phate ⟷ ATP + sulfate, would be most favorable to test for. This reaction was found to be catalyzed by yeast as well as liver extract. In Table I, the separation is shown of the responsible enzyme, ATP-sulfurylase, from APS-kinase, the latter catalyzing the eventual production of PAPS. In addition to the sulfurolysis of ATP, the yeast extract, as shown in the third column of Table I, contains a different enzyme that catalyzes sulfurolysis of ADP, a reaction somewhat analogous to Ochoa's nucleotide diphosphate-linked ribonucleic acid synthesis. Since in the equilibrium

$$\text{ATP} + \text{sulfate} \rightleftarrows \text{APS} + \text{pyrophosphate} \quad (1)$$

TABLE I

SEPARATION OF ATP-SULFURYLASE AND APS-KINASE

Separation of Yeast Enzymes.—National bakers' yeast was used and extracts were prepared essentially according to the method of Jones, *et al.*[7] The formation of ATP from APS was measured by following pyrophosphate disappearance or by measuring ATP formation with hexokinase and glucose-6-phosphate dehydrogenase.[8] PAPS formation was followed by transfer to nitrophenol or by PAP assay. The PAP assay depends on the catalytic activity of PAP in the transfer of sulfate from *p*-nitrophenol to phenol. The rate of nitrophenol formation is measured at 400 mμ in the Beckman DU spectrophotometer.[9] The reaction between APS and P_i was routinely followed by measuring the disappearance of P_i with chromatographic checks on ADP formation.

Fraction	ATP-sulfurylase (APS + PP → ATP) −PP, μM./mg./hr.	APS-kinase (APS + ATP → PAPS) PAPS, μM./mg./hr.	ADP-sulfurylase (APS + P_i → ADP) $-P_i$, μM./mg./hr.
Dialyzed extract, I	2.7	...	1.8
NaCl precipitate, II	10.1	0.55	5.8
17–23% EtOH, III	22.1	3.5	3.0
pH 5.4 precipitate, IVa	85.0	0.6	0
Supernate + 10% EtOH, IVb	0.5	4.1	
40–50% $(NH_4)_2SO_4$, V	0	12.5	

thermodynamically the reverse reaction is favored, the forward reaction as well as the over-all synthesis of PAPS is considerably enhanced by the removal of pyrophosphate by pyrophosphatase. As shown in Table II, in the absence of the APS-kinase, APS can form only on addition of pyrophosphatase. Even so, the yield is very low when compared with the ease with which APS and pyrophosphate reverse to ATP (Table I).

The removal of APS through APS-kinase

$$\text{APS} + \text{ATP} \longrightarrow \text{PAPS} + \text{ADP} \quad (2)$$

likewise favors sulfurolysis, yielding PAPS also in the absence of pyrophosphatase. But the addition of pyrophosphatase still considerably enhances the overall reaction (Table III). Pyrophosphate inhibits strongly (*cf.* also Segal[10]). The equilibrium

(7) M. E. Jones, S. Black, R. M. Flynn and F. Lipmann, *Biochim. Biophys. Acta*, **12**, 141 (1953).

(8) A. Kornberg and W. E. Pricer, Jr., *J. Biol. Chem.*, **193**, 481 (1951).

(9) J. D. Gregory, unpublished method.

(10) H. L. Segal, *Biochim. Biophys. Acta*, **21**, 194 (1956).

TABLE II

ENZYMATIC FORMATION OF APS FROM ATP

The complete incubation mixture contained: 100 μM. tris-(hydroxymethyl)-aminomethane buffer, *p*H 7.5; 5 μM. ATP; 5 μM. $MgCl_2$; 20 μM. K_2SO_4; 10 γ crystalline pyrophosphatase; and 1.0 mg. Fraction IVa, Table I, ATP-sulfurylase, in a final volume of 1 ml. Incubation was for 30 minutes at 37°. The reaction was stopped by heating in a boiling water-bath for 90 seconds. After removing the denatured protein, aliquots were taken for APS assay. APS was determined by conversion to PAPS with APS-kinase in the presence of ATP and $MgCl_2$.

