# Reports

## Coordinate Synthesis of Heme and Apoenzyme in the Formation of Tryptophan Pyrrolase

Abstract. *Reciprocal control mechanisms between hemoprotein and δ-aminolevulinic acid synthetase take part in coordinate synthesis of the heme and apoenzyme moieties of tryptophan pyrrolase. Stimulation of heme biosynthesis increases tryptophan pyrrolase, whereas enhancement of heme binding by apotryptophan pyrrolase secondarily increases the formation of δ-aminolevulinic acid synthetase, the rate-limiting enzyme in heme formation. Tryptophan-mediated induction of δ-aminolevulinic acid synthetase suggests that heme participates in repression of that enzyme.*

One of the problems in the regulation of hemoprotein production is the integration of the synthesis of a specific protein with that of its prosthetic group, heme. In the present studies, the heme enzyme tryptophan pyrrolase (TPO) (*1*) served as a model for the investigation of this problem. Tryptophan pyrrolase is a dissociable heme apoenzyme that requires both moieties for activity (*1, 2*). The amounts of holoenzyme and total TPO may be determined from the activities, with and without added hematin (*2, 3*), of the supernatant fraction obtained from centrifugation at 105,000*g* of liver homogenates. (Concentrations of 1 $\mu M$ hematin and 0.6 m$M$ ascorbic acid were used in these assays.) δ-Aminolevulinic acid synthetase (ALA synthetase) is the rate-controlling enzyme in heme biosynthesis in mammalian liver where it increases in amount after administration of certain compounds (*4–6*).

In our studies, ALA synthetase was assayed in liver homogenates (*7*). The metabolites of tryptophan which react with Ehrlich's reagent to produce colored compounds were removed from 10 ml of rat-liver supernatant containing 5 percent of trichloroacetic acid (derived from the homogenate containing 0.5 g of liver) by passage over a column 10 mm in diameter containing a mixture of 0.35 g of activated charcoal and 0.25 g of celite. The columns were washed with 5 ml of 1$M$ acetic acid, and the washings were combined with the original eluate. Under these conditions, tryptophan metabolites were retained completely on the charcoal, and the aminoketones were quantitatively recovered. The aminoketones present were converted to pyrroles by reaction with acetylacetone at *p*H 4.6 and separated on Dowex-1-acetate (see *8*).

The coordination of protein and heme biosynthesis in the formation of TPO was studied by investigating (i) the response of TPO to increased heme production resulting from induction of ALA synthetase, and (ii) the rate of heme synthesis due to increased binding of heme to the apoenzyme of TPO.

Administration of allylisopropylacetamide (AIA) to fasted rats rapidly and markedly increases both hepatic ALA synthetase (*6*) and TPO (*2*) (Fig. 1).

The increase of ALA synthetase appears to be responsible for the heme saturation of TPO (Table 1). Administration of actinomycin D together with AIA almost completely prevents the AIA-mediated increases of ALA synthetase, heme saturation of TPO, and TPO; this suggests that enzyme induction by AIA is responsible for all three effects. The AIA-stimulated heme saturation of TPO could be caused either by a direct effect of the drug on heme binding by TPO, or by a greater availability of heme. The first possibility is less likely since heme binding to TPO, like that resulting from L-tryptophan administration, is not prevented by actinomycin D (*9*) (Table 1). The induction of ALA synthetase by AIA, which stimulates hepatic heme biosynthesis (*10*), could increase the heme

