Tryptophan Pyrrolase of Liver

II. THE ACTIVATING REACTIONS IN CRUDE PREPARATIONS FROM RAT LIVER*

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SUMMARY

The activation of the apoenzyme of tryptophan pyrrolase in preparations from hydrocortisone-induced rats was shown to consist of two separate, sequential steps, only the second of which is necessary to activate preparations from tryptophan-induced rats. These reactions are as follows. (a) Conjugation of the apoenzyme to the oxidized holoenzyme with hematin supplied by added methemoglobin, a reaction which requires the presence of L-tryptophan or certain analogues and which is inhibited by thiol reagents as well as by globin. (b) Reduction of the oxidized holoenzyme, a reaction which is promoted by L-tryptophan specifically and by ascorbate. It is reversed by oxidation in air in the absence of L-tryptophan.

Conjugation is the more rapid of the two reactions, especially at lower temperatures. The over-all activation is, therefore, limited by the rate of reduction.

The sites on the apoenzyme and the holoenzyme that react with L-tryptophan for conjugation and catalysis, respectively, are different. They are distinguished by the wider specificity and higher affinity of the site on the apoenzyme involved in conjugation.

The tryptophan pyrrolase (L-tryptophan:oxygen oxidoreductase, EC 1.13.1.12, tryptophan oxygenase) in soluble fractions of rat liver is substantially inactive until incubated with tryptophan, methemoglobin, and ascorbate (1). This activation is equally necessary for preparations of the enzyme from rats treated with tryptophan (unless tryptophan is added during the preparation (2, 3)) as well as from rats treated with hydrocortisone. The enzyme as prepared from tryptophan-treated rats is largely conjugated with its hematin prosthetic group, although that from hydrocortisone-treated rats is mostly the apoenzyme, as indicated by the effects on the activities of either omitting methemoglobin (1, 4) or adding globin (3) to prevent the occurrence of conjugation during incubation of the enzyme. These facts indicate that the activation involves a conjugation in the hydrocortisone type of preparation, and also another reaction common to both types of enzyme preparations. This other reaction appears to be a reduction of the oxidized holoenzyme, because ascorbate is needed in the incubation mixture (1, 3) and because the active enzyme is the reduced holoenzyme (ferroporphyrin form) (5). However, it was not established whether the oxidized holoenzyme was a normal intermediate or whether conjugation plus reduction to the reduced holoenzyme occurred in one reaction, because the oxidized holoenzyme could also be formed secondarily by oxidation in air (3). This latter possibility was excluded in the present experiments, and activation was then shown to consist of a separate conjugation reaction involving a thiol group on the protein and forming the oxidized holoenzyme, followed by its reduction to the active reduced holoenzyme.

METHODS

Adult female and male NEDH inbred rats were treated 5 hours before use with 2.5 mg of hydrocortisone acetate or 100 mg of L-tryptophan per 100 g of body weight. The livers were homogenized in 3 volumes of 0.14 M KCl-0.02 M sodium phosphate, pH 7.0, and centrifuged for 1 hour at 78,000 × g. The supernatant fraction was used for all experiments. The liver tryptophan pyrrolase averaged 20 and 30 amoles per hour per g of liver (25°), respectively, in the hydrocortisone- and tryptophan-induced preparations.

The standard assay of tryptophan pyrrolase activity depended upon the increase in absorbance at 360 nm (kynurenine, ε = 4530) in 1 cm cuvettes with a Gilford spectrophotometer at 25°. The mixture contained 0.4 ml of 12.5% liver extract, 0.7 ml of 0.2 M sodium phosphate (pH 7.0), 0.2 mg of methemoglobin, 0.2 ml of 0.05 M L-tryptophan, and 0.1 ml of freshly neutralized 0.3 M ascorbate, in a total volume of 3.0 ml. The activities were calculated from the initial rates during the first 20 min, and expressed as units (micromoles of kynurenine formed per hour) per g of liver (25°).

Prior to assay, incubation of concentrated (12.5%) or dilute (1.7%) liver extracts under a variety of conditions was employed.

