been observed in eight independent experiments, and records taken at intervals of pressure and time show with certainty that the transformation is reversible.

The specific volume of $CaCO_3$-III at 18.7 ± 0.5 kb and 25°C is 0.315 ± .002 cm^3/g. Taking the specific volume of phase II at 15.5 ± 1 kb (transformation pressure I–II) and the same temperature to be 0.360 ± .002 cm^3/g the value for Δv is −0.045 cm^3/g. This is the volume change for the II–III transition as well as that due to any compression that may occur in the interval between 15.5 and 18.7 kb. However, Bridgman (*1*) determined the II–III decrement alone to be −0.00956 cm^3/g and, because of the unknown compressibility in the interval 15.5 to 18.7 kb, a direct comparison of the two decrements cannot be made.

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Tryptophan Synthetase from Neurospora: A Modification in the Reaction Scheme

Abstract. *In addition to indole-3-glycerolphosphate, an unreported reaction product has been detected in incubation mixtures containing tryptophan synthetase from* Neurospora, *indole, and glyceraldehyde-3-phosphate. This product has been tentatively identified as an indole derivative. Since this compound is not formed in incubation mixtures initially containing only enzyme and indole-3-glycerolphosphate, it was concluded that indole-3-glycerolphosphate synthesis and breakdown are not simply the forward and reverse components of a reversible reaction.*

Tryptophan synthetase from both *Neurospora crassa* and *Escherichia coli* has proven extremely useful in genetic and enzymatic studies (*1*, *2*). In interpreting the relation between gene and enzyme in these systems it is important that all the reactions catalyzed by tryptophan synthetase be well understood. The reactions catalyzed by this enzyme are believed to be:

Indole-3-glycerolphosphate plus L-serine → L-tryptophan plus D-glyceraldehyde-3-phosphate. (1)

Indole + L-serine → L-tryptophan + water. (2)

Indole-3-glycerol phosphate $\underset{(R)}{\overset{(F)}{\rightleftharpoons}}$ indole plus D-glyceraldehyde-3-phosphate (3)

where F and R mean forward and reverse. Reactions 1 and 2 require pyridoxal phosphate and essentially are irreversible. Reaction 3, the reaction considered in this report, was believed to be reversible, with the forward reaction (3F) accelerated by pyridoxal phosphate and the reverse reaction (3R), by pyridoxal phosphate and L-serine. Garrick and Suskind (*3*) have noted discrepancies in reaction 3 which are not readily compatible with its formulation as simply the reversible interconversion of indole-3-glycerolphosphate to indole plus D-glyceraldehyde-3-phosphate. The detection of an indole derivative is reported here for reaction mixtures which initially contain tryptophan synthetase, indole, and glyceraldehyde-3-phosphate (reaction 3R), but not for mixtures that initially contain the enzyme and indole-3-glycerolphosphate (reaction 3F).

A lack of stoichiometry between the disappearance of indole and the formation of indole glycerolphosphate (reaction 3R) had been noted earlier for extracts of *Neurospora* tryptophan synthetase mutant td_2 (*4*) and for *Escherichia coli* extracts (*5*). Garrick and Suskind (*3*), after observing different rates for tryptic inactivation of reactions 3F and 3R, examined the stoichiometry for both reactions. The nonstoichiometric relation of indole and indole glycerolphosphate in reaction 3R was confirmed with highly purified extracts of wild-type *Neurospora* tryptophan synthetase, and a nonstoichiometric relation of the two compounds was also observed for reaction 3F (*3*). Data from fractionations, a survey of *Neurospora* tryptophan synthetase mutants (*3*) and quantitative precipitin tests (*6*) eliminated the possibility that the two reactions, 3F and 3R, were catalyzed by separate proteins. Since the activity of reaction 3F was markedly inhibited and precipitated by antibody to the enzyme, and reaction 3R activity was precipitated, but not specifically inhibited by the antibody (*3*), it is possible that reactions 3F and 3R are reactions occurring at different sites on the tryptophan synthetase protein, or that there is differential accessibility of each substrate to the active center in the presence of antibody.

We have examined reaction mixtures which initially contained tryptophan synthetase, indole, and glyceraldehyde-3-phosphate for the presence of reaction products other than indole glycerolphosphate. An enzymatically synthesized indole derivative, not identical to indole, indole glycerolphosphate, indole-3-glycerol, or tryptophan, has been detected in these reaction mixtures. Reaction mixtures, prepared by mixing indole, DL-glyceraldehyde-3-phosphate (prepared from the diethylacetal), and highly purified *Neurospora* tryptophan synthetase were incubated at 37°C (*3*). The components and products of the reaction mixture were separated by ascending paper chromatography for an overnight period with a mixture of isopropanol, ammonium hydroxide, and water (100:10:25) as solvent. For two-dimensional chromatograms, a mixture of butanol, acetic acid, and water (60:15:25) was the additional solvent system. Portions of reaction mixtures were compared chromatographically to known standards of indole, indole glycerol phosphate, indole glycerol, tryptophan, serine, and pyridoxal phosphate by scanning with a Mineralight ultraviolet lamp, model SL-2537, and spray-

