Budapest 2/1963

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NINTH EUROPEAN MEETING OF MEAT RESEARCH WORKERS,

Budapest, September, 1963

THE UTILISATION OF NITRITE BY MITOCHONDRIAL FRACTIONS OF PORK SKELETAL MUSCLE

by

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The work described in this Report is being carried out for the United States Department of Agriculture, with the aid of funds made available under U.S. Public Law 480.

Grateful acknowledgement is made of the financial assistance which has made the work possible.

THE UTILISATION OF NITRITE BY MITOCHONDRIAL FRACTIONS OF PORK SKELETAL MUSCLE

It has already been reported that minces of fresh pig skeletal muscle are able to metabolise sodium nitrite anaerobically at pH 6.0 to yield a gas fraction soluble in alkaline sodium sulphite but not in alkali (presumptive nitric oxide); the presence of nitric oxide in the gaseous incubation products has been confirmed by mass spectrometry and by infra-red spectrophotometry. This ability was not affected by the presence of the broad spectrum antibiotic chloramycetin, and from this and other evidence it was concluded that the reduction of nitrite is mammalian in character, in contrast to that of nitrate, for which bacterial intervention is necessary. (Walters and Taylor, 1963).

As an alternative to the gas production technique, the anaerobic utilisation of nitrite by pig muscle minces has been used as an index of activity. Tests were carried out in Thunberg tubes in the presence of chloramycetin and preliminary displacement of air with argon was used to obtain anaerobic conditions; residual nitrite was determined at the conclusion of the incubation period by the Griess-Ilosvay reagent. Nitrite utilisation was substantially reduced by immersion of the mince in a water bath at 80° C for ten minutes and the nitrite uptake of heat-treated minces was used throughout as a control.

Influence of reduced methylene blue on nitrite-reducing activity of pig muscle minces

Both the production of presumptive nitric oxide and the overall utilisation of nitrite by pig muscle minces under anaerobic conditions were stimulated in the presence of reduced methylene blue (Tables I and II). In the gas production experiments the dye was converted to the reduced form with hydrogen and platinum black before addition to ensure an immediate response; in the later work on nitrite utilisation this precaution was found to be unnecessary since the tests were incubated for 18 hours and the dye, added in the oxidised form, was reduced within about 15 minutes by the unheated muscle minces.

TABLE I. Effect of reduced methylene blue on anaerobic production of presumptive nitric oxide by pig muscle minces at 37°C

/3g. muscle mince;; 3.0 ml. 0.2M phosphate buffer pH 6.0 + 0.5 ml. 3.0 % (w/v) sodium nitrite; chloromycetin present to 8 mg. %; 0.027 mg./ml. reduced methylene blue./

MINCE NO.	GAS EVOLUTION - µl./min.					
	WITHOUT METHYLENE BLUE			WITH METHYLENE BLUE		
	Alkali Absorbent	Alkaline Sulphite Absorbent	Difference (Presumptive Nitric Oxide)	Alkali Absorbent	Alkaline Sulphite Absorbent	Difference (Presumptive Nitric Oxide)
1	0.72	0.45	0.27	1.43	0.71	0.72
2	0.79	0.39	0.40	1.24	0.60	0.64
3	1.16	0.72	0.44	1.36	0.63	0.73

TABLE II. Effect of reduced methylene blue on anaerobic utilisation of sodium nitrite by pig muscle minces

/1 g. muscle mince; 4.0 ml. water + 1.0 ml. 0.05 % (w/v) sodium nitrite; chloromycetin present to 10 mg. %; 0.037 mg./ml. methylene blue; overnight incubation at room temperature on oscillating table./

MINCE NO.	SODIUM NITRITE UTILISED - µg	
	IN ABSENCE OF METHYLENE BLUE	IN PRESENCE OF METHYLENE BLUE
4	5	60
5	7	150

The stimulatory effect of methylene blue on presumptive nitric oxide production and its obvious enhancement of nitrite utilisation by two rather inactive minces suggests that the capacity of muscle to metabolise nitrite under anaerobic conditions is associated with the respiratory processes of the tissue, the electrons passing down the respiratory chain in normal aerobic metabolism being directed towards nitrite in the absence of oxygen. Some evidence to support this postulated competition between oxygen and nitrite for cell respiratory processes is provided by the observations that the endogenous respiration of muscle minces in air is reduced by nitrite (Fig. 1) and that the production of nitrosylmyoglobin from sodium nitrite and the indigenous pigment of minces is greater under anaerobic than under aerobic conditions (Table III).

