of inactivated (heated at 80° for 30 min.) intestinal mucosa of hens, and the optimum pH for the enzyme of mature fowl was not altered by inactivated mucosa of chicks.

3. A mixture of the enzyme preparations of chicks and mature fowl exhibited a pH-activity curve and quantitative reaction velocities which would be expected from a combination of two enzymes possessing different pH optima.

4. In rabbits the shift in pH optima for intestinal phosphatase was to lower values with increasing age, the major change taking place in the first 2 months of life.

5. There was no change in pH optima of the phosphatase of intestinal mucosa of rats as their age increased from 6 weeks to 1 year. The pH optima for the phosphatase of a preparation of the whole duodenum of 1-day-old rats were higher than those of the mucosal enzyme of older rats.

6. The pH optima for the phosphatases of bone, liver and kidney of fowl and rabbits appeared to be constant regardless of their age.

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Protein Synthesis in Mitochondria

REQUIREMENTS FOR THE INCORPORATION OF RADIOACTIVE AMINO ACIDS INTO MITOCHONDRIAL PROTEIN

By D. B. ROODYN, P. J. REIS* AND T. S. WORK
National Institute for Medical Research, Mill Hill, London, N.W. 7

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Keller (1951) observed that after the injection of radioactive leucine into the rat the most radioactive protein in the subcellular components of the liver was in the microsome fraction. After this Siekevitz (1952) found that the most efficient method *in vitro* for obtaining incorporation of radioactive alanine into protein was to incubate a mixture of microsomes (precipitated at pH 5) and mitochondria in a medium that allowed the mitochondria to carry out oxidative phosphorylation. He suggested that the main function of the mitochondria was to provide energy for the microsomal system and this view was strengthened by the observation of Zamecnik & Keller (1954) that the mitochondria could be replaced by cell sap and an adenosine triphosphate-generating system. Because of low rates of labelling of the mitochondria observed in this early work, the high microsomal contamination

in the mitochondrial preparations used, and the high proportion of radioactivity lost from the protein by treatment with ninhydrin (Rutman, Cantarow & Paschkis, 1954) or mercaptoethanol (Peterson & Greenberg, 1952), the mitochondria were ignored for several years as an active site for protein synthesis, and their function was regarded primarily as an energy donor for the microsomal system (see, for example, Brachet, 1958).

More recently, however, evidence has appeared that mitochondria are capable of independent protein synthesis. McLean, Cohn, Brandt & Simpson (1958) found that, in contrast with rat liver, skeletal muscle showed equivalent rates of incorporation of radioactive leucine into mitochondrial and microsomal protein *in vivo*. Equivalent rates of labelling of mitochondria and microsomes *in vivo* have also been observed in ascites cells (E. M. Martin, personal communication). The incorporation of radioactive amino acids into the

* Present address: C.S.I.R.O., Sheep Biology Laboratory, P.O. Box 144, Parramatta, N.S.W., Australia.

protein of isolated plant mitochondria has been reported (Sisakyan & Filippovich, 1957), of mitochondria from *Tetrahymena pyriformis* (Mager, 1960), of mitochondria from *Paracentrotus lividus* (Giudice, 1960), of liver mitochondria (McLean *et al.* 1958; Greengard, 1959; Greengard & Campbell, 1959; Rendi, 1959*a, b*; Korner, 1959) and of skeletal muscle mitochondria (McLean *et al.* 1958). Fraser & Gutfreund (1958) observed similar rates of labelling *in vitro* with microsomal and mitochondrial fractions isolated from mammary tissue. The most striking observations, however, have been the demonstration of the incorporation of radioactive valine into cytochrome *c* by isolated rat-liver mitochondria (Bates, Craddock & Simpson, 1958, 1960) and of the net synthesis of cytochrome *c* over a period of 12 hr. by a preparation of calf-heart sarcosomes (Bates & Simpson, 1959).

Rat-liver mitochondria have been chosen for this study because of the information available as to their isolation and biochemical properties (Schneider, 1959), and in this paper their requirements for incorporation of radioactive amino acids into proteins have been investigated. Some of our preliminary observations have been published elsewhere (Reis, Coote & Work, 1959).

METHODS

Cell fractionation

The cell fractions were isolated between 0° and 4° in 0.3M-sucrose-2 mM-ethylenediaminetetra-acetate (EDTA) (disodium salt)-0.03M-nicotinamide adjusted to pH 7.1 with KOH. Young rats, weighing between 100 and 120 g., were starved for 15 hr. and killed by a blow on the head followed by decapitation. The livers were rapidly removed, washed in 0.15M-NaCl followed by the sucrose-EDTA-nicotinamide medium and then forced through a tissue mincer containing a stainless-steel screen with holes 1 mm. in diameter. The mince was suspended in 3 vol. of medium and homogenized in a Potter-Elvehjem homogenizer with a 1½ in. diam. polytetrafluoroethylene (Teflon) pestle (clearance between pestle and mortar 0.02-0.03 in.) with 8-10 slow strokes, the pestle rotating between 1500 and 2000 rev./min. The homogenate was filtered through plain surgical gauze to remove pieces of fibre and clumps of unbroken cells. Nuclei, whole cells and red blood cells were removed by sedimenting at 700g for 15 min.

Mitochondria were prepared from the resulting supernatant by five successive sedimentations at 8000, 7000, 6000, 8000 and 8000g, each for 10 min. Before the last sedimentation at 8000g, the resuspended mitochondria were centrifuged at 700g for 6 min. to remove any residual nuclei and red blood cells. The mitochondria were resuspended between sedimentations as gently as possible by hand in a Potter-Elvehjem homogenizer, with 5-10 slow strokes of the pestle.

Radioactive microsomal fraction was prepared from the liver of a rat injected 30 min. previously with 10 µC of algal protein hydrolysate. The supernatant, after the

mitochondria had been sedimented, was centrifuged at 105 000g for 60 min. and the pellet washed free of supernatant fluid by gently swirling fresh medium over it. The resuspended microsomal pellet was then centrifuged at 8000g for 10 min. in order to remove any clumped material.

Cell sap was taken as the final supernatant after centrifuging at 105 000g; 4.0 ml. of cell sap was derived from 1 g. wet wt. of liver mince.