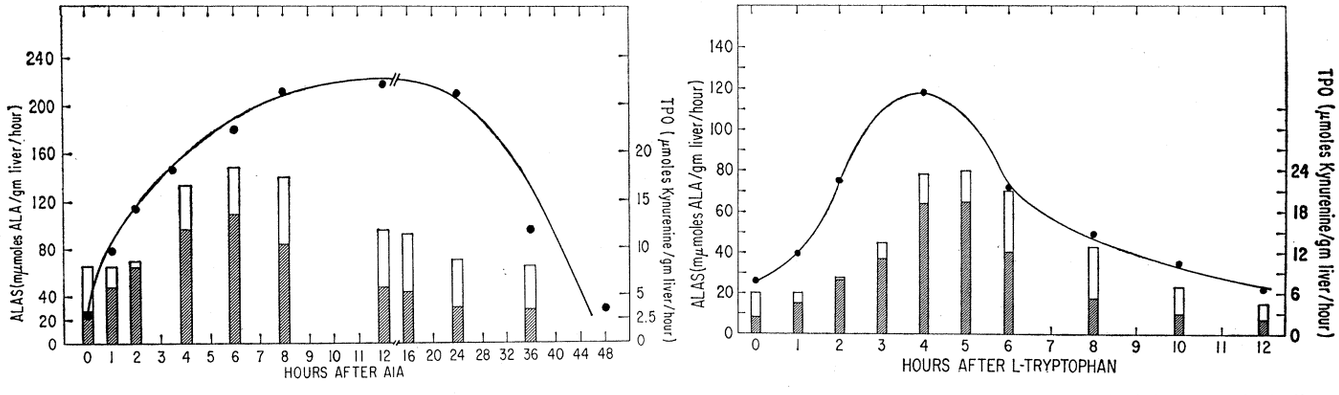


Fig. 1 (left). δ-Aminolevulinic acid synthetase (ALAS) and tryptophan pyrrolase (TPO) in liver, after a single injection of allylisopropylacetamide (AIA). Sprague-Dawley female rats (120 to 150 g) were fasted for 36 hours before subcutaneous administration of AIA (400 mg/kg) and fasting was continued until the end of the experiment. Each point and corresponding bar represents the ALAS and TPO activities respectively of the pooled liver homogenates of a group of six rats. Solid circles, ALAS activity; total bar, total TPO with added heme; shaded portion. TPO without added heme. Fig. 2 (right). δ-Aminolevulinic acid synthetase (ALAS) and tryptophan pyrrolase (TPO) in liver after a single injection of L-tryptophan. Sprague-Dawley female rats (120 to 150 g) were fasted for 36 hours before intraperitoneal administration of L-tryptophan (150 mg/100 g), and fasting continued until the animals were killed. Each point and corresponding bar represents the δ-aminolevulinic acid synthetase and TPO activities of the pooled liver homogenates of a group of six rats. Solid circles, ALAS activity; total bar, total TPO with added heme; shaded portion, TPO without added heme.

Table 1. Effect of actinomycin D on the allylisopropylacetamide (AIA) and tryptophan-mediated increases of δ-aminolevulinic acid synthetase (ALA synthetase) and of tryptophan pyrrolase (TPO). Sprague-Dawley female rats (120 to 150 g) were fasted for 36 hours before administration of the compounds indicated, and the animals were killed 4 hours later. Groups of six rats were studied under each set of conditions. ALA synthetase and TPO assays were performed on the pooled liver homogenates of each group. The values for TPO listed (micromoles of kynurenine per gram of liver per hour) were determined before and after addition of exogenous hematin. The values for ALA synthetase are in millimicromoles of ALA per gram of liver per hour. T, tryptophan; A, actinomycin.

| Substances administered | | | Activities | | |
|---|---|---|---|---|---|
| | | | ALA Synthetase | TPO ($\mu$mole $g^{-1}$ $hr^{-1}$) | |
| AIA | T | A | (m$\mu$mole $g^{-1}$ $hr^{-1}$) | No hematin | Hematin added |
| − | − | − | 26.7, 23.9 | 2.7, 2.3 | 6.9, 5.7 |
| − | − | + | 17.4, 18.3 | 2.1, 2.2 | 5.2, 5.1 |
| + | − | − | 149.3 | 13.7 | 17.1 |
| + | − | + | 35.8 | 3.0 | 6.0 |
| − | + | − | 110.0 | 21.0 | 24.8 |
| − | + | + | 36.2 | 20.1 | 22.5 |

available for the TPO apoenzyme. This mechanism for enhancing the heme saturation of TPO would be sensitive to actinomycin D. The increase in TPO that follows heme saturation of the apoenzyme may result from either increased synthesis or decreased degradation of the enzyme. The latter mechanism is more likely in that hematin influences the stabilization of TPO (*11*). Thus, increased heme production after the AIA-mediated increase of ALA synthetase may increase TPO by decreasing its rate of degradation. The sequence of events after AIA can be postulated as induction of ALA synthetase, heme production enhanced, increased heme saturation of the apoenzyme of TPO, and prolongation of the half-life of TPO leading to an increase in TPO.

The AIA-mediated increases of TPO and δ-aminolevulinic acid synthetase cannot be the result of adrenal cortical stimulation because (i) the pattern of increase of TPO is not characteristic of hydrocortisone-induced stimulation, which is neither preceded by, nor associated with, an increase in the ratio of holoenzyme to apoenzyme such as occurs after AIA (*2*). (ii) In our studies administration of AIA (400 mg/kg) to adrenalectomized, hydrocortisone-treated animals (0.1 mg/100 g intraperitoneally at the time of AIA), resulted in an increase of TPO 58 percent greater than that due to hydrocortisone alone (13.9 $\mu$mole of kynurenine per gram of liver per hour as compared with 8.8). (iii) Although the induction of hepatic ALA synthetase requires hydrocortisone, its administration (5 mg/100 g) did not increase the ALA synthetase in either intact or adrenalectomized female rats (*12*).

