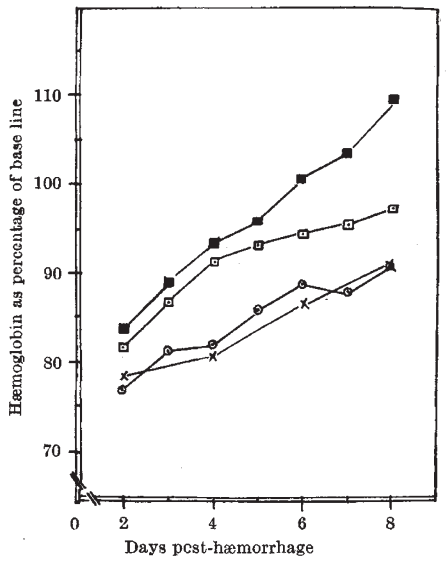

Fig. 1. Mean values for hæmoglobin concentration as a function of time, after a standard hæmorrhage (1 per cent body-weight), in the following groups. ×——×, 14 rabbits injected with isotonic sodium chloride; ⊙——⊙, 16 rabbits injected with normal rabbit plasma; ⊡——⊡, 9 rabbits injected with water extract of 'anæmic plasma'; ■——■, 4 rabbits injected with acetone extract of 'anæmic plasma'

The purified acetone extract of 'anæmic plasma' shows (Fig. 2) an absorption spectrum in the ultra-violet, similar to that of the extract of 'anæmic urine'. It is interesting to note that exposure to oxygen—which diminishes the activity of the extract—changes the ultra-violet absorption spectrum notably. However, further chemical purification is necessary before it can be determined whether the ultra-violet absorption spectrum is characteristic of the active factor in 'anæmic plasma' or is due to impurities.

Preliminary chemical studies, carried out on purified acetone extracts, show that these only give a positive colour reaction with sudan IV (fatty acid esters) and negative reactions with ninhydrin (amino-acids), mercuric nitrate reagent (purines), and ammoniacal silver nitrate (sugars) on Whatman No. 1 paper.

Our observations suggest that the factor isolated from 'anæmic plasma' is not identical with the 'erythropoietic hormone' isolated by Contopoulus *et al.*[4] from sheep anterior pituitary, which is probably a polypeptide.

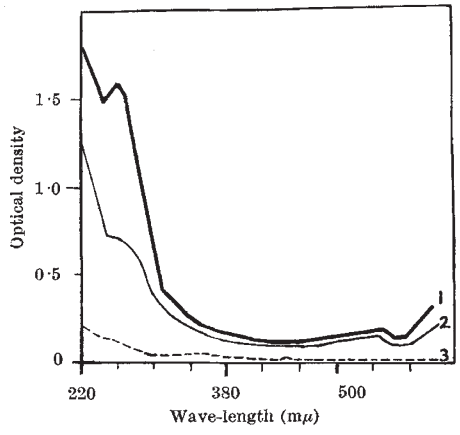

Fig. 2. Ultra-violet absorption curve of purified acetone extract of 'anæmic plasma'; curve 1 before, and curve 2 after, exposure to oxygen. Curve 3 refers to a blank experiment

Further details of the extraction processes and results of the biological tests will be published elsewhere. This work was aided by a grant from the Consejo de Investigación Científica de la Universidad de Concepción. We thank Messrs. A. Fernández and H. Varela for technical assistance.

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Reconstruction of Coupled Phosphorylation in the Succinic Oxidase System

Calcium ions are known to inhibit oxidative phosphorylation in isolated mitochondria. As reported previously[1], in the case of substrates oxidized via diphosphopyridine nucleotide, this inhibition can be reversed by the addition of the pyridine nucleotide, adenosine triphosphate and manganous ions. Treatment with the pyridine nucleotide alone partially restores only the respiration; but addition of all three factors is necessary in order to re-establish both oxidation and phosphorylation.

The phosphorylation accompanying the oxidation of succinate to fumarate is likewise inhibited by calcium treatment; but, since pyridine nucleotides are not involved in this reaction, the oxidation does not become dependent on the addition of the co-enzyme. Calcium treatment thus produces a mitochondrial system catalysing the single-step oxidation of succinate. The addition of adenosine triphosphate and manganous ions to such a system results in a coupled phosphorylation at a phosphorus to oxygen ratio (moles of esterified phosphate to atoms of oxygen consumed) of about unity.

Mitochondria were obtained from rat liver, homogenized in sucrose–versene (0·25 and 0·01 *M*, respectively) and washed once with the same solution. The preparation from one liver was suspended in 20 ml. of a saline-buffer solution (*p*H 7·5) containing potassium chloride, potassium phosphate, adenylic acid and glucose, in concentrations specified in Table 1. The suspension was supplemented with yeast

Table 1. Effect of Calcium Ions on the Oxidation of Different Substrates in Rat Liver Mitochondria

Each Warburg vessel contained: substrate, 30 micromol.; ortho-phosphate, 76 micromol.; adenylic acid, 5·7 micromol.; glucose, 200 micromol.; potassium chloride, 230 micromol.; magnesium chloride, 10 micromol.; yeast hexokinase (prepared and diluted as described in ref. 1), 0·1 ml.; calcium ions (where indicated), 1·15 micromol. *p*H 7·5. Final volume, 2·5 ml. Gas phase, air. Temp., 30° C. Substrate added from side-arm after 5 min. of incubation. Total incubation time, 29 min.