TABLE III. Nitrosylmyoglobin formation on incubation of fresh muscle minces with sodium nitrite under aerobic and anaerobic conditions at 37°C

/3 g. muscle mince; 3.0 ml. 0.2 M phosphate buffer pH 6.0 + 0.5 ml. 3.0 % (w/v) sodium nitrite; chloromycetin present to 8 mg. %; 90 minutes incubation./

MINCE NO.	AVERAGE NITROSYLMYOGLOBIN* FORMED - mg.	
	ANAEROBICALLY /ATMOSPHERE - ARGON/	AEROBICALLY /ATMOSPHERE - AIR/
6	2.5	0.8
7	5.2	2.2

*Nitrosylmyoglobin estimated by extraction into 80 % (v/v) acetone and determination of optical density at 540 mμ. (Hornsey, 1956)

Fractionation of pig muscle tissue

These considerations have prompted the attempted isolation of the portion of the cell responsible in the main for its respiratory capacity, namely the mitochondrion, in order that the components of the respiratory chain responsible for nitrite reduction may be identified and substrates, etc., contributing to or antagonising the action may be established.

The mitochondria of soft tissues such as liver and brain have been studied extensively whilst those of skeletal muscle have received only scant attention because of the difficulties of homogenization of the fibrous tissue and the lower metabolic activities of the products. As a marker enzyme for the location of mitochondria within cellular fractions, succinic dehydrogenase has been adopted as it is associated almost exclusively with this portion of the cell and it is remarkably stable; it is, in fact, necessary to damage cellular particles by immersion in hypotonic solution and/or by freezing and thawing to obtain maximum activity in this respect (Aldridge and Johnson, 1959).

Initial attempts to isolate the mitochondria of pig skeletal muscle were made using the conventional homogenization medium for other tissues, namely an isotonic solution of sucrose, with and without the addition of versene to chelate harmful trace metals, and an ordinary blade homogenizer. A change of medium to a tris (hydroxymethyl)-aminomethane /tris/-KCl buffer pH 7.4 and of homogenizer to a type in which the tissue was forced past closely fitting and rapidly rotating plastic plungers in a smooth glass tube (Aldridge, Emery and Street, 1960) firstly with a clearance of 0.02" (0.51 mm.) and then with one of 0.01" (0.25 mm.) led to improvement in the dispersion of the tissue and consequently in the localization of the succinic dehydrogenase activity.

Figure 2 illustrates the fractional centrifugation at 2°C to which pig skeletal muscle homogenates were subjected, the first fraction sedimenting at 850 x g for 10 minutes consisting of whole cells and other heavy debris. Two mitochondrial fractions have been isolated by centrifugation for 15 minutes at 5000 x g and 14000 x g These fractions contained only 2-3% and 0.5-2.0 % of the original protein respectively, overall protein recoveries being in the range of 90-95 %.

For the determination of the succinic dehydrogenase activities of the cellular fractions, the manometric method of Quastel and Wheatley (1938) was adopted; in this method potassium ferricyanide acts as the electron acceptor and H^+ ions simultaneously released upset the equilibrium of the CO_2/bicarbonate buffer with the production of CO_2 gas. In order to obtain maximum activity, the original homogenates and their fractions were frozen overnight at -20°C and allowed to thaw before use and a hypotonic medium was employed. Concentrations of

each fraction were chosen to maintain CO_2 output to within the range 2-5 µl./min. as far as possible.