Acetone-dried powders were prepared by slowly adding a suspension of mitochondria in water to 10 vol. of AnalaR acetone at -15°. The precipitate was washed with cold acetone and dried. The dry powder was homogenized in buffer and the suspension centrifuged at 8000g for 10 min. For demonstration of adenosine triphosphate (ATP)-pyrophosphate exchange (Table 2) the buffer used was 0.1M-2-amino-2-hydroxymethylpropane-1:3-diol (tris)-HCl, pH 7.6, and for separation of soluble and insoluble protein for radioactivity determinations (Fig. 1) 0.1M-potassium phosphate, pH 7.0, was used.

For measuring bacterial contamination, suitable quantities of the homogenate, mitochondrial fraction or final reaction mixture were diluted with sterile 0.9% NaCl soln. and 0.1 ml. was plated on blood-agar plates. Colonies were counted after 18 hr. incubation at 37°.

Incubation conditions for studying amino acid incorporation

The mitochondria were incubated in 3-6 ml. of the appropriate medium generally at 30° under O₂, in 50 ml. conical flasks shaken at 100 cyc./min.; 10-20 mg. of mitochondrial protein was added per 3 ml. of reaction mixture. The reaction was stopped by addition of carrier amino acid, followed by trichloroacetic acid to a final concn. of 5% (w/v), and the protein then isolated for plating by the method of Simkin & Work (1957*a*).

The composition of the reaction mixture was modified as the work developed. However, all experiments were done with a basal medium (*Medium A*: this contained 0.1M-sucrose, 0.04M-KCl, 1.3 mM-EDTA, 0.02M-nicotinamide and 0.01M-potassium succinate) supplemented with the additions described in the legends to the appropriate figures and tables. In some experiments 0.01M-potassium α -oxoglutarate was used as an energy source instead of succinate, and this is indicated in the legends to the appropriate tables and figures. Many of the later experiments were done with a more complex medium (*Medium B*: this was basal medium A + 0.016M-potassium phosphate, 4 mM-adenosine monophosphate, 0.5 mM-diphosphopyridine nucleotide (DPN), 8 mM-MgSO₄ and 50 µg. of synthetic amino acid mixture/ml.). The synthetic amino acid mixture had similar molar proportions to that found in cell sap (alanine 1.0, arginine 0.125, aspartic acid 0.5, asparagine 0.25, cystine 0.5, glutamic acid 1.0, glutamine 0.75, glycine 1.0, histidine 0.25, isoleucine 0.375, leucine 0.375, lysine 0.5, methionine 0.5, phenylalanine 0.125, proline 0.25, serine 0.75, threonine 0.5, tryptophan 0.125, valine 0.5 and tyrosine as a saturated solution) and was passed through the resin Amberlite IRC-50 (K⁺ form) before use in order to remove heavy metals. The pH of all the incubation media was adjusted to pH 7.0-7.2 with KOH. In some experiments 0.02M-glucose and (crude) hexokinase (80 µg./ml.) were added in order to lower the concentration of the extramitochondrial ATP and hence minimize the effect of microsomal contamination. With α -oxoglutarate as an

energy source this did not affect the incorporation, but, for a reason as yet unknown, some inhibition was obtained when succinate was used.

Enzyme preparations and assay methods

pH 5 Enzyme was prepared by the method of Keller & Zamecnik (1956).

Two samples of ribonuclease were used, one from Worthington Biochemical Corp., Freehold, N.J., U.S.A., and the other, a highly purified sample, kindly provided by Dr R. R. Porter.

Glucose 6-phosphate dehydrogenase was obtained from C. F. Boehringer und Söhne GmbH., Mannheim, Germany.

A crude sample of hexokinase was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. Determination of P:O ratios was carried out with a sample of twice recrystallized yeast hexokinase kindly provided by Dr R.A. Darrow and Dr S. P. Colowick.

Glucose 6-phosphatase was estimated by incubating the tissue at 30° for 10 min. in the presence of 4 mM-glucose 6-phosphate-0.03M-sodium succinate, pH 6.6. The liberation of inorganic phosphate was followed.

Succinoxidase was estimated manometrically by the method of Schneider & Potter (1943), except that AlCl_3 was omitted from the reaction mixture.

Adenosine triphosphate-pyrophosphate exchange was followed by measuring the incorporation of [^{32}P]pyrophosphate into ATP. The incubation conditions were as described by Hoagland (1955) (with an equimolar mixture of 12 amino acids) and ATP was isolated from the reaction mixture by the method of Crane & Lipmann (1953). Percentage exchange was taken as

$$\frac{\text{Total counts/min. in ATP}}{\text{Counts/min. in } [^{32}\text{P}]\text{pyrophosphate added}} \times 100.$$

Oxidative phosphorylation was measured by the appearance of glucose 6-phosphate after incubation in the presence of glucose and hexokinase (Slater, 1953). Glucose 6-phosphate was estimated spectrophotometrically with triphosphopyridine nucleotide (TPN) and glucose 6-phosphate dehydrogenase, according to the method of Horecker & Wood (1957). For the measurement of P:O ratios, crystalline hexokinase was used, and O_2 uptake was measured manometrically and extrapolated to the time the mitochondria were added, according to the method of Slater & Holton (1953).

Chemical estimations

Acid-soluble material, ribonucleic acid (RNA), lipid and protein were separated essentially according to the method of Schneider (1945).

Inorganic phosphate and total phosphate were determined by the method of Fiske & Subbarow (1925).

Phospholipid was estimated by its total phosphate content and it was assumed that 1 mg. of phospholipid P corresponded to 25 mg. of phospholipid (Siekevitz & Watson, 1956).

Ribonucleic acid was determined by its pentose content with the orcinol reagent (Mejbaum, 1939) as described by Umbreit, Burris & Stauffer (1957). Adenosine monophosphate was used as a standard and it was assumed to give

twice the extinction of an equivalent amount of RNA (Dische, 1955). It was also assumed that 1 μmole of RNA-pentose was equivalent to 31 $\mu\text{g.}$ of RNA-phosphorus (RNA-P).

Protein was estimated by its N content by using a micro-Kjeldahl procedure.

α -Amino nitrogen of cell sap and mitochondria. Cell sap was heated at 100° for 5 min. and the coagulated protein removed by centrifuging. α -Amino N was determined directly on the clear supernatant. Mitochondria were extracted with cold 5% (w/v) trichloroacetic acid and the extract was shaken with ether several times. The aqueous phase was taken for analysis. The Van Slyke technique was used (Van Slyke, 1929; Van Slyke, Dillon, MacFadyen & Hamilton, 1941).

