From the data thus far obtained it may be concluded that the pink protein possesses a molecular weight of the order of 100,000; the numerical differences between the values found for Fractions *A* and *B* are not considered to be significant. It might be mentioned that the sedimentation constant of Fraction *B*, when examined at 0.285% concentration, was found to be 4.6 *S*, indicating a small or negligible concentration dependence.

As judged by all available criteria, the pink protein represents a well-defined chemical compound; *viz.*, a cobalamin protein, rather than an adsorption complex. The vitamin B_{12} content is compatible with the assumption that each molecule of the conjugated protein contains one cobalamin residue. The protein moiety appears to be identical with the so-called "vitamin B_{12}-binding factor" present in hog gastric mucosa, and probably also in human gastric juice. Colour reactions performed on paper strip electrophoresis diagrams of Fraction *B* as well as the presence of hexosamine among the hydrolysis products of Fraction *A*, lend support to the assumption that this material is a mucoprotein.

From the absorption spectrum one may conclude that the cyano-group most probably is still present in the molecule. This would indicate that the cobalamin protein described herein is a substance that as such probably does not occur in nature under physiological conditions (cobalamin in normal food being linked to polypeptides or proteins rather than to a cyano-group).

The clinical activity in pernicious anemia patients in relapse upon daily oral administration, was followed at various stages throughout the purification. Partially purified preparations from which Fractions *A* and *B* were obtained showed such clinical activity; *Fraction I.F. 762*, the absorption spectrum of which is given in Fig. 1, and which has a vitamin B_{12} content of 3.7 μg per mg as determined with *L. Leichmannii*, provided a very good clinical response in two patients given doses of 2.0 and 3.0 mg per day respectively.

Fractions *A* and *B*, however, failed to show any clinical effect after the daily oral administration of amounts corresponding to 5 μg of bound vitamin B_{12}.

Although it would thus appear that the cobalamin protein herein described does not, *by itself*, exhibit hematopoietic activity, the question whether it represents a *component* of Castle's "Intrinsic Factor" is at present under investigation.

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EVIDENCE FOR A NEW INTERMEDIATE IN THE PHOSPHORYLATION COUPLED TO α-KETOGLUTARATE OXIDATION*

by

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The oxidation of KG** to succinyl CoA (reaction 1)[1] has been linked to the phosphorylation of ADP (reaction 2)[2,3]. The phosphorylation system has now been resolved into two separate enzymes,

$$KG + DPN^+ + CoA \rightarrow \text{Succinyl CoA} + DPNH + H^+ + CO_2 \quad (1)$$

$$\text{Succinyl CoA} + ADP + P_i \rightleftarrows \text{Succinate} + CoA + ATP \quad (2)$$

designated A and B, which catalyze consecutive reactions and involve a new intermediate. Enzyme preparation A is prepared from an acetone powder extract of beef heart mitochondria or of washed pig kidney residue by fractionation with ammonium sulfate and ethanol. Preparation B is obtained from an acetone powder extract of beef heart mitochondria. The activity of the individual and combined preparations in two different assays is shown in Table I.

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** The following abbreviations will be used: KG for α-Ketoglutarate, CoA or CoA–SH for coenzyme A, ADP for adenosine diphosphate, ATP for adenosine triphosphate, P_i for orthophosphate, DPN for diphosphopyridine nucleotide, THAM for trishydroxymethyl aminomethane, and –SH for sulfhydryl.

TABLE I

SEPARATION OF THE PHOSPHORYLATION SYSTEM INTO TWO ENZYMES

DPNH assay		*CoA-SH assay*	
Enzyme	*Δ D/min*	*Enzyme*	*Δ-SH/10 min μmoles*
A (0.17 mg)	0.006	A (0.042 mg)	0.014
B (0.85 mg)	0.000	B (0.55 mg)	0.000
A + B	0.142	A + B	0.180

The DPNH assay is carried out spectrophotometrically as previously described[3] and represents the rate of continued DPN reduction. For the CoA-SH assay, 0.32 μmoles of reduced CoA, 1 μmole of KBH_4, 10 μmoles of succinate, 1 μmole of ATP and 3 μmoles of THAM in 0.25 ml at pH 7.5 were incubated for 10 minutes at 30°. The -SH-disappearance, resulting from the synthesis of succinyl CoA, was determined by the nitroprusside reaction[4].