On the basis that succinic dehydrogenase is exclusively located in the mitochondria, the debris fractions still contained up to 40 % of either unbroken cells or mitochondria, but activity in the heavy mitochondrial fractions was 6-10 times that of the original homogenate on the basis of protein content. Details of a typical fractionation procedure are presented in Table IV.

TABLE IV. The distribution of protein and succinic dehydrogenase activity on differential centrifugation of an homogenate of pig skeletal muscle (24 g.)

Fraction	Total protein mg.	Succinic dehydrogenase activity : µl. CO_2 per hr		Enrichment (Activities per mg. protein per hour)
		per g. original tissue	per mg. protein	
Homogenate	3240	6600	48	-
Debris (up to 850 x g for 10 min.)	1580	2570	36	x 0.75
Mitochondria - heavy (up to 5000 x g for 15 min.)	92	1600	375	x 7.8
Mitochondria - light (up to 14000 x g for 15 min.)	50	640	290	x 6.0
Supernatant	880	1140	29	x 0.6
Washings	318	990	69	x 1.4

% recovery of protein: 91 %

% recovery of activity: 105 %

In general, the recoveries of activity were of the order of 90 %, probably because of the difficulties involved in taking representative samples of the thick debris fraction, but in all cases the succinic dehydrogenase activity has been concentrated in the mitochondrial fractions.

Utilisation of nitrite by mitochondrial fractions

Both heavy and light mitochondrial fractions have been investigated for their capacities to utilise sodium nitrite in comparison with control determinations including these tissue preparations previously heat inactivated. Table V records the amounts of nitrite consumed anaerobically by preparations of pig skeletal muscle mitochondria over the course of one hour at 37° C and pH 6.7. The supporting medium contained hexokinase, which is lost during the separation of the mitochondria by centrifugation; this enzyme is necessary for the maintenance of oxidative phosphorylation. Pyruvate and fumarate were also added as substrates capable of supporting oxidative phosphorylation; a small quantity of fumarate is required in addition to pyruvate since the Krebs cycle does not proceed efficiently in its absence as the supply of oxaloacetate then becomes a limiting factor. The utilisation of nitrite in air by two mitochondrial preparations was 63 and 74 % of that observed anaerobically.

TABLE V. Nitrite utilisation by mitochondrial fractions

Fraction	Tissue protein present: mg.	Sodium nitrite available: µg.	Sodium nitrite utilised: µg.
Heavy mitochondria	1.9	12.5	5.4
" "	1.9		6.6
Heavy "	3.8	25.0	4.0
Light "	1.3		3.8
Heavy "	2.9		11
Light "	0.61		9.8
Heavy "	4.6		13
Light "	2.4		8.9

In the undamaged mitochondrion the process of electron transport, i.e. oxidation is accompanied by the concurrent esterification of inorganic phosphate into adenosine triphosphate, etc., so that the supply of high energy phosphate bonds is maintained. The utilisation of nitrite by mitochondria under conditions suitable for the maintenance

of oxidative phosphorylation thus provides further evidence of the association of nitrite metabolism with respiratory processes. It is hoped in future work to utilise mitochondria in studies of factors promoting nitrite utilisation and hence colour development.

SUMMARY

Under anaerobic conditions, both the utilisation of sodium nitrite by minces of fresh pig skeletal muscle and the production of a nitric oxide gas fraction arising from such incubations at pH 6.0 and 37° C were stimulated by reduced methylene blue.

The separation of mitochondria, the cellular component mainly associated with respiratory processes, from pig skeletal muscle has been accomplished by differential centrifugation of tissue homogenates at 2° C. Under conditions suitable for the maintenance of oxidative phosphorylation and using pyruvate and fumarate as substrates, such mitochondrial preparations have been found to metabolise sodium nitrite under anaerobic conditions.