ing with indole color reagent (a fresh solution of 0.25 g of *p*-dimethylaminobenzaldehyde in 50 ml of ethanol, hydrochloric acid, and water—94:1:5) or with tryptophan color reagent (a fresh solution of 0.50 g of *p*-dimethylaminobenzaldehyde in 50 ml of acetone and HCl—90:10). With the isopropanol-ammonia solvent system and the indole color reagent, a compound (X) was detected which did not correspond in R_F or spot color to any of the standards (Table 1). The quantity of this compound (and of indole glycerol phosphate) synthesized was directly related to the time of incubation and the tryptophan synthetase concentration. The relation between the synthesis of compound X and the activity of reaction 3R was demonstrated with altered tryptophan synthetase from several sources as follows. (i) Extracts of a tryptophan synthetase mutant, which lack reaction 3R activity, are unable to catalyze the formation of compound X from indole and glyceraldehyde-3-phosphate. (ii) Extracts of a mutant, which catalyze both reaction 3F and reaction 3R, also catalyze the formation of compound X. (iii) When tryptophan synthetase is treated with trypsin until at least 85 percent of all tryptophan synthetase-associated reactions except 3R are inactivated, the enzyme is still able to catalyze the formation of compound X. In addition, the synthesis of compound X is completely dependent on the presence of both glyceraldehyde-3-phosphate and indole in the reaction mixture.

The substrate requirements suggest that the formation of compound X is related to the combination of indole with glyceraldehyde-3-phosphate. The addition of crystalline triose phosphate isomerase to the reaction mixture either before or during incubation with tryptophan synthetase markedly reduced the activity of reaction 3R. When dihydroxyacetone phosphate is used in place of glyceraldehyde-3-phosphate, there is no indole uptake, no indole glycerolphosphate formation, and no synthesis of compound X. The presence of an indole moiety in compound X eluted with water from a paper chromatogram has been confirmed by its ultraviolet-absorption spectrum, and fluorescence-excitation and emission spectra. The visible absorption spectrum of the X spot after spraying with the indole color reagent and elution with 95 percent ethanol is similar to that for eluted indole glycerolphosphate. It differs from that of indole in that indole exhibits an additional peak at 540 to 570 mμ. The presence of the glyceraldehyde phosphate moiety in compound X is suggested, in part, by its solubility in water, but not in toluene, diethyl ether, or acetone. After periodate oxidation of the reaction mixture, the usual spot for compound X cannot be detected. Consequently, it seems likely that compound X possesses *cis*-hydroxyl or some similar grouping derived from the glyceraldehyde phosphate.

Table 1. Paper chromatography of compound X. An ascending chromatographic system was used with the isopropanol-ammonia solvent. The chromatogram was sprayed initially with indole color reagent which developed all compounds but tryptophan, and subsequently was sprayed with the tryptophan color reagent. For the standards, 0.1 μmole of indole, 0.1 μmole of tryptophan, 0.05 μmole of indole glycerol phosphate, and 0.008 μmole of indole glycerol were used. For compound X we used a reaction mixture which initially contained 0.6 μmole of indole, 1.2 μmole of DL-glyceraldehyde phosphate, 10 μg of tryptophan synthetase protein, and 10 μmole of potassium phosphate buffer (*p*H 6.2) in a volume of 0.2 ml. After 60 minutes incubation at 37°C followed by 1 minute in a boiling water bath, the indole, indole glycerolphosphate, and compound X in the entire reaction mixture were detected chromatographically.

Compound	R_F
Indole	0.93
Tryptophan	0.45
Indole glycerol phosphate	0.09
Indole glycerol	0.56
Compound X	0.23

If the incubation is halted with the addition of acid or base, compound X still appears at its characteristic chromatographic position. The depth of coloring with the indole color reagent is enhanced when the reaction mixture has been acidified prior to chromatographic separation. Compound X reacts with $FeCl_3$ reagents (*7*), but may be distinguished from indole glycerolphosphate since compound X shows a deeper color with $FeCl_3$-H_2SO_4 reagent than with $FeCl_3$-$HClO_4$ reagent, while indole glycerolphosphate gives the opposite pattern. The nonidentity of indole glycerolphosphate and compound X may be demonstrated by treatment with hot alkali which results in the formation of indole from indole glycerolphosphate but not from compound X. There is little likelihood that compound X is an intermediate in tryptophan synthesis since two-dimensional chromatography of reaction mixtures which initially contained indole, indole glycerolphosphate, glyceraldehyde-3-phosphate, pyridoxal phosphate, L-serine, and tryptophan synthetase demonstrated that first indole and then indole glycerolphosphate disappeared (with concomitant tryptophan synthesis), while compound X remained.

Thus tryptophan synthetase from *Neurospora* catalyzes the formation of two indole derivatives from indole and glyceraldehyde-3-phosphate. Some properties of one of these compounds (X) are presented here; the other has been previously identified as indole-3-glycerolphosphate (*1*). We were unable to detect compound X when tryptophan synthetase and indole glycerolphosphate were incubated together. Therefore, the suggestion (*3*) that reactions 3F and 3R are not simply the forward and reverse components of a reversible reaction is confirmed. Since Yanofsky (*5*) has noted a similar lack of stoichiometry in the case of reaction 3R with tryptophan synthetase from *E. coli* it is likely that compound X is also synthesized in that system.

Recently, tryptophan synthetase from *E. coli* has been reported to catalyze the dehydration of L-serine (*8*). However, this does not appear to be true for the *Neurospora* enzyme (*9*). When all of the substrates and products taking part in the reactions catalyzed by tryptophan synthetase have been fully identified, the wealth of genetic and enzymatic data on this multifunctional protein may be better evaluated.

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