The first reaction in the phosphorylation sequence, catalyzed by enzyme A alone, can be demonstrated spectrophotometrically. In Fig. 1, curve 1, a small increment in the amount of DPNH is produced on addition of enzyme A after reaction 1 is completed. This change is consistent and

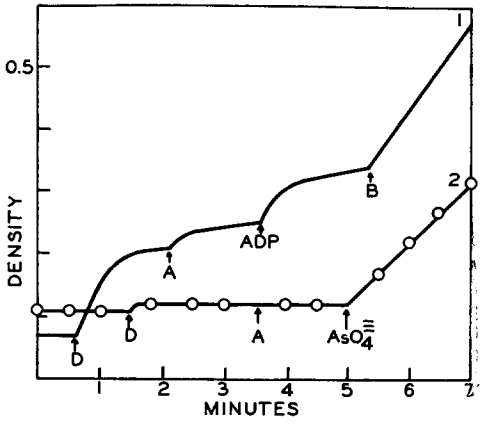

Fig. 1. Curve 1 — 0.01 ml of KG dehydrogenase[6] was added at the time shown by arrow D to a mixture of 5 μmoles of KG, 0.17 μmole of CoA, 10 μmoles of cysteine, 1 μmole of DPN, 10 μmoles of $MgCl_2$ and 100 μmoles of phosphate in 3.0 ml at pH 7.4. The other arrows indicate addition of enzyme A (0.17 mg), ADP (2 μmoles) and enzyme B (0.85 mg) in the order shown. The density at 340 mμ was followed continuously in the automatically recording Beckman spectrophotometer. Curve 2 — The reaction mixture was as above except that 20 μmoles of THAM instead of phosphate, 0.02 μmoles of CoA and 0.85 mg of enzyme A were used. Arsenate (40 μmoles) was added at 5 minutes.

reproducible, and depends upon the presence of P_i and Mg^{++} in the medium. A more pronounced increase in DPNH is produced when ADP is subsequently added. The amount of DPNH thus formed is a function of ADP concentration. The DPN reduction is resumed on adding enzyme B, and, with limiting concentrations of ADP, proceeds until all the ADP is consumed. The liberation of CoA in the presence of ADP and enzyme A (implied by the DPN reduction in Fig. 1) has been directly confirmed with succinyl CoA[5] by the nitroprusside reaction. This reaction also suggests the coincident formation of another product which cannot be ATP since coupling with the hexokinase system requires enzyme B also. Furthermore, succinate and ATP do not influence the initial rate of DPN reduction with enzyme A and ADP although the overall reaction with B also present is inhibited strongly.

Enzyme A catalyzes the arsenolysis of succinyl CoA (Fig. 1, curve 2) producing continued DPN reduction without ADP and enzyme B. Mg^{++} is required for the reaction and there is a constant proportionality between the rate of arsenolysis by enzyme A and its activity in the DPNH assay. The stoichiometry of the arsenolysis agrees with equation 3. It must be pointed out that enzymes

$$\text{Succinyl CoA} \xrightarrow[\text{Arsenate}]{\text{A}} \text{Succinate} + \text{CoA-SH} \qquad (3)$$

A and B are not interchangeable in any of the above reactions.

The reaction catalyzed by enzyme A may be due to a trace of contaminant in commercial ADP. However, rate measurements indicate that the intermediate formed by enzyme A is an essential link in the sequence for ATP formation.

