A new procedure for staining lipoproteins in ionographic separations

During very recent years there has been an upsurge of interest shown in the study of lipoproteins, largely because of their implication in the genesis of atherosclerosis. One of the most convenient ways of separating the α- and β-lipoproteins from blood is to fractionate serum ionographically[1]. In previous studies of lipids and lipoproteins by this method, in paper-stabilized electrolytes, the chief difficulties encountered were in the inadequate staining of the lipoprotein bands, in the retention of the stain by the paper itself, and in the undue length of time often required for the staining procedure,—sometimes a matter of several hours. By the new procedure described here, involving pre-staining of the lipids before application of the serum sample to the paper, all of these difficulties have been overcome.

Of the several stains which have been proposed for lipoproteins[1], the most favorable experience in this laboratory with the conventional method of staining the ionograms, namely, after a run, was obtained with Sudan Black B[2], or so-called acetylated Sudan Black B[3], which produce ionograms having dark blue bands corresponding to the areas covered by lipoproteins, against a light blue background. With the acetylated dye, the color intensity of the lipoprotein bands was increased and the background lightened, but the result still left much to be desired.

By the new procedure, the lipoproteins are pre-stained directly in the blood serum sample before application to the paper, as follows: To 1 ml of blood serum in a small test tube there is added slowly (to avoid precipitation) 0.1 ml saturated solution of acetylated Sudan Black B in 95% ethanol (or for smaller relative amounts, the same 10 to 1 ratio). The tube containing the serum is now shaken and allowed to stand at room temperature for 30 minutes (although even 3–5 minutes will yield fair results). The excess alcohol is evaporated by applying a suction to the tube and allowing a fine stream of nitrogen (or air) to be pulled over the serum through a thinly-drawn capillary tube.

Five microliters of the serum containing the stained lipoproteins are now applied from a micropipet to the filter paper (Whatman No. 1, 0.5 inch width) as a streak across the paper ribbon. The horizontal open-strip method, employing the Precision Ionograph, was used in making the separations. The other experimental conditions generally used for optimal results were: veronal buffer; pH, 8.6; ionic strength 0.05; potential gradient 6 volts/cm; atmosphere, water-saturated helium at 0–5° C, although runs in a simple water-saturated air atmosphere at room temperature were also quite satisfactory. An adequate separation is obtained in 4–5 hours. Both the α- and the β-lipoproteins migrate in the usual manner and appear as blue zones against a white background.

Since the dye is physically dissolved in the lipoproteins and is insoluble in water, there is no smearing of the colored zones. No dissociation or break-up of the colored materials was evident, as is observed when serum proteins are pre-stained with bromophenol blue[4]. This can be explained as follows: the Sudan Black B dissolves physically in the lipid-protein moiety whereas the protein-bromophenol blue complex involves an ionic bond and an equilibrium which can be upset due to the leaking away of the bromophenol blue ions from the complex, under the influence of the electric field. Using the same technique, except that the Sudan Black B was replaced by Sudan III, Sudan IV and Oil Red O, the results were much less satisfactory.

The new technique eliminates a long and tedious staining procedure, including washing of the ionogram, conserves rather costly reagents and yields crisp, well-demarked zones which enable the relative amounts of α- and β-lipoproteins to be determined more accurately.

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Some components of the oxidative phosphorylation system*

Attempts at fractionation of the mitochondrial phosphorylative oxidation system, outlined in this communication, have led to the identification of Mn^{+2} and cytochrome *c* as essential components in the over-all process. ERNSTER AND LINDBERG[1] have recently reported that the inhibition of coupled

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phosphorylation by Ca^{+2} is relieved by Mn^{+2}, and have concluded that Mn^{+2} is necessary for the system.

The Mn^{+2} requirement for phosphorylation becomes apparent after preincubation of the mitochondria in phosphate buffer (Table I). During the pretreatment a considerable amount of protein becomes soluble but the rate of succinate oxidation remains constant or sometimes increases slightly. The coupled phosphorylation is lost, but can be partially (50%) restored by Mn^{+2}. Activation of phosphorylation by Mn^{+2}, to a lesser extent, has been observed after other types of treatment.

TABLE I

Mn^{+2} REQUIREMENT FOR PHOSPHORYLATION

Expt.	*Mn^{+2}*	*Oxygen μ atoms*	*P/O*
1	None	14.7	0.00
	$1.7 \cdot 10^{-4}$ *M*	16.2	0.82
	$1.7 \cdot 10^{-4}$ *M* *	18.0	0.87
2	None	21.0	0.23
	$0.67 \cdot 10^{-4}$ *M*	19.5	0.73
	$1.7 \cdot 10^{-4}$ *M*	18.2	0.84
	$3.3 \cdot 10^{-4}$ *M*	18.6	0.82

The mitochondria were suspended in 0.03 *M* phosphate, pH 7.0, and incubated at 30° for 1 hour. The reaction mixture contained digested mitochondria (0.5 ml from 0.5 g rat liver), succinate (50 μmoles), Mg^{+2} (10 μmoles), ATP (5 μmoles), KF (20 μmoles), glucose (100 μmoles), hexokinase (0.05 ml) and phosphate (48 μmoles) in 3.0 ml of 0.25 *M* sucrose. The P/O ratios with the untreated mitochondria were 1.5 and 1.6, and the oxidation rates 14.7 and 14.2 in Expts. 1 and 2 respectively (20 min at 30°).

* Mn^{+2} was present during the pretreatment.