Substrate	Respiration (microatoms oxygen)	
	Calcium ions absent	Calcium ions present
α-Ketoglutarate	24·6	2·3
Succinate	28·7	21·7
Fumarate	12·4	1·0

Table 2. EFFECT OF ADENOSINE TRIPHOSPHATE AND MANGANESE IONS ON PHOSPHATE ESTERIFICATION COUPLED TO THE OXIDATION OF SUCCINATE TO FUMARATE IN RAT LIVER MITOCHONDRIA TREATED WITH CALCIUM IONS

Experimental conditions as in Table 1. Succinate (30 micromol.), adenosine triphosphate and manganese ions added from side-arm after 5 min. incubation. Total incubation time, 25 min.

Additions: at 0 time	Additions: after 5 min.		Phosphorus to oxygen ratio
Calcium ions (1·15 μmol.)	Manganese ions (1·5 μmol.)	Adenosine triphosphate (μmol.)	
–	–	–	1·70
+	–	–	0·12
+	–	0·1	0·07
+	–	0·2	0·07
+	–	0·5	1·03
+	–	1·0	1·02
+	+	–	0·05
+	+	0·1	1·10
+	+	0·2	1·17
+	+	0·5	1·12
+	+	1·0	1·09

hexokinase and magnesium chloride and diluted with isotonic potassium chloride to 25 ml. 2 ml. of this suspension was pipetted into Warburg vessels and incubated for 5 min., before addition of the substrate from the side-arm.

If this pre-incubation is performed in the presence of calcium ions (5×10^{-4} *M*), the system loses its ability to oxidize those substrates requiring the participation of pyridine nucleotides, while it is still capable of oxidizing succinate. This is illustrated in Table 1. The nearly theoretical molar relationship between succinate and oxygen consumed (Fig. 1) substantiates the fact that we are here dealing with the single-step oxidation of succinate to fumarate.

In the above system no esterification of orthophosphate accompanies the one-step oxidation of succinate (Table 2). The effect of added adenosine triphosphate and manganous ions is shown in this table. Adenosine triphosphate alone, when added in sufficiently high concentration (the factors determining this critical concentration of adenosine triphosphate are under further investigation), induces a phosphorylation at a maximum phosphorus to oxygen ratio of about unity. Manganous ions alone, at the concentration tested, had no effect; but the involvement of manganese in the phosphorylative mechanism is indicated by the fact that it potentiates the effects of limiting concentrations of adenosine triphosphate.

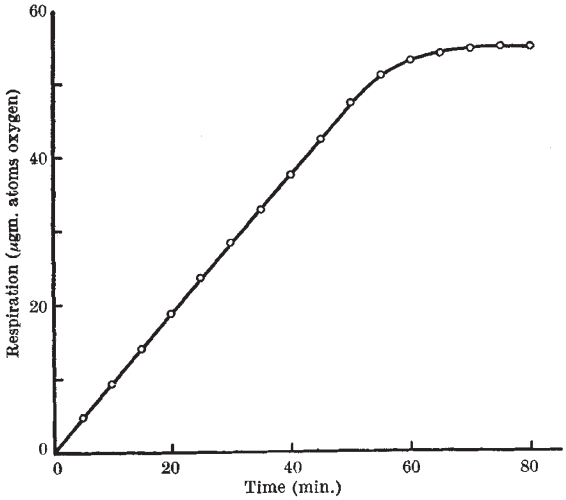

Fig. 1. One-step oxidation of succinate in rat liver mitochondria treated with calcium ions. Added succinate, 50 micromol. Other experimental conditions as in Table 1

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Inactivation and Activation of Liver Phosphorylase

THE level of phosphorylase in liver slices represents a balance between inactivation and resynthesis of the active form; epinephrine and glucagon rapidly increase the amount of active phosphorylase in pre-incubated liver slices[1,2]. In order to study the nature of the change in the phosphorylase molecule, liver phosphorylase and the enzyme inactivating it from dog liver have been prepared in purified form. The liver phosphorylase was purified approximately 400-fold, a value higher than previously reported[3]; this purified enzyme was very soluble in water and when inactivated enzymatically showed little or no activity when tested in the presence of adenylic acid. The inactivating enzyme from dog liver was also purified about 400-fold, and this purified enzyme was sufficiently active for microgram quantities to be used in most experiments. Preliminary tests using several proteins as possible substrates have revealed no proteolytic activity.

In searching for possible small fragments released during inactivation, liver phosphorylase inactivated enzymatically was dialysed and the dialysates were analysed. Phosphate was found in the dialysate of liver phosphorylase fractions incubated with the inactivating enzyme, and identical values were found when measurements were made for stable phosphate, or for phosphate as determined by the method of Fiske and SubbaRow[4], or by the Lowry–Lopez method[5]. The phosphate present in the liver phosphorylase was not released when liver phosphorylase was precipitated with cold trichloroacetic acid, so subsequent analyses were conducted by sampling reaction mixtures into trichloroacetic acid and measuring phosphate released enzymatically in the trichloroacetic acid supernatant. Fig. 1 shows the results of such an experiment where the enzymatic inactivation of liver phosphorylase is accompanied by a release of phosphate. 9·5 mgm. of liver phosphorylase in 1·3 ml. of water was incubated with and without 0·038 mgm. of inactivating enzyme (IE), which had been purified approximately 400-fold. At the times indicated along the solid lines, 0·01-ml. aliquots were removed and were assayed for liver phosphorylase activity in a test system which contained adenosine-5-phosphate. At the times indicated along the broken lines, 0·2-ml. aliquots were treated with trichloroacetic acid, and the supernatants after centrifugation were analysed for phosphate. Results are expressed as micrograms of phosphorus released per 9·5 mgm. of liver phosphorylase. The inactivating enzyme alone released no measurable amount of phosphate into trichloroacetic acid supernatants.

Using both the dialysis and trichloroacetic acid precipitation techniques, about 0·25 μgm. phosphorus was found to be released per milligram of liver phosphorylase, or 31 gm. phosphorus per 124,000 gm.