Isolation of amino acids from cell sap. The supernatant from boiled cell sap was desalted by loading on the ion-exchange resin Zeo-Karb 225 (H^+ form). The amino acids were eluted with aq. N- NH_3 soln. and the eluate was evaporated to dryness. Samples were taken for studying the effect on amino acid incorporation (Table 1) and the remainder was analysed by two-dimensional descending paper chromatography with aq. 71% (v/v) phenol, in an atmosphere saturated with NH_3 in the first direction and butan-1-ol-acetic acid-water (63:10:27, v/v) in the second. The chromatograms were developed by spraying with 0.1% (w/v) ninhydrin in 98% butanol and the relative amounts of amino acids estimated from the size and colour intensity of the spots.

Radioactivity determinations

^{32}P content of adenosine triphosphate. This was determined in a liquid counter (Veall, 1948) and suitable precautions were taken to allow for the decay of ^{32}P in calculation of the results.

Radioactive protein. This was counted at infinite thickness as compressed disks, with either 1 sq.cm. polythene or 0.3 sq.cm. polymethyl methacrylate (Perspex) planchets. End-window Geiger-Müller counters were used and standard Perspex sources of radioactivity obtained from The Radiochemical Centre, Amersham, Bucks., were counted in parallel with the protein samples. A 1 sq.cm. standard containing 1000 $\mu\text{mc/g.}$ gave 950 counts/min. with the apparatus used. In all experiments a zero-time assay was carried out by adding carrier amino acid before adding the mitochondria and then immediately adding trichloroacetic acid. This zero-time value was then subtracted from all the values found for radioactivity of protein. With 0.33 $\mu\text{c/ml.}$ of algal protein hydrolysate the zero-time value was 10.6 $\mu\text{mc/g.}$ (average of 24 determinations). With 1.0 $\mu\text{c/ml.}$ of [^{14}C]valine it was 4.2 $\mu\text{mc/g.}$ (average of six determinations).

Radioactive chemicals. [^{32}P]Pyrophosphate was prepared by pyrolysis of inorganic [^{32}P]phosphate obtained from The Radiochemical Centre, Amersham, Bucks. (Kornberg & Pricer, 1951). Uniformly labelled L-[^{14}C]valine (1 mc/17.9 mg.) and [^{14}C]algal protein (1 mc/4.5 mg.) were also obtained from The Radiochemical Centre. The algal protein was hydrolysed and the amino acids obtained were purified by the method of Simkin (1958).

Where possible all reagents were recrystallized before use. All solutions were made up in de-ionized distilled water.

RESULTS

Development of incubation medium for studying amino acid incorporation into mitochondrial protein

Effect of cell sap. If mitochondria isolated in 0.25M-sucrose (Schneider, 1948) were incubated in the presence of radioactive amino acids in relatively simple media (e.g. tris-Mg²⁺ ions-succinate mixtures) little or no incorporation was observed. The incorporation was greater if EDTA (Cleland & Slater, 1953) and nicotinamide [to prevent DPN breakdown (Kaplan, 1955)] were added to the isolation medium and under these conditions cell sap was necessary for incorporation. However, if

Table 1. *Effect of various treatments of cell sap on incorporation of radioactive amino acids into mitochondrial proteins*

Mitochondria were incubated at 37° under the following conditions. Expt. 1: medium A plus 0.01M-potassium phosphate, 0.07M-tris-HCl, 0.012M-MgSO₄, 6 mM-adenosine monophosphate, 0.5 mM-DPN, 0.15 ml. of cell sap and 1.25 µC of algal protein hydrolysate/ml.; incubation in air. Expt. 2: medium A plus 0.01M-potassium phosphate, 0.07M-tris-HCl, 5 mM-MgSO₄, 0.15 ml. of cell sap and 1.25 µC of algal protein hydrolysate/ml. Expt. 3: as in Expt. 2 except with 1.5 µC of [¹⁴C]valine/ml. Expt. 4: medium B plus glucose and crude hexokinase, and α-oxoglutarate as energy source; 0.3 ml. of cell sap and 1.0 µC of algal protein hydrolysate/ml.

Expt. no.	Time of incubation (min.)	Radioactivity in protein (µmc/g.)		
		Whole cell sap	Boiled cell sap	Amino acids from boiled cell sap
1	30	84, 86, 88	87, 89	—
	60	112	110	—
2	15	73	96	—
	30	92	97	—
	60	100	109	—
3	30	—	21.5	19.0
4	60	—	60	59

the whole cell sap was heated at 100° for 5 min. and the coagulated protein centrifuged down, the clear supernatant was as effective as the untreated cell sap in maintaining incorporation (Table 1). Treatment of whole cell sap with ribonuclease was not inhibitory, nor was treatment with ribonuclease followed by boiling. These results suggested that the effect of cell sap was due to substances of low molecular weight. Examination revealed the presence of considerable quantities of adenosine monophosphate and DPN and when these were added improvement in amino acid incorporation and in phosphorylation were observed. However, the main effect of the cell sap seemed to be due to the amino acids present. Van Slyke analysis showed 81 µg. of α-amino nitrogen (approx. 0.7 mg. of amino acid/ml.) and isolation on Zeo-Karb 225 (H⁺ form) followed by chromatography showed a full complement of amino acids in the approximate molar proportions given in the Methods section (see 'Incubation conditions for studying amino acid incorporation'). These isolated amino acids could effectively replace the boiled cell sap in maintaining incorporation (Table 1).

Role of amino acid-activating enzymes. Since the effect of cell sap is not destroyed by boiling, it is unlikely that added activating enzymes can stimulate mitochondrial incorporation, and it has not been possible to observe any effect with added pH 5 enzyme. It is possible, however, that mitochondrial amino acid-activating enzymes are involved. It was difficult to demonstrate any ATP-pyrophosphate exchange that is dependent on amino acids if intact or disrupted mitochondria were used, because of the high level of non-specific exchange. However, some amino acid dependence was observed in extracts of acetone-dried powders, which showed much less non-specific exchange, but the enzyme activity was smaller than with pH 5 enzyme (Table 2).