REFERENCES

[1] D. R. SANADI AND J. W. LITTLEFIELD, *J. Biol. Chem.*, 201 (1953) 103.
[2] S. KAUFMAN, C. GILVARG, O. CORI AND S. OCHOA, *J. Biol. Chem.*, 203 (1953) 869.
[3] H. HIFT, L. OUELLET, J. W. LITTLEFIELD AND D. R. SANADI, *J. Biol. Chem.*, 204 (1953) 565.
[4] R. R. GRUNERT AND P. H. PHILLIPS, *Arch. Biochem.*, 30 (1951) 217.
[5] E. J. SIMON AND D. SHEMIN, *J. Am. Chem. Soc.*, 75 (1953) 2520.
[6] D. R. SANADI, J. W. LITTLEFIELD AND R. M. BOCK, *J. Biol. Chem.*, 197 (1952) 851.

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INFLUENCE OF THIOURACIL INCORPORATION IN THE RIBONUCLEIC ACID MOIETY OF TOBACCO MOSAIC VIRUS ON ITS MULTIPLICATION

by

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We have recently shown[1] that the ribonucleic acid of tobacco mosaic virus can incorporate i mportant amounts of thiouracil in the form of an unidentified component, chromatographically different from thiouracil and from the normal constituents of ribonucleic acid. At the same time, MATTHEWS[2] gave evidence for the similar incorporation of 8-azaguanine in the ribonucleic acid of the same virus.

Tobacco mosaic virus[3], as well as turnip yellow mosaic virus[4], is not infective if it does not contain ribonucleic acid; this suggests that the ribonucleic acid of plant virus plays an essential role in their multiplication, as does desoxyribonucleic acid in the case of bacteriophage[5]. A change in the composition of ribonucleic acid obtained by incorporation of thiouracil could be expected to have a marked influence on the multiplication of tobacco mosaic virus; the following experiment was thus attempted.

Ten tobacco leaves are used for each experiment. Each leaf is divided longitudinally along its principal vein in two halves. One half is infected with a solution of virus cultured *in vitro* according to the technique of COMMONER[6]. The other half is infected with a solution of virus of the same concentration, cultured simultaneously and purified by the same method; but in this virus 10–12% of the uracil present in the ribonucleic acid has been replaced by thiouracil. The half leaves thus infected are washed and placed for 3 to 8 days on the surface of VICKERY's nutrient medium. The newly formed virus is extracted quantitatively and titrated either by addition of an antiserum under conditions of maximum precipitation, or by the method described by COMMONER[7]. The two methods give comparable results. Those obtained by the immunological method are described in the following table. The amount of precipitated protein is always smaller in the case of leaves infected with thiouracil-containing virus. The fall in concentration of infective particles, which was to be expected from the presence of thiouracil, was determined by comparing these results with those of parallel experiments in which solutions of normal virus were applied, at various dilutions, on physiologically comparable leaves and in which the newly formed virus was titrated as above. An example of the results is given in Fig. 1. As can be seen in the last column of Table I, the fall in concentration amounted to 28–92%.

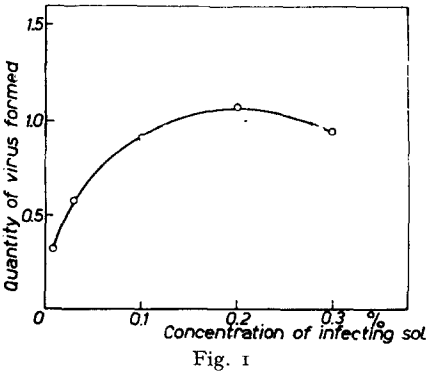

Fig. 1

It should be possible to demonstrate such an important drop in the number of infective particles by applying the virus solution on leaves of *Nicotiana glutinosa* and counting the number of lesions produced. However COMMONER[7] has stated that virus cultivated in the presence of thiouracil retains a normal infectivity when submitted to this test. Thus it appears that a particle of virus which has incorporated thiouracil in its ribonucleic acid remains infectious. Our results show, on the other hand, that the amount of virus it can produce in a short time is considerably reduced. This apparent