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to activate the tryptophan pyrrolase. Incubation of the former for 30 min at 37\(^\circ\) in air ("concentrated incubation") provided the standard activating system to which other activating systems were compared. The final concentrations in the concentrated incubation mixture were 12.5\(\%\) liver extract, 2.5 mM L-tryptophan, 0.5 mg of methemoglobin per ml, and 30 mM freshly neutralized ascorbate. At least 1 ml of this mixture was incubated at a time in test tubes. The same type of incubation was also done in nitrogen and in two stages by use of Thunberg tubes with side arms.

The "diluted incubation" of 1.7\(\%\) liver extract consisted of the mixture used in the standard assay, placed in evacuated and nitrogen-filled Thunberg tubes when anaerobic, and with necessary additions made from the side arm. L-Tryptophan was present or added for protection of the reduced holoenzyme before the tubes were opened to the air after the incubation period. The mixture was poured into cuvettes and immediately assayed.

The degree of conjugation of a preparation was measured by the fraction of the total activity found when 0.5 mg of globin per ml was added to the concentrated incubation mixture and methemoglobin was omitted. A larger amount of globin, sufficient to prevent further conjugation, was added to the second of two sequential incubations of concentrated (Table IV) and dilute (Tables III and VI, Figs. 3 and 4) mixtures. In this way the conditions for conjugation during the first incubation could be determined separately from the conditions for reduction in the second incubation.

**RESULTS**

**Oxidation of Reduced Holoenzyme**—The possibility that the oxidized holoenzyme is an intermediate in the activation of the apotryptophan pyrrolase could not be assessed in the kind of aerobic activations previously used (1), because of the alternative possibility that oxidized holoenzyme could be formed by oxidation in air of the reduced holoenzyme (3). As shown in Table I, aerobic dialysis of the reduced holoenzyme to remove L-tryptophan resulted in the loss of enzyme activity. The inactive, oxidized holoenzyme that was formed was previously identified by its reactivation in the presence of globin (3). The activity loss was prevented by the presence of L-tryptophan, but not by \(\alpha\)-methyltryptophan. The loss was also prevented in the absence of L-tryptophan by dialysis under anaerobic conditions with \(\text{N}_2\) or CO, which preserved the reduced holoenzyme.

After anaerobic dialysis in the absence of L-tryptophan, the reduced holoenzyme was still oxidized promptly when exposed to air, unless tryptophan had been added for protection, as was done in the experiments of Table I. The rapidity of this aerobic oxidation of the reduced holoenzyme and the concentration of L-tryptophan necessary to prevent this oxidation were measured by adding the dialyzed, reduced holoenzyme to aerobic assay mixtures containing different concentrations of L-tryptophan. The reduced holoenzyme was prepared from a fully activated enzyme dialyzed free of tryptophan under nitrogen as in Table I. It was added to the aerobic assay mixtures without prior exposure to air and promptly assayed. The reactions began immediately at linear rates.

The full activity of the reduced holoenzyme was preserved when it was added to assay mixtures containing more than 1 mM L-tryptophan. With less tryptophan present, there was an immediate loss of activity. The remaining activity for at least 15 min was proportional to the tryptophan concentration present in the assay mixture. The loss was not immediately reversible by tryptophan since excess tryptophan (1.66 mM) added 2 min later did not change the reaction rate for the next 15 min. The inactivation was reversible, but only by excess tryptophan and the removal of oxygen in a short anaerobic incubation. The effect of L-tryptophan concentration on the preservation of the reduced holoenzyme activity under aerobic conditions is shown in Fig. 1 as a double reciprocal plot. The half-maximal protection occurred with \(3.7 \times 10^{-4}\) M L-tryptophan, a value comparable to those previously reported.

![Graph showing the relationship between L-tryptophan concentration and activity of reduced holoenzyme.](http://www.jbc.org/DownloadedFrom)
Relative activation of tryptophan pyrrolase in diluted and concentrated liver extracts with anaerobic and aerobic incubation

Hydrocortisone-induced supernatant fractions were kept frozen for several days with activities averaging 20 units per g of liver were incubated for 30 min at 37° in wide test tubes (aerobic) or in evacuated and nitrogen-filled Thunberg tubes (anaerobic) with the quantities of additions described under "Methods." The dilute incubation mixtures were equivalent to the standard assay mixtures (0.2 ml of 25% liver extract in 3 ml). The additions missing from the incubation were added just before assay (in the anaerobic incubations from a side arm before opening the Thunberg tubes to air). Results are the averages of at least four preparations, expressed as the percentage of activity obtained in the complete concentrated system incubated aerobically.