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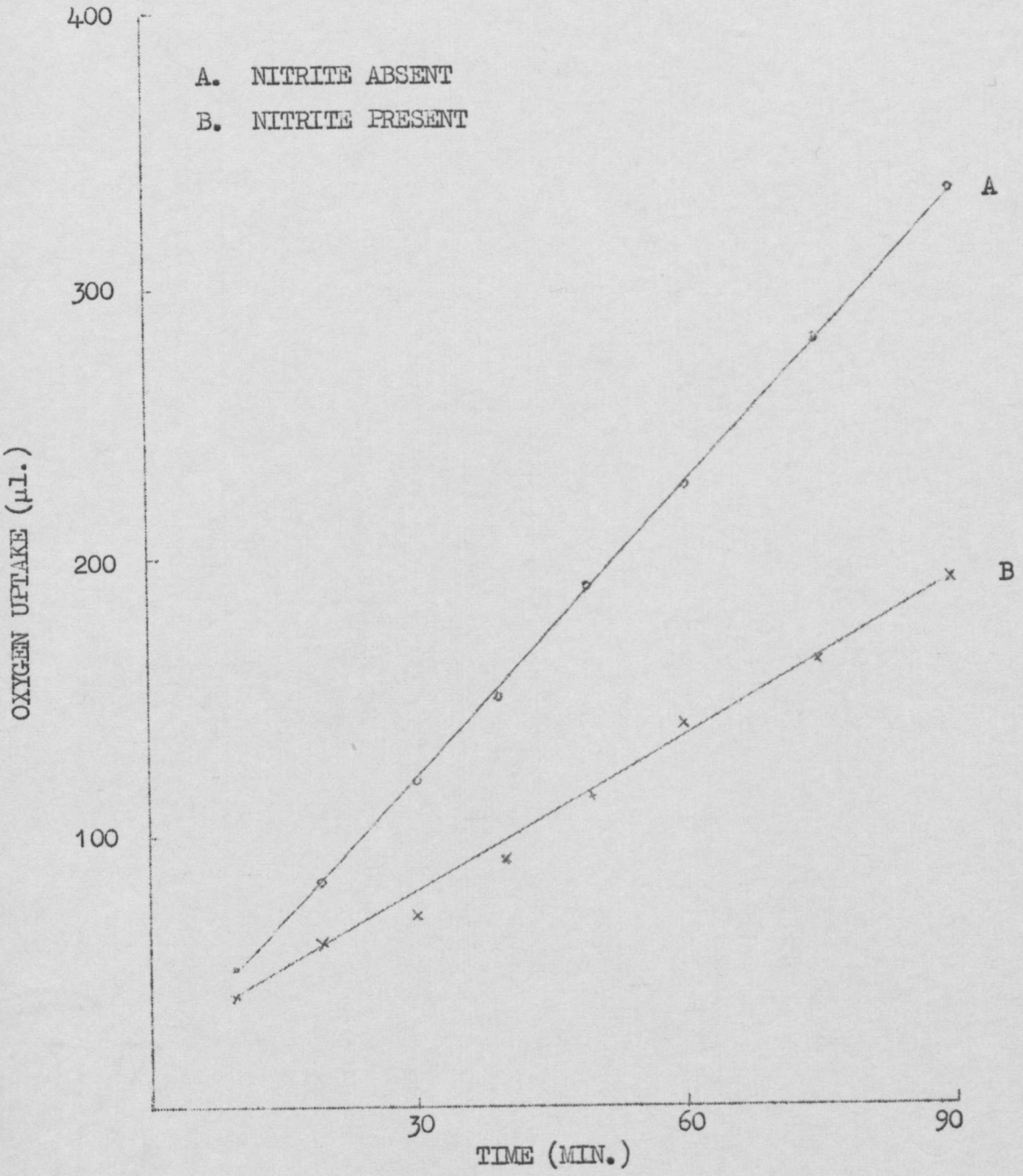

Fig. 1.