Amino acid concentration in mitochondria and medium. Van Slyke analysis and chromatography showed the presence of a full complement of free

Table 2. *Amino acid-dependent exchange between adenosine triphosphate and pyrophosphate in various preparations*

Mitochondria were disrupted with ultrasonic vibrations (25 kc./sec. for 2 min. at 0°). Acetone-dried powder was extracted with 0.1M-tris-HCl, pH 7.6.

Prep.	Wt. of liver used to obtain preparation (mg.)	Exchange (%)		
		No amino acids	Plus amino acids	Increment due to amino acids
Intact mitochondria	310	20.4	20.9	+0.5
Disrupted mitochondria	280	23.8	24.7	+0.9
Extract of acetone-dried powder	350	4.8	6.4	+1.6
	920	10.7	14.0	+3.3
pH 5 enzyme	120	19.0	37.5	+18.5

amino acids in the mitochondria (3.3 $\mu\text{g.}$ of α -amino nitrogen or approx. 28 $\mu\text{g.}$ of amino acid/mg. of protein). Since 10–20 mg. of mitochondrial protein was added to the incubation flasks, it would be expected that milligram quantities of radioactive amino acid would be needed to saturate the mitochondrial pool. In confirmation of this it was observed that maximum labelling of the mitochondrial protein was not obtained until 2 mg. of amino acid/ml. was added, and even then only in insoluble and not in soluble proteins (Fig. 1).

Unfortunately the amount of incorporation obtained with the mitochondria does not permit the routine use of such high concentrations of amino acid, since exorbitant amounts of radioactivity would have to be added to obtain measurable counts. We have found that adding 150 $\mu\text{g.}$ of synthetic amino acid mixture and 1 μC of [^{14}C]-algal hydrolysate/3 ml. of reaction mixture gives a convenient final radioactivity in the protein of 50–100 counts/min. after incubation for 1 hr., but these amounts are entirely empirical and the

system is far removed from saturation with respect to amino acid concentration.

If it is assumed that the intramitochondrial amino acids equilibrate freely with those in the medium, the average specific radioactivity of the amino acids in the system can be calculated from the amounts of mitochondria, [^{14}C]amino acid and [^{14}C]amino acid added, assuming the presence of 28 $\mu\text{g.}$ of amino acid/mg. of protein. If the effect of adding low concentrations of radioactive amino acid (0–200 $\mu\text{g./ml.}$) is studied in this way, it is observed that the final radioactivity of the protein is directly proportional to the (calculated) specific radioactivity of the amino acids (Fig. 2). Over this range of amino acid concentration, therefore, alterations in the amount of amino acid added do not affect the net rate of protein synthesis, but merely alter the extent of labelling of the pool.

Energy source. In all the experiments, we have relied upon oxidative phosphorylation by the mitochondria to provide energy for the incorporation process. The dependence of the system on oxidative phosphorylation is shown by greatly reduced incorporation on incubation under anaerobic conditions, in the presence of 0.2 mM-2,4-dinitrophenol, 1 mM-potassium cyanide, or in the absence of inorganic phosphate or an oxidizable substrate (succinate or α -oxoglutarate) (Table 3). The optimum concentrations of inorganic phosphate, α -oxoglutarate and succinate are shown in Fig. 3. On several occasions it was possible to observe simultaneous failure in incorporation and phosphorylation (see Fig. 1 in Reis *et al.* 1959).

Fig. 1. Effect of amino acid concentration on radioactivity of mitochondrial protein. ●, Residual protein; ○, extracted protein; ■, calculated radioactivity of pool. Mitochondria were incubated for 1 hr. in medium B in the presence of glucose and crude hexokinase and α -oxoglutarate as energy source. Mixtures of algal protein hydrolysate and synthetic amino acid mixture were added in constant proportion (9.1 $\mu\text{mc/}\mu\text{g.}$) in the amounts shown. Radioactivity of pool was calculated from amounts of amino acid added and present in mitochondria and assuming free equilibration between mitochondria and medium. Acetone-dried powders were prepared at end of incubation and extracted with 0.1 M-potassium phosphate buffer, pH 7.0.

Fig. 2. Relationship between labelling of mitochondrial protein and (calculated) specific radioactivity of pool. Mitochondria were incubated for 30 min. in medium A plus 0.01 M-potassium phosphate, 0.02 M-tris-HCl, 6 mM-MgSO₄ and mixtures of algal protein hydrolysate and synthetic amino acid mixture in constant proportion (50 $\mu\text{mc/}\mu\text{g.}$) added in amounts ($\mu\text{g./ml.}$) shown in parentheses. Specific radioactivity of pool were calculated as in Fig. 1.

Nucleotides and Mg^{2+} ions. Addition of DPN results in some improvement in incorporation (Table 3) but there appears to be no absolute requirement, probably because of the presence of endogenous nucleotide. The effect of addition of adenosine monophosphate and $MgSO_4$ is more

Fig. 3. Effect of different concentrations of succinate, α -oxoglutarate and inorganic phosphate on amino acid incorporation. $\circ$, Succinate; Δ , α -oxoglutarate; $\bullet$, phosphate. Mitochondria were incubated for 1 hr. in medium B in the presence of $0.33 \mu\text{C}$ of algal protein hydrolysate/ml. Maximum incorporation with succinate was $115 \mu\text{mc/g./hr.}$, α -oxoglutarate $104 \mu\text{mc/g./hr.}$ and phosphate $121 \mu\text{mc/g./hr.}$

Table 3. Effect of inhibitors and of omission of components of incubation medium on incorporation of amino acids

Mitochondria were incubated in medium B plus $0.33 \mu\text{C}$ of algal protein hydrolysate/ml. Results are expressed as percentage of radioactivity in protein after incubation for 1 hr. in the complete system.

	Radioactivity in protein	
	1 hr. incubation	2 hr. incubation
Complete system	100.0	127.0
- DPN	72.0	97.0
- AMP	59.8	77.0
- $MgSO_4$	16.2	13.4
- α -Oxoglutarate*	16.4	14.8
- Succinate	4.4	19.0
- Phosphate	11.3	33.3
Sucrose medium only	15.2	17.3
+ 0.2 mM 2,4-Dinitrophenol	11.0	—
+ 1 mM KCN	8.3	11.8
Incubation under N_2	10.8	—

* Complete system contained α -oxoglutarate plus glucose and crude hexokinase. Succinate was used as energy source in all other experiments.

impressive, however. The optimum concentrations of $MgSO_4$ and adenosine monophosphate are shown in Fig. 4 and a ratio of approx. 2 moles of $MgSO_4$ to 1 mole of adenosine monophosphate was most satisfactory.

