concentrations of non-haem iron and that this effect was removed when adequate NADH was generated suggests that the iron protein acts as an electron carrier in the coupled nitrate reductase system. By using electron spin resonance technique, NICHOLAS *et al.*[8] have shown that a non-haem iron protein in Azotobacter particles was markedly reduced by dithionite. These particles also catalyse H_2 evolution and N_2 fixation provided an ATP-generating system and dithionite are added[9,10].

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Department of Agricultural Biochemistry,
Waite Agricultural Research Institute,
University of Adelaide,
Adelaide (Australia)

M. S. NAIK
D. J. D. NICHOLAS

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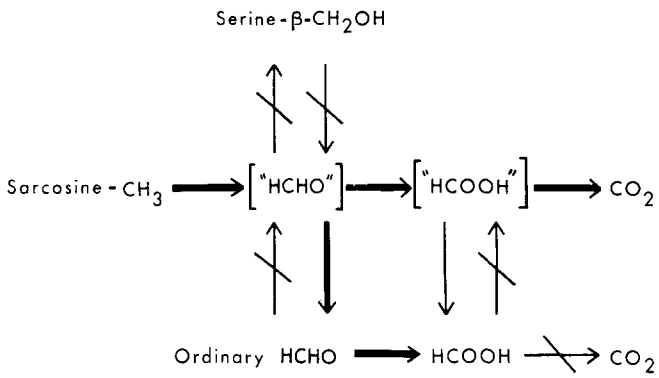
Oxidative phosphorylation in the metabolism of sarcosine and formaldehyde

It had been shown previously that the *N*-methyl groups of both sarcosine and dimethylglycine are metabolized to "active formaldehyde" in phosphate-washed mitochondria[1–3]. In these preparations, the "active formaldehyde" does not undergo further oxidation but is converted either to ordinary formaldehyde or to the β-carbon of serine. Subsequent studies, described below, have demonstrated that the metabolism of sarcosine in sucrose-washed mitochondria results in oxidative phosphorylation and also that the oxidation of the methyl group under these conditions proceeds beyond the level of "active formaldehyde" to formate and carbon dioxide.

With mitochondria washed 3 times with 0.25 M sucrose and then incubated with 0.01 M phosphate in sucrose, as described in Table I, the methyl group is oxidized beyond the level of formaldehyde to formate and carbon dioxide, and serine is a

minor product. Under the same conditions, exogenous formaldehyde is also oxidized, but only to the level of formate. Exogenous formate and serine are not oxidized. The foregoing results can be summarized by the following sequence of reactions:



The data of Tables I and II show that the oxidation of both sarcosine and formaldehyde give a P/O ratio approaching 2. This identity of the ratios for the two substrates provided presumptive evidence that each oxidative step for the conversion of the sarcosine-methyl group to carbon dioxide, including the initial demethylation, results in phosphorylation.

Corroborative evidence for phosphorylation resulting from the initial demethylation step was obtained by carrying out the oxidation of sarcosine in the presence of semicarbazide. Our earlier studies[12] had shown that this reagent can trap the "active formaldehyde" as ordinary formaldehyde. In the experiments described in Table III the oxidized methyl groups were not trapped completely by the semicarbazide in phosphorylating mitochondria, but formaldehyde accumulation was increased more than 6-fold over the control and exceeded the quantities of either formate or carbon dioxide by three to one. However, the observed P/O ratio under

TABLE I

OXIDATIVE PHOSPHORYLATION IN MITOCHONDRIAL METABOLISM OF SARCOSINE

Mitochondria were prepared in 0.25 M sucrose[4–6]. Methods for phosphate and ^{14}C analyses are given in refs. 7–11. 0.5 ml of mitochondrial suspension was added last to the reaction mixture. Other components: 80 μmoles glycylglycine buffer (pH 7.4, 0.2 ml); 20 μmoles potassium phosphate (pH 7.4, 0.2 ml); 5 μmoles ADP (0.2 ml); 0.4 mg hexokinase *plus* 48 μmoles glucose (0.1 ml); 10 μmoles $MgCl_2$ (0.1 ml); 10 μmoles [*Me*-^{14}C]sarcosine and sufficient 0.25 M sucrose to bring the total reaction volumes to 2.0 ml. All solutions, except the mitochondrial suspension, were adjusted to pH 7.4. After a 10 min equilibration period the hexokinase–glucose solution, in the side arm, was tipped into the main compartment, containing all other components. All incubations were in air at 37°.