### Table II

<table>
<thead>
<tr>
<th>Additions</th>
<th>Condition of incubation at 37° for 30 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concentrated (12.5%)</td>
</tr>
<tr>
<td></td>
<td>% standard activity</td>
</tr>
<tr>
<td>None</td>
<td>12</td>
</tr>
<tr>
<td>Methemoglobin</td>
<td>11</td>
</tr>
<tr>
<td>Methemoglobin + L-tryptophan</td>
<td>57</td>
</tr>
<tr>
<td>Methemoglobin + L-tryptophan + ascorbate</td>
<td>100</td>
</tr>
</tbody>
</table>

The first column in Table III shows the activities obtained after anaerobic incubation of diluted enzyme with different additions, like those in Table II. The second column shows the result of reincubating the same preparations in the presence of ascorbate and tryptophan and with added globin. This second incubation did not change the activity of the already fully supplemented preparations (last line) but increased the activity of all the others. Since globin prevented further conjugation, only reduction could have occurred during the second incubation. The difference between Columns 2 and 1, shown in Column 3, represents that fraction of the enzyme which became conjugated but not reduced during the first incubation. Since it was subsequently activated in the presence of globin and the complete system, this fraction was the oxidized holoenzyme. This fraction was often greater than that which was both conjugated and reduced in the first incubation. In

as the $K_m$ for the tryptophan pyrrolase reaction (6-8), which presumably also measured the protection of the active form. The presence of $\alpha$-methyltryptophan in the assay mixtures did not prevent this inactivation, just as it did not protect the reduced holoenzyme during aerobic dialysis (Table I).

### Requirements for Activation in Anaerobic, Concentrated and Dilute Systems

The requirements for the activation were restudied with the precautions of anaerobiosis and addition of L-tryptophan before air outlined above. The inadequacy of methemoglobin alone and the need for tryptophan and ascorbate for activation were evident from previous studies in the usual aerobic incubation of concentrated (12.5%) liver extracts (1) (first column of Table II). With anaerobic incubation of the concentrated liver extract, slightly more activity was obtained. However, anaerobiosis decreased the need for L-tryptophan and eliminated the need for ascorbate (second column), making impossible the separation of conjugation from reduction. As seen in the last column, dilute (1.7%) systems were also almost fully activated under anaerobic conditions, and had the advantage of requiring both tryptophan and ascorbate. It was not attempted to show these requirements in the dilute system aerobically, because this system could not be fully activated (third column).

### Dissociation of Conjugation from Reduction

In the dilute, anaerobic system of Table II, omission of ascorbate resulted in less than maximal reduction. Anaerobiosis prevented the alternative formation of oxidized holoenzyme from reduced holoenzyme. It was therefore possible in this system to determine whether the oxidized holoenzyme was an intermediate in the activation of the apotryptophan pyrrolase by letting it accumulate in a first incubation in the absence of ascorbate and then determining the conjugated enzyme by the extra activity obtained after reduction in a second incubation where globin prevented further conjugation.

Added globin can be used to prevent conjugation without resolving the already conjugated enzyme (3). It was only necessary to show that globin, without preventing reduction, would stop conjugation by the amount of methemoglobin added for optimal conjugation in the first incubation. As shown in Fig. 2, quite small concentrations of added methemoglobin (0.1 mg per ml) were sufficient for nearly maximal conjugation of both the hydrocortisone- and tryptophan-induced preparations from perfused livers. The addition of 0.5 mg of globin per ml of the incubation mixture prevented the conjugation by endogenous hematin and also largely prevented the conjugation by added methemoglobin. The globin nearly neutralized the effect of an equal weight of methemoglobin, yet it did not interfere with the reductive activation of the oxidized holoenzyme present in the tryptophan-induced preparations.