Mitochondrial concentration. If the amount of radioactive amino acid added is kept constant the final radioactivity of the protein falls as the mito-

Fig. 4. Effect of different concentrations of $MgSO_4$ and adenosine monophosphate on amino acid incorporation. $\circ$, $MgSO_4$; $\bullet$, adenosine monophosphate. Mitochondria were incubated for 1 hr. in medium B in the presence of $0.33 \mu\text{C}$ of algal protein hydrolysate/ml. Maximum incorporation with $MgSO_4$ was $98 \mu\text{mc/g./hr.}$, with adenosine monophosphate $48 \mu\text{mc/g./hr.}$

Fig. 5. Effect of concentration of mitochondrial suspension on incorporation of amino acids into mitochondrial protein. $\bullet$, Incubation for 1 hr.; $\circ$, incubation for 2 hr. Mitochondria were incubated in medium B plus $0.33 \mu\text{C}$ of algal protein hydrolysate/ml.

chondrial concentration is raised (Fig. 5). This is to be expected if the mitochondria contain a significant pool of endogenous amino acid. However, it also appears from Fig. 5 that the duration of the incorporation (as judged by the difference between incubation for 1 and 2 hr.) is affected by mitochondrial concentration, the system being most active at a concentration of 4 mg. of protein/ml.

Behaviour of mitochondria in final medium used (medium B)

Oxygen uptake and phosphorylation. Medium B, which was developed to obtain the best amino acid incorporation, bears a strong resemblance to media generally used for the study of oxidative phosphorylation. With this medium, lower oxygen uptakes were observed with succinate as a substrate than in the succinoxidase assay of Schneider & Potter (1943) [280 μ l. of O_2 /hr./mg. of protein N compared with 2100 μ l. of O_2 /hr./mg. of protein N], indicating that the mitochondria do not exhibit their full oxidative capacity. The oxygen uptake is very low if 0.01M-glucose is used instead of the succinate (19 μ l. of O_2 /hr./mg. of protein N). The inability of the preparations to oxidize glucose has been of use in studying the problem of bacterial contamination (Fig. 9). Under the conditions normally used for studying amino acid incorporation, P:O ratios of 0.9–1.1 were generally obtained if crystalline hexokinase was used in the assay. If the mitochondria were incubated for short periods of time (10–20 min.) in more dilute suspension than was normally used, more satisfactory P:O ratios could be obtained (1.5–1.7).

Amino acid incorporation. Considerable variation was observed in the initial rate of incorporation and in the shape of the progress curve. In general, incorporation is linear for about 1 hr. and then falls off steadily, finally ceasing after incubation for 1½–2 hr. at 30°. In some experiments, however, the incorporation ceased abruptly after 1 hr., whereas in others it continued for 2½ hr. With medium B and succinate as an energy source the rate of labelling of mitochondrial protein was $83.0 \pm 7.9 \mu\text{mc/g./hr.}$ (eight determinations), in the presence of 0.33 μ C of algal protein hydrolysate/ml. Addition of glucose and crude hexokinase lowered this ($57.5 \pm 8.5 \mu\text{mc/g./hr.}$) (13 determinations). With medium B and 1.0 μ C of [^{14}C]valine/ml. as a label the rate of labelling was $62.7 \pm 8.0 \mu\text{mc/g./hr.}$ (6 determinations). In the absence of direct measurements of the specific radioactivity of the intramitochondrial pool of amino acids, the observed radioactivity in the protein cannot be expressed in terms of weight of amino acid incorporated. However, by using the figure of 28 μ g. of amino acid/mg. of protein for the size of the

pool, from the amount of amino acid added and the mitochondrial concentration, and assuming free equilibration of amino acids across the mitochondrial membrane, it can be calculated that the mean rate of incorporation with algal protein hydrolysate as a label ($83 \mu\text{mc/g./hr.}$) is equivalent to 45 μ g. of amino acid/g. of protein/hr., i.e. one amino acid residue in 22 000.

Improvement in this figure depends on increasing the time for which incorporation can continue. Preliminary experiments suggested that alteration in the composition of the medium was a more likely cause for failure than inactivation of the mitochondria. Preincubated mitochondria were almost as active as fresh mitochondria if they were resuspended in fresh medium. However, fresh mitochondria were less active in the medium obtained from preincubated mitochondria than in fresh medium. It was then found that if the medium was changed every hour, there was considerable improvement in the stability of the system. Table 4 shows that both phosphorylation and amino acid incorporation continue for 3 hr. under these conditions.

Site of labelling of mitochondria. Fig. 1 shows that the proteins that are extracted by dilute phosphate buffer from acetone-dried powders of mitochondria are less radioactive than the residual protein. Also, during incubation a certain amount of protein is lost from the mitochondria into the medium and this protein has a lower radioactivity than that retained within the mitochondria.

Disruption of mitochondria with the non-ionic detergent Triton X-100 (Rohm and Haas Co., Philadelphia, Pa., U.S.A.) confirmed that the insoluble proteins (sedimented at 105 000g for 60 min.) are more radioactive than the soluble

Table 4. *Stability of amino acid incorporation and phosphorylation in mitochondria incubated in medium B*

Results are expressed as a percentage of the value after incubation for 1 hr. (45.0 $\mu\text{mc/g.}$ and 5.82 μmoles of glucose 6-phosphate/ml. of reaction mixture). Mitochondria were incubated in medium B in the presence of 0.33 μ C of algal protein hydrolysate/ml., 0.02M-glucose and 80 μ g. of crude hexokinase/ml. Medium was changed by centrifuging the suspension at 8000g for 10 min. after incubation for 1 and 2 hr. and re-suspending the pellet in fresh medium B.

	Time of incubation at 30° (hr.)		
	1	2	3
Amino acid incorporation			
Medium not changed	100	134	139
Medium changed	100	159	236
Phosphorylation			
Medium not changed	100	67.5	17.8
Medium changed	100	192	284

proteins (Fig. 6). More detailed analyses showed that the protein of highest specific radioactivity appears in a fraction sedimenting between 10 000g

for 30 min. and 105 000g for 60 min. This fraction is also rich in phospholipid, RNA and succinoxidase activity (Table 5).