Incubation time (min)	*$H^{14}CHO$ (μmole)*	*$H^{14}COOH$ (μmole)*	*$^{14}CO_2$ (μmole)*	*O requirement (theoretical) (μatoms)*	*Oxygen uptake (observed) (μatoms)*	*ΔP (μmoles)*	*P/O equiv. to products*	*P/O (manometric)*
40	0.02	0.73 (0.70–0.75)	0.74 (0.68–0.80)	3.7 (3.5–3.9)	3.0 (2.7–3.2)	5.7 (4.5–6.9)	1.5	1.9

TABLE II

OXIDATIVE PHOSPHORYLATION IN THE MITOCHONDRIAL METABOLISM OF FORMALDEHYDE

4 μmoles of formaldehyde or 10 μmoles of sarcosine were incubated with sucrose-prepared mitochondria as described in Table I.

Incubation time (min)	*Substrates*		*Oxygen uptake (μatoms)*	*ΔP (μmoles)*	*P/O*
	Formaldehyde	*Sarcosine*			
20	+	−	3.7 (3.7–3.8)	6.1 (5.9–6.3)	1.7
	−	+	5.8 (6.4–5.2)	10.1 (9.9–10.3)	1.7

TABLE III

[*Me*-^{14}C]SARCOSINE AS A SUBSTRATE FOR OXIDATIVE PHOSPHORYLATION IN THE PRESENCE OF SEMICARBAZIDE

10 μmoles of [*Me*-^{14}C]sarcosine, with and without 20 μmoles of semicarbazide, were incubated for 50 min as described under Table I.

	Products			*ΔP (μmoles)*	*O uptake (μatoms)*	*P/O*
	$H^{14}CHO$ (μmoles)	*$H^{14}COOH$ (μmoles)*	*$^{14}CO_2$ (μmoles)*			
− Semicarbazide	0.61	0.86	0.42	12.4 ± 1.9	5.0 ± 0.3	2.5
+ Semicarbazide	3.9	1.35	1.1	22.6 ± 3.6	11.9 ± 1.0	2.1

these circumstances was not appreciably different from that in the absence of semicarbazide. This finding supports the conclusion that all three of the sequential reactions for converting the sarcosine-methyl group to carbon dioxide result in oxidative phosphorylation.

Oxidative phosphorylation with sarcosine is completely abolished in the presence of 2,4-dinitrophenol and pentachlorophenol. It may be concluded from these results that the oxidation of the sarcosine does not entail a "substrate-level" phosphorylation.

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Department of Biochemistry,
The University of Colorado School of Medicine,
Denver, Colo. (U.S.A.)

WILHELM R. FRISELL
NOEL C. SORRELL

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The replacement of cytochrome c in digitonin fragments from beef-heart sarcosomes

TSOU[1] and others[2–5] using Keilin–Hartree heart muscle particle preparations, have demonstrated that cytochrome *c*-deficient preparations could be made and that exogenous cytochrome *c* has much less enzymatic activity than endogenous. In mitochondria the removal of cytochrome *c* (ref. 6) produces greater reactivity with exogenous cytochrome *c* (ref. 7). This communication deals with the extraction and reinsertion of cytochrome *c* in phosphorylating digitonin submitochondrial fragments.

Digitonin fragments were prepared and stored according to the HAAS AND ELLIOTT[8,9] modifications of the methods of COOPER AND LEHNINGER[10] and DEVLIN AND LEHNINGER[11].

Aliquots of digitonin fragments (2.5 ml) were placed into each of four precooled polypropylene 30 rotor (Spinco) ultracentrifuge tubes and 5.0 ml of 0.10 M phosphate buffer (pH 7.4) were added. All tubes were kept at 2° for 30 min. The tubes were centrifuged for 40 min at 105000 × *g*. The supernatants were collected and stored (—20°). The pellet from one tube was suspended in 0.25 M sucrose *plus* 0.01 M Tris buffer (pH 7.4).

The pellets from the other two tubes were homogenized in 2.5 ml of $2.5 \cdot 10^{-4}$ M cytochrome *c*, held at 2° for 30 min, centrifuged at 105000 × *g* for 40 min, and one pellet was suspended in 0.1 M phosphate buffer (pH 7.4) and was immediately centrifuged as before and resuspended in sucrose–Tris medium (2.5 ml). The other pellet was resuspended in sucrose–Tris medium. Treated digitonin fragments were stored as previously described[8].

Respiratory activity was observed as described by STRICKLAND, ZIEGLER AND ANTHONY[12] using a reaction medium containing 20 ml 0.02 M phosphate buffer (pH 7.4); 20 ml of the 75 mM sucrose–225 mM mannitol–0.1 mM EDTA solution; 4 ml 1 M KCl; and 2 ml 1 M $MgCl_2$ and bovine albumin (1 % final concentration). Protein concentrations were determined by the method of LOWRY *et al.*[13].

Abbreviation: PMS, phenazine methosulphate.