The first column in Table III shows the activities obtained after anaerobic incubation of diluted enzyme with different additions, like those in Table II. The second column shows the result of reincubating the same preparations in the presence of ascorbate and tryptophan and with added globin. This second incubation did not change the activity of the already fully supplemented preparations (last line) but increased the activity of all the others. Since globin prevented further conjugation, only reduction could have occurred during the second incubation. The difference between Columns 2 and 1, shown in Column 3, represents that fraction of the enzyme which became conjugated but not reduced during the first incubation. Since it was subsequently activated in the presence of globin and the complete system, this fraction was the oxidized holoenzyme. This fraction was often greater than that which was both conjugated and reduced in the first incubation. In

![Fig. 2. The activity of tryptophan-induced (Trp) (•) and hydrocortisone-induced (HC) (□) tryptophan pyrrolase (TP) after concentrated, aerobic incubation at 37° for 30 min with increasing concentrations of methemoglobin (---), plus additions of 0.5 mg of globin per ml (----). Preparations were made from livers perfused with cold 0.9% NaCl immediately after death to minimize their blood content.](http://www.jbc.org)
the presence of tryptophan, without ascorbate 61% was only conjugated and later reduced. In the presence of \( \alpha \)-methyltryptophan (with or without ascorbate) at least 71% was conjugated and only 22% was conjugated and also reduced.

Inspection of Table III reveals that there was very little activity after the first incubation with \( \alpha \)-methyltryptophan, even in the presence of ascorbate, although with L-tryptophan and ascorbate the activation was complete. Yet with \( \alpha \)-methyltryptophan, the activation after the second incubation in the presence of globin and L-tryptophan was nearly complete. It is apparent that \( \alpha \)-methyltryptophan permitted conjugation but not reduction, and that the second incubation with L-tryptophan and ascorbate was necessary for the reduction. Thus both \( \alpha \)-methyltryptophan and L-tryptophan facilitated conjugation, but only L-tryptophan permitted the reduction of the inactive, oxidized holoenzyme formed by the conjugation. Ascorbate was not necessary for the conjugation, but facilitated the reduction. The nature of the measurements made in Table III also established that the conjugation of the apotryptophan pyrrolase in the first incubation preceded the reduction in the second incubation.

**Affinities of Tryptophan Analogues for Conjugation and Reduction—** \( \alpha \)-Methyltryptophan and some other tryptophan analogues did not replace L-tryptophan for the over-all activation in the concentrated, aerobic system (1). Since \( \alpha \)-methyltryptophan replaced L-tryptophan for the conjugation, the other compounds were also tested for their ability to permit conjugation. The two-step experiments were like those of Table III but in aerobic, concentrated incubations like the earlier experiments. The tested compounds permitted conjugation. They did not activate because they did not promote reduction. The concentrations of several compounds giving half-maximal conjugation as determined by double reciprocal plots are given in Table IV. L-Tryptophan was effective for conjugation \( (K_m = 2.6 \times 10^{-5} \text{ M}) \) in one-tenth the concentration, aerobic system (1). The \( K_m \) values of \( \nu \)-tryptophan and \( \alpha \)-methyltryptophan were twice that for L-tryptophan, suggesting that the \( \nu \) isomers were relatively ineffective and that the \( \nu \) isomers of tryptophan and \( \alpha \)-methyltryptophan were equally effective for conjugation. This specificity and affinity distinguishes the site of tryptophan catalysis. \( \nu \)-Tryptophan itself also permitted conjugation, but it was tested only at the high concentration of 5 mM. 5-Methyl-\( \nu \)-tryptophan also permitted conjugation, but it was also required in a high concentration.

The concentration of L-tryptophan needed for reduction was also determined. Oxidized holoenzyme, prepared by dialysis in air of a fully activated preparation as in Table I, was incubated in nitrogen with ascorbate and L-tryptophan. It was activated with the same small concentrations of L-tryptophan that were effective in conjugation. The results, shown by a double reciprocal graph in Fig. 3, indicate a \( K_m \) for reduction of \( 1.7 \times 10^{-5} \text{ M} \) L-tryptophan. This was not significantly different from the value for the over-all activation of conjugation plus reduction in the same experiments, and not different from the observed \( K_m \) for conjugation that was also determined in concentrated mixtures \( (2.6 \times 10^{-5} \text{ M}) \), Table IV. Miss Jeanne Li kindly determined the \( K_m \) of L-tryptophan for reduction in dilute mixtures, after conjugation with \( \alpha \)-methyltryptophan and addition of globin and ascorbate, and this was \( 7.1 \times 10^{-4} \text{ M} \). Both values were clearly less than the apparent \( K_m \) for catalysis and for the prevention of oxidation \( (3.7 \times 10^{-4} \text{ M}) \), Fig. 1) that were determined in dilute solutions.