Fig. 6. Rate of incorporation of radioactive amino acids into protein of mitochondrial subfractions. ■, Intact mitochondria; ●, sediment after 60 min. at 105 000g; ○, supernatant after 60 min. at 105 000g. Mitochondria were incubated in medium B plus glucose and crude hexokinase and 1 μ C of [14 C]valine/ml. At the times shown they were sedimented at 10 000g for 10 min. and disrupted with 0.2% (v/v) Triton X-100. Disrupted mitochondria were then centrifuged at 105 000g for 60 min.

Artifacts in studying mitochondrial incorporation of radioactive amino acids

Microsomal contamination. The mitochondria used in these experiments have been well washed and probably have a low microsomal contamination, as indicated by their low RNA content. There was considerable variation in the amount of RNA found in the various preparations and the mean value was $8.85 \pm 0.62 \mu$ g. of RNA-P/mg. of protein N (15 determinations), corresponding to approx. 1.4 mg. of RNA/100 mg. of protein. This variation was probably not due to the method of chemical fractionation, since phospholipid analyses gave more constant values [$54.5 \pm 0.31 \mu$ g. of phospholipid P/mg. of protein N (8 determinations) corresponding to approx. 21 mg. of phospholipid/100 mg. of protein]. It was also observed that the amount of RNA in the various preparations bore no obvious relationship to the amount of incorporation obtained in different experiments.

To confirm the efficiency of the washing procedure unwashed mitochondria were mixed with a radioactive microsomal preparation obtained from a rat previously injected with [14 C]algal protein hydrolysate. Four successive washings reduced the radioactivity in the protein from 50.3 to 1.4 μ mc/g. Repeated washing also greatly reduced the amounts of RNA and glucose 6-phosphatase present, as would be expected from the removal of microsomal material. If the mitochondria at various stages of the washing procedure were incubated with radioactive amino acids, however, the

Table 5. Biochemical properties of fractions obtained by disruption of mitochondria with Triton X-100

Radioactivity in protein was determined after incubation of intact mitochondria for 1 hr. in medium B with 1.0 μ C of [14 C]valine/ml. Mitochondria were centrifuged down, disrupted with 0.2% (v/v) Triton X-100 and sedimented at the speeds shown. Relative concentration is value/unit protein of fraction divided by value/unit protein in intact mitochondria. Values for mitochondria were 7.29 μ g. of RNA-P and 53.9 μ g. of phospholipid P/mg. of protein N, radioactivity 71.6 μ mc/g./hr. and succinoxidase 328 μ l. of O_2 /hr./mg. of protein.

Fraction	...	...	...	Sediment after 10 000g for 30 min.	Sediment after 105 000g for 60 min.	Final supernatant
% of total amount in mitochondria						
Phospholipid				13.0	31.2	45.5
RNA				14.2	48.5	21.1
Protein				9.8	22.4	58.5
Radioactivity in protein				17.2	45.6	16.1
Relative concentration						
Phospholipid				1.32	1.40	0.78
RNA				1.45	2.07	0.36
Succinoxidase				0.58	1.14	0.01
Radioactivity in protein				1.73	2.05	0.28

Table 6. *Effect of ribonuclease on mitochondrial incorporation*

In Expts. 1-3 mitochondria were incubated in medium A plus 0.01M-potassium phosphate, 0.07M-tris-HCl, 8 mM-MgSO₄, 0.15 ml. of cell sap and 1.25 μ C of algal protein hydrolysate/ml. at 37° for 1 hr. In Expt. 1, 20 μ g. of Worthington ribonuclease/ml. was added. In Expt. 2, whole cell sap was treated with 100 μ g. of Worthington ribonuclease/ml. for 15 min. at 37°; part of it was then boiled and used for Expt. 3. In Expt. 4 mitochondria were incubated for 1 hr. in medium B plus 0.33 μ C of algal protein hydrolysate/ml., 0.2 μ g. of highly purified ribonuclease (obtained from Dr Porter) was added/ml.

Expt. no.	Additions to medium	Radioactivity in protein (μ mc/g./hr.)	Change (%)
1	Whole cell sap	62.0	—
	Whole cell sap + ribonuclease	74.0	+19
2	Whole cell sap	100.0	—
	Whole cell sap pretreated with ribonuclease	124.0	+24
3	Boiled cell sap	109.0	—
	Cell sap pretreated with ribonuclease, then boiled	125	+14
4	Synthetic amino acid mixture	61	—
	Synthetic amino acid mixture plus ribonuclease	85	+39

Fig. 7. Effect of washing on mitochondria. ●, Incorporation into protein after incubation for 2 hr. in medium B plus 0.33 μ C of algal protein hydrolysate/ml.; ○, total RNA of suspension; △, total glucose 6-phosphatase of suspension. Unwashed mitochondrial suspension was obtained by centrifuging homogenate at 8000g for 10 min., after removal of nuclei and whole cells. They were washed by repeated sedimentation at 8000g for 10 min. After each washing, amino acid incorporation in medium B, RNA and glucose 6-phosphatase were determined. Initial values were: amino acid incorporation, 88.5 μ mc/g./2 hr.; RNA, 126 μ g. of RNA-P/ml. (36.2 μ g. of RNA-P/mg. of protein N); glucose 6-phosphatase, 109 μ moles of glucose 6-phosphate split/hr./ml.

incorporation observed was actually increased by repeated washing (Fig. 7).

Addition of amounts of ribonuclease sufficient to hydrolyse two to three times the amount of RNA present in the system had no inhibitory effect and

in fact was slightly stimulatory (Table 6). It cannot be concluded that RNA is not required, however, since, although an excess of ribonuclease was added, only 25-30 % of the RNA present was destroyed by this treatment.

Bacterial contamination. Although there was no detectable contamination in the isolation and incubation media used, significant numbers of bacteria were found both in the mitochondrial suspensions and in the homogenates, although the numbers varied considerably from experiment to experiment. Removal of lobes of liver under sterile conditions suggested that most of the bacteria came from the liver itself and that very few were introduced during the preparation of the mitochondria.