After administration of L-tryptophan, there is an initial increase in the ratio of holo- to total enzyme with a subsequent increase in TPO (*2*) (Fig. 2). There is a rapid increase of ALA synthetase in association with the increasing heme saturation of TPO (Fig. 2). This increase is not a nonspecific response to a large amount of amino acid. Glycine, or L-histidine (0.75 mmole/100 g) failed to induce hepatic ALA synthetase. Actinomycin D has no effect on the elevation of TPO after tryptophan administration (Table 1), an effect consistent with demonstrations by Greengard, Smith, and Acs (*9*), but the rise of ALA synthetase is reduced by about 85 percent. Therefore, unlike the tryptophan-mediated increase of TPO, the increase of ALA synthetase after tryptophan is probably RNA dependent. These data also indicate that if these two effects of L-tryptophan administration, that is, (i) the increased heme bound by TPO and (ii) the rise of ALA synthetase, are interdependent, then (i) is responsible for (ii). This relation is further supported by the fact that in our study administration of α-methyl-DL-tryptophan (0.5 mmole per 100 g of body weight) raised hepatic ALA synthetase three- to fourfold in 2 hours. This compound, which is not metabolized to kynurenine (*13*), can, like tryptophan, increase both heme binding (*14*) to apotryptophan pyrrolase and TPO (*13, 14*). On the other hand, 5-methyl-DL-tryptophan, a compound which does not increase heme binding by TPO (*14*), failed to raise hepatic ALA synthetase when given in doses of 0.5 to 1.0 mmole per 100 g.

That an increase in the binding of heme to apotryptophan pyrrolase leads to induction of ALA synthetase would be consistent with the concept that heme is involved in the repression of ALA synthetase. Although heme inhibits its bacterial ALA synthetase activity (*15*), there is no evidence that the activity of hepatic ALA synthetase is significantly affected by physiologic concentrations of heme (*5*). In our studies, homogenates of liver with increased levels of ALA synthetase were incubated with equal amounts of normal liver homogenates or with 0.05 m*M* heme, without significant inhibition of ALA synthetase (ALA synthetase was ± 10 percent of control activity). Furthermore, 0.05 m*M* heme did not inhibit soluble ALA synthetase prepared from liver mitochondria of porphyric rats (*16*). Although the substance or mechanism that directly represses ALA synthetase formation remains unknown, our studies suggest that heme participates in that regulating function.

The sequence of events following administration of L-tryptophan can be postulated as (i) increased binding of heme to the apoenzyme of TPO producing, in turn (ii), increased TPO resulting from its decreased rate of degradation (*11*), (iii) decreased availability of heme for repression, and (iv) induction of ALA synthetase resulting from (iii). One would expect that only the fourth step would be prevented by actinomycin D, and this is indeed the case (Table 1).

Hydrocortisone induces TPO (*2, 9*), but it does not significantly increase ALA synthetase (*12*). The fact that tryptophan induces ALA synthetase and hydrocortisone does not, even though both elevate TPO, is probably explained by the relatively greater amount of heme bound to the TPO apoenzyme after tryptophan treatment, compared to that after hydrocortisone treatment (*2*).

Thus, reciprocal control mechanisms between hemoprotein and ALA synthetase levels appear to be present in the hepatocyte and could be prototypes of control mechanisms in other cells, including the formation of hemoglobin in the erythrocyte.

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31 August 1966

## Pyrimidine Dimers: Effect of Temperature on Photoinduction

Abstract. *Ultraviolet-induced pyrimidine dimer formation in DNA and polyuridylic acid was inhibited on irradiation at 77°K. Enhancement of thymine dimer formation in solutions of DNA occurred upon addition of ethylene glycol. Low temperature measurements of absorbance of polyuridylic acid at low temperature showed that no significant alteration of the residues occurred after irradiation at 77°K and before thawing.*

Bacteriophage (*1*) and bacteria (*2, 3*) are much more sensitive to inactivation by ultraviolet light when irradiated in the frozen state than when irradiated at room temperature. There is considerable evidence (*4*) that DNA is the ultraviolet-sensitive target and that pyrimidine dimers in the DNA can account for much of the biological inactivation at room temperature. Therefore, we compared the light-induced information of pyrimidine dimers at room temperature and at 77°K in *Escherichia coli* DNA and in polyuridylic acid (polyU). That many fewer dimers were formed at the lower temperature in both those systems suggests that factors other than enhanced pyrimidine dimer formation are responsible for the increased sensitivity of bacteria and phage in the frozen state.