**Kinetics of Activation of Tryptophan Pyrrolase—** The soluble liver fractions from 12.5% homogenates incubated in air with

### Table III

**Conjugation without reduction of tryptophan pyrrolase demonstrated by second incubation in presence of globin**

A hydrocortisone-induced preparation (0.2 ml) was first incubated anaerobically for 30 min at 37° with 0.2 mg of methemoglobin and 0.7 ml of 0.02 M sodium phosphate (pH 7.0) in a total volume of 2.7 ml, plus the additions listed in the table (0.2 ml of 0.05 M L-tryptophan or \( \alpha \)-methyltryptophan, 0.1 ml of 0.3 M ascorbate). At the end of this incubation 0.3 ml containing 1.5 mg of globin plus the additions omitted during the first incubation period were tipped in from a side arm. The solutions were aerated and immediately assayed (first column). Duplicate tubes with the additions after the first incubation were kept unopened, and were incubated for a second 30-min period at 37°, and then assayed (second column). The results are expressed as percentage of the activity obtained under these conditions with the complete system of methemoglobin, L-tryptophan, and ascorbate (28.8 units per g). The difference (third column) represents the enzyme that was conjugated but not reduced in the first incubation.

<table>
<thead>
<tr>
<th>Additions</th>
<th>After first incubation</th>
<th>After second incubation (with globin)</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methemoglobin only</td>
<td>100</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Methemoglobin + ascorbate</td>
<td>26</td>
<td>11</td>
<td>15</td>
</tr>
<tr>
<td>Methemoglobin + ( \alpha )-methyltryptophan</td>
<td>52</td>
<td>11</td>
<td>41</td>
</tr>
<tr>
<td>Methemoglobin + ( \alpha )-methyltryptophan + ascorbate</td>
<td>108</td>
<td>22</td>
<td>86</td>
</tr>
<tr>
<td>Methemoglobin + L-tryptophan</td>
<td>39</td>
<td>11</td>
<td>28</td>
</tr>
<tr>
<td>Methemoglobin + L-tryptophan</td>
<td>93</td>
<td>22</td>
<td>71</td>
</tr>
<tr>
<td>Methemoglobin + L-tryptophan</td>
<td>108</td>
<td>47</td>
<td>61</td>
</tr>
<tr>
<td>Methemoglobin + L-tryptophan + ascorbate</td>
<td>100</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

### Table IV

**Concentrations for half-maximal conjugation of tryptophan pyrrolase**

Hydrocortisone-induced preparations (12.5% liver extract) were incubated 30 min at 37° with 0.4 mg of methemoglobin per ml and with methemoglobin plus various concentrations of tryptophan or its analogues. The incubations with L- and \( \nu \)-tryptophan were anaerobic to avoid loss of substrate. At the end of the incubation, 0.5 mg of globin per ml, excess L-tryptophan, and ascorbate were added and the incubation was continued for 30 min in air. The concentrations of analogues and the activities, less that with methemoglobin alone, were plotted in double reciprocal graphs to determine the \( K_m \) values.

<table>
<thead>
<tr>
<th>Compound</th>
<th>( K_m ) for conjugation</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Tryptophan*</td>
<td>26</td>
</tr>
<tr>
<td>( \nu )-Tryptophan*</td>
<td>52</td>
</tr>
<tr>
<td>( \alpha )-Methyl-( \nu )-tryptophan</td>
<td>53</td>
</tr>
<tr>
<td>5-Methyl-( \nu )-tryptophan</td>
<td>2500</td>
</tr>
</tbody>
</table>

* Anaerobic incubations.
tryptophan, methemoglobin, and ascorbic acid at 37° become fully activated in 30 to 60 min (1). In a large number of experiments, the initial rate of the activation during the first 15 min of incubation was found to be proportional to the total enzyme activity formed, and this rate (average 71% of total) was not different for enzymes from tryptophan-treated rats (approximately 80% conjugated) or enzymes from hydrocortisone-treated rats (approximately 10% conjugated). The rates of activation in the concentrated, aerobic incubation for two such enzymes with low and high degrees of conjugation are shown in Fig. 4. Less than 20% increase in activity occurred in the first 5 min, and the curves were not significantly different for the two kinds of preparations, indicating that the limiting reaction under these conditions is not the conjugation. These results suggested that the conjugation must precede the rate-limiting step, which is presumably the reduction.