System	APS, μM./ml.
Complete	0.23
No sulfurylase	0
No pyrophosphatase	0.01
No $SO_4^=$	0

TABLE III

PYROPHOSPHATE INHIBITION OF OVER-ALL ACTIVATION REACTION,

$$2\text{ATP} + SO_4^= \longrightarrow \text{PAPS} + \text{ADP} + \text{PP}$$

The basic incubation mixture contained: 100 μM. tris-(hydroxymethyl)-aminomethane buffer, pH 8.5; 10 μM. ATP; 5 μM. $MgCl_2$; 20 μM. K_2SO_4; 100 γ Fraction IVa, Table I, ATP-sulfurylase; and 100 γ Fraction V, APS-kinase, in a final volume of 1 ml. Incubation at 37°. The reaction was stopped by heating in a boiling water bath for 90 seconds. PAPS concentration was determined by means of the PAP assay, as described in Table I.

	PAPS, μM./ml./hr.
Basic	0.27
Basic + 10 γ pyrophosphatase/ml.	0.81
Basic + 1 μM. pyrophosphate/ml.	0.02

concentration of APS in the over-all system should therefore be rather low. Accordingly, the affinity of the second enzyme for APS was found to be high. It may be significant that the highest rate of PAPS formation is observed with 10^{-5} *M* APS while higher concentrations cause inhibition. Both enzymes, ATP-sulfurylase and APS-kinase, require as cofactors Mg^{++} or other divalent cations.

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A ROLE OF BIOTIN IN THE INTERACTION OF ORNITHINE AND CARBAMYL PHOSPHATE

Sir:

The loss in ability of rat liver homogenates to convert ornithine, bicarbonate and ammonium ion to citrulline as a result of biotin deficiency has been reported to be restored by supplements of N-carbamylglutamate but not by biotin.[1,2] N-Carbamylglutamate has a role in the conversion of carbonate and ammonium ion to a compound X which interacts with ornithine to form citrulline.[3] Evidence that compound X is carbamyl phosphate

(1) P. R. MacLeod, S. Grisolia, P. P. Cohen and H. A. Lardy, *J. Biol. Chem.*, **180**, 1003 (1949).

(2) G. Feldott and H. A. Lardy, *ibid.*, **192**, 447 (1951).

(3) S. Grisolia and P. P. Cohen, *ibid.*, **198**, 561 (1952).

has been recently presented.[4,5] These various results would suggest that biotin has a role in the synthesis of a catalytic factor necessary for the formation of carbamyl phosphate.

In the present investigation, biotin deficiency in *Streptococcus lactis* 8043 has been found to result in greatly diminished ability to convert carbamyl phosphate and ornithine to citrulline, so that a role of biotin is indicated in the utilization of carbamyl phosphate for citrulline synthesis.

Cells of *S. lactis* were harvested after 24 hours of growth at 30° in a previously described medium[6] containing 200 mγ per ml. of calcium pantothenate and either 2 mγ or 0.01 mγ of biotin per ml. The cells were washed twice in fresh medium which was modified by eliminating arginine, purines, pyrimidines and biotin and adjusted to *p*H 8. To obtain cell-free preparations, the cells were suspended in the wash medium, 0.5 mg. of cells per ml., and exposed to sonic oscillations for 40 minutes. The disrupted cell suspensions were centrifuged at 5000 g. to obtain the cell-free supernatant as the enzyme preparation.

For assay, 0.1 ml. of this cell-free preparation (derived from 0.05 mg. of whole cells) was incubated for 2 hours at 30° with carbamyl phosphate (dilithium salt), 20 μM: magnesium chloride, 2.5 μM.; DL-ornithine, 20 μM.; and tris-(hydroxymethyl)-aminomethane buffer, 10 μM. at *p*H 8, in a total volume of 1 ml. The amount of citrulline produced was determined by a previously described method.[7] From the results (Table I), it is apparent that enzyme preparations from biotin deficient cells have considerably less ability to convert ornithine and carbamyl phosphate to citrulline than preparations from normal cells. In order to show that the decrease of activity of the biotin-deficient preparations was the result of biotin deficiency and not of reduced growth, cells were harvested after growth in medium in which the pantothenic acid concentration was lowered from 200 mγ to 2 mγ per ml. so that only half-maximal growth was obtained. Cell-free enzyme preparations from the pantothenic acid deficient cells showed no decrease in ability to form citrulline from carbamyl phosphate and ornithine.