It is unlikely that the amount of bacteria present contributed significantly to the observed amino acid incorporation, at least with incubation for 3 hr. First, there was no correlation between the amount of contamination and the incorporation (Table 7). Secondly, the incorporation was not reduced by incubation in the presence of penicillin and streptomycin (Table 7, Expt. 2). Thirdly, if the mitochondria were incubated in medium B for only 20 min. in the absence of an oxidizable substrate, the incorporation was reduced by 75 % (Fig. 8), the inhibition being irreversible. It is highly unlikely that in the presence of sucrose, inorganic phosphate, nucleotides, a complete mixture of amino acids and a high concentration of mitochondria, the contaminant bacteria would show a specific requirement for succinate. If the succinate is replaced by glucose, the incorporation is still greatly reduced, in parallel with the oxygen uptake (Fig. 9). The shape of the incorporation curve in the presence

Table 7. *Lack of correlation between amino acid incorporation and bacterial contamination in mitochondrial suspensions*

Mitochondria were incubated in medium B with 0.01 M-potassium succinate or 0.01 M-glucose in the presence of 0.33 μ C of algal protein hydrolysate/ml.

Expt. no.	$10^{-3} \times$ No. of bacterial colonies			Amino acid incorporation (μ mc/g./hr.)	
	Colonies/ml. of reaction mixture	Colonies/g. of liver taken		Succinate	Glucose
		Homogenate	Mitochondria		
1	47.0	—	—	54.0	—
2	33.0	36.1	72.0	49.0	4.0
				46.0*	—
3	12.8	65.1	33.9	144.0	—
4	3.32	20.8	5.51	64.0	—
5	1.39	—	3.23	75.0*	8.0*
6	0.95	2.02	2.61	76.0	7.0

* 100 units of penicillin G and 60 μ C of streptomycin sulphate/ml.

Fig. 8. Effect of omission of succinate from reaction mixture on amino acid incorporation. Mitochondria were incubated in medium B in the presence of 1.0 μ C of [14 C]-valine/ml. Succinate was added at 0 min. (A), 20 min. (B), 40 min. (C) and at end of experiment (D).

of succinate argues against bacterial contamination contributing to the system, since a definite plateau is obtained after incubation for 1½ hr., which would not be expected if the incorporation were due to bacteria. The low incorporation in the presence of glucose, however, appears to continue with time and it is possible that at least part of this is due to bacteria in the system, although even here there is no correlation between the incorporation observed and the amount of bacterial contamination (Table 7). The dependence of the system on oxidative phosphorylation, the inhibition by cyanide and 2:4-dinitrophenol (Table 3), the requirement for adenosine monophosphate (Table 3, Fig. 4), the response to added amino acids (Fig. 1) and the improvement in incorporation obtained by replen-

Fig. 9. Amino acid incorporation and oxygen uptake in the presence of succinate and glucose. ●, Oxygen uptake; ○, radioactivity in protein. A, Mitochondria incubated in medium B with succinate as energy source; B, 0.01 M-glucose instead of succinate. Media contained 0.33 μ C of [14 C]algal protein hydrolysate/ml.

ishing the medium (Table 4) provide additional arguments against the importance of bacterial contamination.

Artifacts in labelling. Treatment of radioactive mitochondrial protein, obtained after incubation in the presence of [14 C]algal protein hydrolysate, with performic acid (Peterson & Greenberg, 1952), ninhydrin (Peterson & Greenberg, 1952) or thio-glycollic acid (Siekevitz, 1952) resulted in the release of less than 5% of the total radioactivity, indicating that the radioactive amino acids were not attached by S-S bonds or present as bound amino acid. Protein treated with 1-fluoro-2:4-dinitrobenzene (Sanger, 1945) was hydrolysed in

6.0N-hydrochloric acid at 105° for 15 hr. in a sealed tube and the hydrolysate extracted with ether. About 1% of the radioactivity was extracted, indicating that only a small proportion of the radioactive amino acids were present as *N*-terminal residues.

DISCUSSION

In studies on mitochondrial protein synthesis, particularly those requiring long periods of incubation, the problem of possible bacterial contamination takes on a special significance. First, any bacteria either present in the tissue taken, or introduced during the isolation procedure, will tend to become concentrated in the mitochondrial fraction, because of their similarity in size to mitochondria. Secondly, the media used for studying mitochondrial protein synthesis are ideal for bacterial growth, containing protein, amino acids, nucleotides, inorganic ions and oxidizable substrates. Thirdly, because of the much greater synthetic rate in micro-organisms, compared with the systems under study, a very small contamination could contribute significantly to the observed effect. In this work bacteria have indeed been detected in reasonable numbers, both in the final mitochondrial suspension and in the original liver taken. It has been possible to show, however, that this contamination does not contribute significantly to the observed incorporation for the times of incubation we have used. This agrees with the observation of Lamborg & Zamecnik (1960) with *Escherichia coli*, that, even with bacteria in the exponential phase of growth, 100 000 cells/ml. are needed before significant counts can be detected.

The mitochondrial and microsomal systems for amino acid incorporation differ in several respects. First, mitochondria do not require cell sap (Zamecnik & Keller, 1954) nor the pH 5 fraction obtained from it (Keller & Zamecnik, 1956), at least in our experiments. There is unfortunately some confusion in the literature about the importance of cell sap in the mitochondrial system. Greengard (1959) showed greater stimulation with whole cell sap than with boiled cell sap, as well as an effect with the pH 5 fraction. Korner (1959) found that cell sap improves the incorporation of radioactive leucine, but lowers that of radioactive tryptophan. Both these workers used conditions which would favour microsomal incorporation, however, and at least part of their results might have been due to microsomal contamination. McLean *et al.* (1958) reported that the pH 5 fraction can replace cell sap but in a later publication this group confirmed our observation (Reis *et al.* 1959) that cell sap is not inactivated by boiling (Bates, Craddock & Simpson, 1960). It is possible that intramitochondrial amino acid-

activating enzymes are involved, although difficult to demonstrate satisfactorily (Table 2; Craddock & Simpson, 1960).

The second major difference between the two systems is in the effect of ribonuclease, the microsomes being inactivated by this enzyme (Allfrey, Daly & Mirsky, 1953; Zamecnik & Keller, 1954), whereas the mitochondria are either unaffected or actually stimulated (Table 6; McLean *et al.* 1958; Rendi, 1959*a*; Mager, 1960). The insensitivity to ribonuclease does not preclude RNA from the incorporation process, however, since only a part of the mitochondrial RNA is degraded by the enzyme, probably because of inaccessibility between enzyme and substrate. Certainly in two reports where amino acid incorporation was observed in fractions isolated from disrupted mitochondria (Rendi, 1959*a*; Kalf & Simpson, 1959) sensitivity to ribonuclease was observed.