The rates of conjugation and reduction were measured separately in dilute, anaerobic incubations by determining the activation at intervals in two sequential steps like those of Table III. Conjugation in the presence of α-methyltryptophan was interrupted at intervals by the addition of globin from a side arm, followed by treatment with the complete activating system of L-tryptophan and ascorbic acid. To measure the rate of reduction, an enzyme already fully conjugated by anaerobic incubation with α-methyltryptophan and methemoglobin was reduced for various periods of time in the presence of globin and the complete activating system. The results at 37° (Fig. 4) show that conjugation occurred faster (an increase of 62% in 5 min) than the reduction (an increase of 33% in 5 min). This was expected from the earlier observations which indicated that the second step of activation was rate-limiting. Both reactions in the dilute, anaerobic system were somewhat faster than the over-all activation in the concentrated, aerobic system, suggesting that dilution and anaerobiosis accelerated the rate-limiting step.

With incubation at 25°, the rate of conjugation was only slightly slower than at 37°, but the rate of reduction was considerably less. The initial rates, as the percentage of reaction during the first 5 min for conjugation and reduction, respectively, were 62% and 33% at 37°, and 42% and 12% at 25°. The over-all activation rates in the concentrated, aerobic system at 37° and 25° were in the ratio of 2.0 (1), but in the dilute incubation the ratio of rates at 37° and 25° for conjugation was 1.5 and for reduction 2.8.

**Effect of Sulfhydryl Reagents on Conjugation**—Certain thiol reagents inhibited the activation of the hydrocortisone-induced tryptophan pyrrolase more than the tryptophan-induced enzyme. The effects of three different reagents were therefore studied on the separate conjugation and reduction reactions. That the reagents could act at one and not the other step in the activation, and not on the catalytic reaction, was evident from comparing the inhibition of the activations of hydrocortisone-induced enzyme with low conjugation and of tryptophan-induced enzyme with high conjugation (Table V). The percentage of inhibition by globin of the two types of enzyme preparations, which measure the percentage of apoenzyme present before activation, is included in Table V for com-

![Fig. 3. Concentrations of L-tryptophan necessary for reduction of the oxidized holoenzyme. Three activated preparations, 25 units per g of liver, were dialyzed in air as in Table I. They were incubated at 12.5% concentration in nitrogen for 30 min at 37° with ascorbate and the indicated concentrations of L-tryptophan (S = 10^{-4} M). Excess L-tryptophan was added before admission of air and the activity immediately determined. The activity, less the blank activation in the absence of L-tryptophan of 50 to 60% (Table II), was expressed as the percentage of full activation (v).](http://www.jbc.org/)

