 days germination (Hanson, et al, 1959). However, our experiments to date do not clearly distinguish between the action of ribonuclease as a basic protein and as an enzyme, and some other uncoupling mechanism may be involved.

If our extrapolation to *in vivo* conditions is sound, it appears that the biochemical basis for respiratory senescence in plant cells may lie in those events leading to large increases in ribonucleases and/or other basic proteins. Plant ribosomes contain ribonucleases (Matsushita and Ibuki, 1960), and ribosomes appear to be lost from maturing cells (Lund, et al, 1958; Setterfield, 1961). Cherry and Hageman (1961) found corn scutellum to be depleted of microsomal RNA by 3-4 days. Perhaps basic proteins are freed as the RNA of ribosomes is lost.

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