The systems also differ in their stability. In our experiments incorporation proceeds for 1½–2 hr. if the medium is unchanged and for at least 3 hr. if it is replenished. Bates & Simpson (1959) have reported stability of calf-heart sarcosomes for 12 hr. In general the microsomal system is less stable, incorporation stopping after 15–30 min. (Simkin & Work, 1957*b*).

From these considerations it is unlikely that the incorporation observed in our experiments is due to microsomal contamination. For example, repeated washing of the mitochondria, although obviously removing the bulk of the microsomal contamination, actually increases the incorporation into the mitochondria. Other workers agree that the effect is not due to microsomes (McLean *et al.* 1958; Greengard, 1959), and Bates *et al.* (1960) have shown that microsomes could not synthesize cytochrome *c* under the conditions used to demonstrate mitochondrial synthesis of this protein. Mager (1960) has distinguished the two systems by the effect of chloramphenicol, the mitochondria being inhibited (see also Rendi, 1959*b*; Bates *et al.* 1960) and the microsomes not.

Since one can exclude microsomal contamination, studies on the role of RNA in the mitochondrial system might provide valuable evidence about the true significance of the small amounts of this nucleic acid found in washed mitochondria (see Harel, Jacob & Moulé, 1957; Novikoff, 1959). Disruption with Triton X-100 releases a certain amount of RNA into solution (Table 5), and this might include a mitochondrial soluble RNA. However, the bulk of the RNA remains attached to material rich in lipid and respiratory enzymes and sedimenting at 100 000*g*. By analogy with the results of other workers on disrupted mitochondria (for example, Cooper & Lehninger, 1956; Siekevitz & Watson, 1956, 1957; Watson & Siekevitz, 1956;

Abood & Alexander, 1957) this fraction is probably rich in mitochondrial membranes and also an important site for oxidative phosphorylation. The observation that the protein in this fraction is the most rapidly labelled (Table 5, Fig. 6) could therefore be attributed either to the membrane system or to ribonucleoprotein particles, as suggested by Rendi (1959a). The electron-microscope evidence in favour of the existence of intramitochondrial ribonucleoprotein particles is poor at the moment, however, and Siekevitz & Watson (1956) ascribed those particles present in fractions from disrupted mitochondria to microsomal contamination. More work is clearly required to establish the precise localization and function of RNA in the mitochondria.

It is difficult to distinguish the true requirements for mitochondrial amino acid incorporation from secondary effects on the efficiency of oxidative phosphorylation, and there is little doubt that many of the observed properties (e.g. dependence on inorganic phosphate, an oxidizable substrate, inhibition by cyanide, 2,4-dinitrophenol or anaerobic incubation) are primarily those of the phosphorylation system. For example, Bates *et al.* (1958) found that succinate, adenosine monophosphate, cytochrome *c* and inorganic phosphate could be partially replaced by adenosine diphosphate and creatine phosphate, showing that these substances are required primarily for ATP production and not for the incorporation process. If this is so, the general impression appears that the mitochondrion is highly autonomous with respect to protein synthesis, provided that it is incubated under conditions favouring continuous energy production. In fact, it bears more resemblance in its behaviour to an intact cell than to an isolated cell fragment. Since isolated mitochondria have been shown in the last decade to possess considerable structural and biochemical organization, it is not improbable that they also contain the complex equipment for protein synthesis.

SUMMARY

1. Washed rat-liver mitochondria incorporate radioactive amino acids into their protein if incubated aerobically in the presence of an oxidizable substrate, inorganic phosphate, adenosine monophosphate, magnesium ions, diphosphopyridine nucleotide and a full complement of amino acids.

2. The system is slightly stimulated by ribonuclease and does not require cell sap or pH 5 enzyme. It has been shown that the observed incorporation is not due to microsomal contamination.

3. The incorporation is dependent upon the maintenance of oxidative phosphorylation by the mitochondria.

4. The optimum concentrations of adenosine monophosphate, magnesium ions, α -oxoglutarate, succinate and phosphate required for amino acid incorporation were determined.

5. Replenishment of the medium during incubation considerably improves the duration of incorporation.

6. Fractionation of radioactive mitochondria showed the highest rate of labelling to be in the protein of an insoluble fraction rich in ribonucleic acid and phospholipid. The soluble proteins, however, were poorly labelled.

7. Significant bacterial contamination was observed in the final mitochondrial fraction and in the livers originally taken. However, this contamination contributes negligible amounts to the observed incorporation at least during incubation for 3 hr.

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The Regulation of Pathways of Glucose Catabolism in Maize Roots

BY V. S. BUTT AND H. BEEVERS

Department of Botany, Oxford University, and Department of Biological Sciences, Purdue University, Lafayette, Indiana, U.S.A.

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The operation of the pentose phosphate pathway in glucose catabolism, as demonstrated by low C-6/C-1 ratios (the yield of $^{14}\text{CO}_2$ from [6- ^{14}C]glucose relative to that from [1- ^{14}C]glucose), can now be regarded as a feature of many plant tissues (Gibbs, 1959). On the other hand, the internal and external conditions which determine the extent of its activity have received little attention. In the work described here, the action of oxidizing agents on the yield of $^{14}\text{CO}_2$, when maize root-tips were provided with glucose specifically labelled in various carbon atoms, has been examined and compared with nitrite as a likely natural electron acceptor, and with 2:4-dinitrophenol as an uncoupling agent.

Maize root-tips, in common with other young developing plant tissues (Gibbs & Beevers, 1955), show a C-6/C-1 ratio of unity (Beevers & Gibbs, 1954), suggesting that hexose breakdown proceeds

primarily through the classical pathways of glycolysis and the tricarboxylic acid cycle. However, Beevers (1956) has shown that such tissues break down labelled gluconate, ribose, xylose and sedoheptulose in air and under nitrogen to give products with the radiochemical distribution expected from the operation of the pentose phosphate pathway, so that they must possess the necessary enzymic equipment, which appears to be inoperative when glucose is the substrate at this stage. Any induction of the alternative oxidation pathway should therefore be recognized readily by a decrease in the natural C-6/C-1 ratio.

The stimulation of the oxygen consumption and carbon dioxide production of plant tissues by artificial hydrogen acceptors, especially by methylene blue (Turner, 1940), has been recognized for some time (James, 1953), and recent work with carrot disks (ap Rees & Beevers, 1960) has shown