TABLE I

EFFECT OF BIOTIN DEFICIENCY ON CITRULLINE SYNTHESIS

Cell-free enzyme preparations	Additional supplements	Per cent. conversion of ornithine to citrulline[c]
I[a]	None	7.7
I	N-Carbamyl-L-glutamic Acid (20 μM.)	9.2
I	N-Acetyl-L-glutamic Acid (20 μM.)	8.8
I	Biotin (1 γ)	7.7
I	Heated II (0.5 ml.)	7.2
II[b]	None	60.0

[a] I derived from cells cultured in a medium deficient in biotin (0.01 mγ/ml.). [b] II derived from cells cultured in a medium containing an excess of biotin (2 mγ/ml.). [c] Based on L-ornithine.

Supplements of biotin, biocytin, N-carbamylglutamic acid, N-acetylglutamic acid, glutamine, bicarbonate and adenosine triphosphate singly or in various combinations did not restore normal activity to the biotin-deficient preparations. Also, addition of a heated cell-free preparation from normal cells, hot water extract of pig liver or a heated residue of rat liver homogenate similarly failed to enhance the activity of the deficient preparations. Although biotin deficient cell-free preparations were not activated by these supplements, normal activity could be restored to biotin deficient cells in a biotin supplemented growth medium in a few hours.

Whether biotin functions directly as a component or indirectly in the synthesis of the enzyme system and whether a common factor related to biotin is necessary for both the formation and transfer of a carbamyl group remains to be determined.

(4) M. E. Jones, L. Spector and F. Lippmann, THIS JOURNAL, **77**, 819 (1955).

(5) J. M. Lowenstein and P. P. Cohen, *J. Biol. Chem.*, **220**, 57 (1956).

(6) J. M. Ravel, L. Woods, B. Felsing and W. Shive, *ibid.*, **206**, 391 (1954).

(7) R. M. Archibald, *ibid.*, **156**, 121 (1944).

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PARAMAGNETISM OF THE SYSTEM S_8–SO_3–H_2O

Sir:

Yellow to blue solutions are formed when sulfur is dissolved in oleum,[1] and a blue solid, known as sulfur sesquioxide, arises when sulfur reacts with anhydrous liquid sulfur trioxide.[2] Recent evidence[3] indicates that the blue solid is a molecular compound with the formula $(SO_3.S)_x$. We have found that both the colored solutions and the blue solid are paramagnetic and contain at least two different paramagnetic species. The paramagnetism was detected by means of a paramagnetic resonance spectrometer operating at a wave length of 3.2 cm.[4]

Both the colored solutions and the blue solid in an excess of sulfur trioxide gave resonance spectra consisting of two well-resolved lines. For 0.01 *M* solutions of S_8 in 30% oleum, the total paramagnetic resonance intensity corresponds very roughly to ten unpaired electrons per S_8 molecule; but for 1 *M* S_8 in 30% oleum the intensity corresponds to only of the order of one unpaired electron per hundred S_8 molecules. The line widths (defined as the separation in gauss between inflection points of the absorption curve) and spectroscopic splitting factors (*g*-values) were the same for all the systems investigated. The low field line (I) had a width of 4 gauss and a *g*-value of 2.026 ± 0.003; and the high field line (II) had a width of 8 gauss and a *g*-value of 2.016 ± 0.003. The assignment of these two lines to two different species is justi-

(1) W. Ostwald and R. Auerbach, *Kolloid-Z.*, **38**, 336 (1926).

(2) L. Wohler and O. Wegwitz, *Z. anorg. allgem. Chem.*, **213**, 129 (1933).

(3) R. Appel, *Naturwissenschaften*, **40**, 509 (1953).

(4) J. M. Hirshon and G. K. Fraenkel, *Rev. Sci. Instr.*, **26**, 31 (1955).