The respiration of a fresh mince of pig skeletal muscle at pH 6.0 with and without the presence of sodium nitrite (0.43 %)

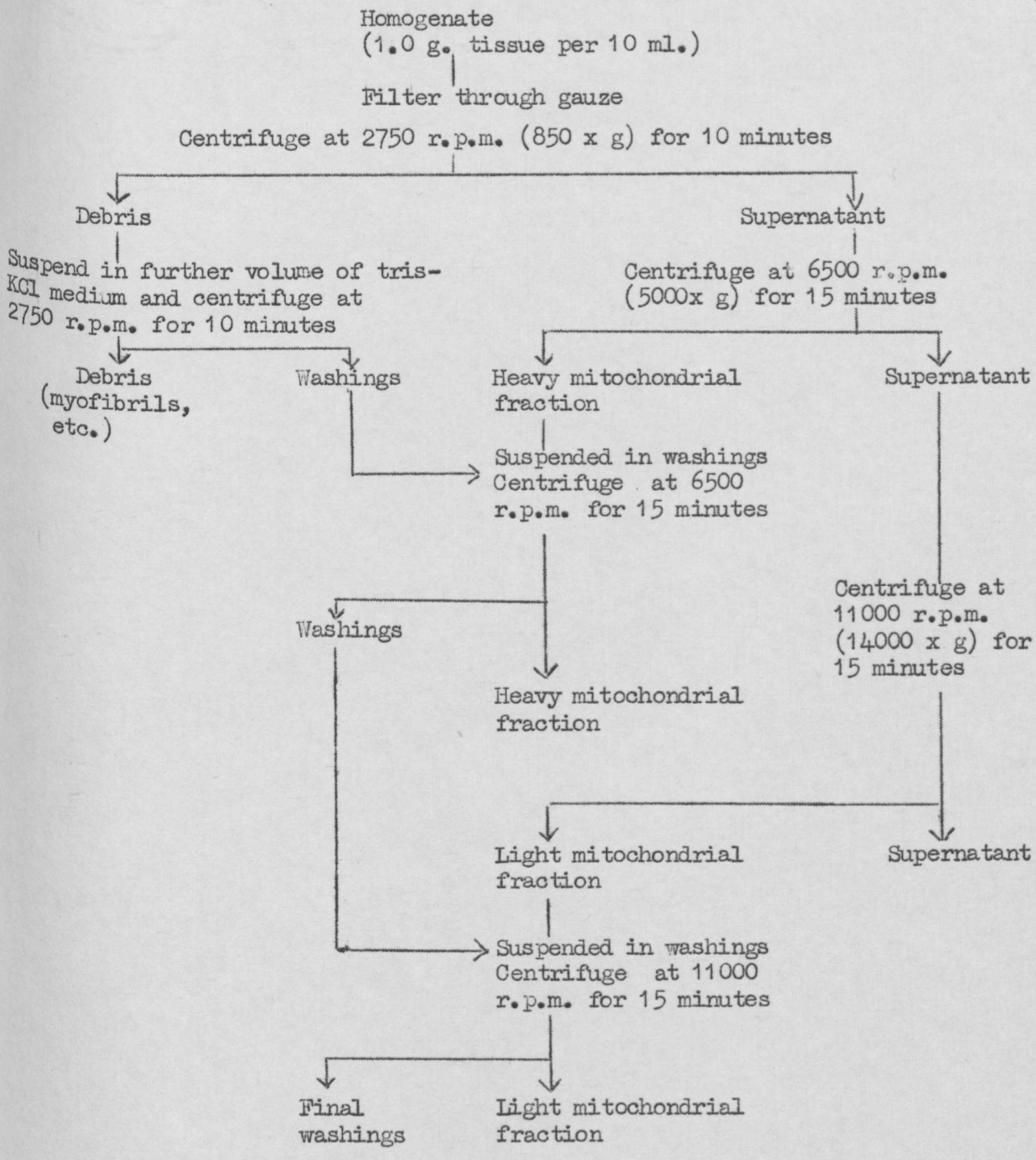

Fig. 2.

Flow sheet of pig muscle tissue fractionation by differential centrifugation at 2° C.