*Escherichia coli* DNA labeled with $^3$H-thymidine was dissolved in a mixture of ethylene glycol and water (1:1); this mixture forms an optically clear glass at 77°K. A *p*H of 7, measured at room temperature, was maintained with 0.005*M* phosphate. Irradiations were carried out on samples (0.65 $\mu$g/ml) in quartz electron-spin-resonance (ESR) tubes which were held in a fingertip, quartz Dewar. The Dewar was rotated during irradiation to ensure even irradiation of the sample. Monochromatic ultraviolet radiation (±50 Å) was obtained from a Hilger quartz-prism monochromator and a 500-watt Phillips high-pressure mercury lamp. The results presented here were obtained with 2800-Å irradiation at about 50 erg mm$^{-2}$sec$^{-1}$. After irradiation, the samples were subjected to hydrolysis by formic acid, and the products of hydrolysis were chromatographed on paper with a solvent mixture of butanol, acetic acid, and water to separate the thymine photoproducts from thymine (*5*). The distribution of radioactivity was measured and used to calculate the percentage of thymine photoproduct.

From the large difference in the initial slopes (Fig. 1), thymine photoproducts are formed at a much slower rate at 77°K. Although no $\overline{T}\overline{T}$ ($R_F = 0.31$) or $\overline{U}\overline{T}$ ($R_F = 0.22$) (dimers of thymine and of uracil and thymine, respectively) formation was observed at 77°K, there was some indication of a thymine photoproduct with $R_F = 0.40$, corresponding to that obtained for irradiated dry DNA or in spores (*3, 6*).

Inhibition of thymine dimer formation at 77°K was also observed in ice. As indicated in Table 1, for an incident dose of 3 × 10$^4$ erg/mm$^2$ the percentage of thymine photoproduct formed at 77°K was about the same in water as in the mixture of ethylene glycol and water, between 1 and 2 percent. The effective radiation dose in ice was less than that in the ethylene glycol and water because of light-scattering by ice crystals. A rough estimate of the amount of light lost by scattering in ice, based on measurements by Füchtbauer and Mazur (*7*), is about 50 percent. The reduction of thymine photoproduct in ice, then, seems only partially due to scattering.

The formation of thymine photoproduct was also measured for heat-denatured DNA both in water and in ethylene glycol and water at 77°K. Although there was no significant difference in yields between native and denatured DNA in water at 77°K, the formation of thymine photoproducts, mostly dimer, was slightly greater for denatured DNA irradiated in the ethylene glycol and water mixture.

The loss in transforming activity of *Hemophilus influenzae* DNA (*4*) upon 2800-Å irradiation at 77°K was also measured. The persistence of activity (survival) both in water and in ethylene glycol and water at room temperature and at 77°K is given in Table 1. For a dose of 3 × 10$^4$ erg/mm$^2$, the percentage of activity remaining at 77°K was several orders of magnitude greater than at room temperature, consistent with the absence of dimer formation at 77°K in *Escherichia coli* DNA. Furthermore, the activity persisting at room temperature was less in the presence of glycol (0.02 percent) than in water (0.12 percent). This result is in accord with the increased rate of dimer formation found for *E. coli* DNA irradiated in the mixture of ethylene glycol and water. Freezing alone has no effect on the transforming activity.

Polyuridylic acid (Miles) was irradiated in quartz ESR tubes as described for DNA except that a 2650-Å interference filter (Baird Atomic) with a 150-Å bandpass was used in place of the monochromator. Samples were dissolved in the mixture of ethylene glycol and water, and no salt was added. The formation of photoproduct was followed by measuring the corresponding changes in the optical absorbance, the initial absorbance at 2600 Å being around 0.50. The absorbance measurements were

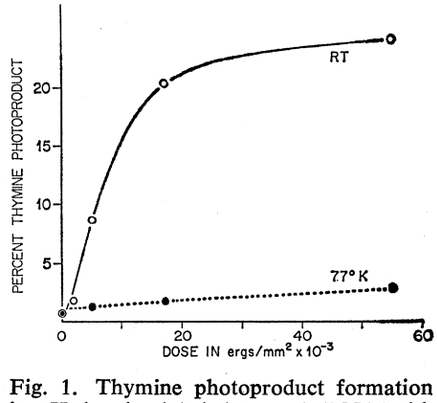


Fig. 1. Thymine photoproduct formation in $^3$H-thymine-labeled *E. coli* DNA with 2800-Å radiation at room temperature and at 77°K. Solvent was ethylene glycol and water (1:1), 0.005*M* phosphate, *p*H 7. The percentage of thymine photoproduct was calculated from radioactivity in the $\overline{T}\overline{T}$ and $\overline{U}\overline{T}$ regions of the chromatograms ($R_F$ between 0.16 and 0.40) relative to the total radioactivity.