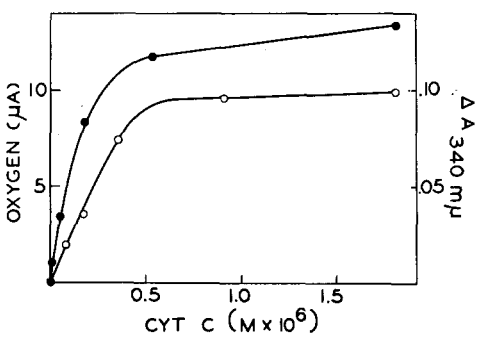

Fig. 1. *Effect of cytochrome* c *on the oxidation of succinate and DPNH.* Closed circles represent succinate oxidation measured in terms of μatoms of oxygen uptake, as in Table I, in the presence of $1.7 \cdot 10^{-4}$ *M* Mn^{+2}. Open circles represent DPNH oxidation measured by the decrease in absorbancy ($\log I_0/I$) at 340 mμ as follows. The reaction was started by adding the enzyme preparation (0.5 mg protein) to a mixture of phosphate (10 μmoles), DPNH (0.2 μmoles), $MgCl_2$ (5 μmoles) and cytochrome *c* in 3.0 ml at pH 7.2. The rate was measured at 30°. For the experiment with succinate the mitochondria were suspended in 0.001 *M* KCl for 30 minutes at 0° followed by washing with 0.12 *M* KCl. DPNH oxidation was measured with a preparation exposed to 0.005 *M* KCl and washed. For details see Table II.

When rat liver mitochondria, isolated in 0.25 μM sucrose[2], are exposed to distilled water[3] or preferably to low concentrations of salt, the endogenous cytochrome *c* becomes extractable with 0.12 *M* KCl. The only hemoprotein that is easily detectable in the KCl extract is indistinguishable spectroscopically from cytochrome *c*. The concentrations of cytochrome *c* necessary to restore the oxidation of succinate and DPNH are extremely low (Fig. 1) and of the same order of magnitude as the cytochrome *c* that is taken out by the washing. The washed residue, obtained under carefully controlled conditions, retains much of the phosphorylative activity coupled to succinate oxidation when supplemented with cytochrome *c* (Table II). Approximately one-half of the protein in the mitochondria is lost during the pre-treatment with no loss in potential oxidative activity. This system is apparently different from the DPNH oxidase purified from beef heart[4]. Our studies suggest that oxidative phosphorylation, under appropriate conditions, may be stable enough to be studied by classical methods of fractionation.

TABLE II

COUPLED PHOSPHORYLATION IN FRACTIONATED MITOCHONDRIAL RESIDUE

Pretreatment	*Protein mg*	*Cyt. c* $9 \cdot 10^{-7}$ *M*	*Oxygen µatoms*	*P/O*
None	11	—	16.9	>1.5
Dist. water	5.0	—	2.0	—
		+	10.6	0.71
0.005 *M* KCl	7.6	—	2.0	—
		+	16.7	1.19
0.001 *M* Phosphate, pH 7.0	5.1	—	1.2	—
		+	17.0	0.89
0.005 *M* Phosphate, pH 7.0	7.2	—	1.9	—
		+	17.0	1.14

The mitochondria in 0.25 *M* sucrose were centrifuged at 20,000 × *g*, and the residue was suspended in the above media for 5 minutes at 0°. One-tenth the volume of 2.5 *M* sucrose was then added and centrifuged. The residue was washed twice with 0.12 *M* KCl and resuspended in the *original* volume of sucrose. The phosphorylation coupled to succinate oxidation was measured as in Table I in the presence of $1.7 \cdot 10^{-4}$ *M* Mn^{+2}, with 0.5 ml of enzyme preparation. The protein concentration is indicated in the second column.

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Flavoproteins involved in the first oxidative step of the fatty acid cycle

It was recognized two years ago that the first oxidative step of the fatty acid cycle, the dehydrogenation of the saturated to the unsaturated fatty acyl CoA, is catalyzed by an enzyme system of the flavoprotein class[1-4]. Evidence was also available at that time that two distinct flavoproteins, one specific for substrates of short carbon chain and the other for substrates of intermediate or long carbon chain, are involved in this catalysis[1,2,5]. The former enzyme, a green copper-containing flavoprotein, had been obtained in high purity and was described in detail[6,7].

In attempts to isolate the flavoprotein specific for CoA derivatives of longer carbon chain it became apparent that altogether four flavoproteins are participating in the dehydrogenation of fatty acyl CoA's in pig liver. These four enzymes have now been separated from each other and obtained in a state of high purity. There is no evidence that additional enzymes are implicated in the primary dehydrogenation step. The functional relationships of the four flavoproteins are outlined in the following scheme, the arrows indicating direction of hydrogen transfer or electron flow:

$$\text{fatty acyl CoA} \begin{cases} \text{NC}_4\text{–C}_6 \longrightarrow \text{G} \searrow \\ \text{NC}_4\text{–C}_{16} \longrightarrow \text{Y}_1 \rightarrow \text{ETF} \longrightarrow \text{acceptors} \\ \text{NC}_6\text{–C}_{16} \longrightarrow \text{Y}_2 \nearrow \end{cases}$$

G is a green copper protein similar to that described earlier[6,7]. Y_1 and Y_2 are yellow flavoproteins. Y_1 has a broad specificity range[8], whereas Y_2 does not react significantly with butyryl CoA. G, Y_1 and Y_2 accept hydrogen from saturated fatty acyl CoA's. The coincident reduction of their prosthetic