![Fig. 4. Rates of conjugation (○) and reduction (×) in dilute, anaerobic incubations at 25° (■) and 37° ( ). Each curve is an average of two or three experiments on hydrocortisone-induced preparations with total activities, determined in the same system after incubation for 60 min at 37°, of 19.6 to 28.0 units per g. The values are expressed as the percentage of the total activity of the preparation. Thunberg tubes contained, in the main vessel, 0.2 ml of 25% liver fraction, 0.7 ml of 0.02 M phosphate (pH 7.0), 0.2 mg of methemoglobin, 0.1 ml of 0.05 M α-methyltryptophan, and 1.2 ml of water; and in the side arm, 0.2 ml of 0.05 M L-tryptophan, 0.3 ml of solution containing 1.5 mg of globin, and 0.1 ml of 0.3 M ascorbate. The tubes at 0° were evacuated and filled with nitrogen then incubated at 37° for two successive periods of up to 30 min. To measure conjugation, the tubes were first incubated for the indicated times, the side arm contents were added, and the incubation was continued for 30 min more. The contents were then quickly equilibrated at 25°, centrifuged, and assayed. To measure reduction, the tubes were first incubated for 30 min to complete conjugation, then the side arm contents were added, and the incubation was continued for the indicated times. The initial activity of the contents was assayed. After the first 6 to 8 min of the reaction, the rate increased because of increased reduction. For comparison, the rates of over-all activation in concentrated aerobic incubations of a 10% conjugated hydrocortisone-induced (■■■■■■) and a 70% conjugated tryptophan-induced (■■■■■■■) preparation with total activities after 60 min of 22.5 and 28.3 units per g, respectively, are shown at 37°.)
Inhibitors were added to 25% liver extracts at 37°, 5 min before dilution to 12.5% with the components of the complete activating system. The concentrations are those during the aerobic incubation at 37° for 30 min. The results are given as the percentage of inhibition (for the number of preparations indicated in parentheses), compared to the activity of the preparations not treated with the inhibitors.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Globin (0.5 mg per ml)</th>
<th>N-Ethylmaleimide (5 X 10^{-6} M)</th>
<th>Formamidine disulfide (100 μg per ml)</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrocortisone-induced</td>
<td>85 (5)</td>
<td>87 (5)</td>
<td>71 (3)</td>
<td>19</td>
</tr>
<tr>
<td>Tryptophan-induced</td>
<td>30 (5)</td>
<td>32 (5)</td>
<td>20 (1)</td>
<td>5</td>
</tr>
</tbody>
</table>

**Table VI**

Inhibition by thiol reagents of catalysis and of conjugation, and reduction in dilute activating system

There were two 30-min incubations at 37° of dilute (1.7%) liver extract in nitrogen. The first (with a-methyltryptophan) conjugated and the second (with L-tryptophan) reduced the apo-tryptophan pyrrolase of a hydrocortisone-induced preparation (as in Table III), then the solution was assayed. The third reagents, in the final concentrations indicated, were added just before the first (conjugation) or second (reduction) incubations or the assay (catalysis). Results are expressed as the percentage of inhibition of activity compared to the preparations treated in the same way without the addition of inhibitors.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>N-Ethylmaleimide (5 X 10^{-6} M)</th>
<th>Formamidine disulfide (5 μg per ml)</th>
<th>p-Mercuribenzoate (5 X 10^{-4} M)</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conjugation</td>
<td>90</td>
<td>86</td>
<td>80</td>
<td>10</td>
</tr>
<tr>
<td>Reduction</td>
<td>5</td>
<td>4</td>
<td>60</td>
<td>10</td>
</tr>
<tr>
<td>Catalysis</td>
<td>3</td>
<td>3</td>
<td>54</td>
<td>10</td>
</tr>
</tbody>
</table>

*The effect of GSH on the inhibition of activation was measured in the concentrated, aerobic system. GSH (3.3 X 10^{-4} M) added 5 min after p-mercuribenzoate reversed all but 6% inhibition of conjugation plus reduction, and all of the inhibition of catalysis.

Discussion

For routine measurements of the tryptophan pyrrolase activity in crude liver extracts, the prior incubation of concentrated mixtures in air or nitrogen provides the optimal activation (Table II). However, incubation of diluted mixtures in nitrogen was necessary to separate and identify the individual steps of the activation process, the conjugation of the apoenzyme to the oxidized holoenzyme, and its reduction to the active, reduced holoenzyme.

In air, in the absence of tryptophan, the reduced holoenzyme is rapidly oxidized. The concentration of L-tryptophan which prevented half of this oxidative inactivation (3.7 X 10^{-4} M, Fig. 1) was the same as that previously reported as the apparent Kᵢ of the catalytic reaction (6, 7), and as calculated for this value from the data of Schimke, Sweeney, and Berlin (8). This indicates that in the presence of oxygen the reduced holoenzyme that is combined with L-tryptophan will oxidize it, although that not combined will itself be oxidized. The oxidative inactivation was reversible by addition of tryptophan and removal of oxygen. The two measurements are equivalent ways of determining the affinity of tryptophan for the enzyme in the presence of oxygen. The oxidative formation of the oxidized holoenzyme was easily prevented in nitrogen, so long as L-tryptophan was added before subsequent admission of air (Table I).

Therefore, oxidized holoenzyme formed during anaerobic experiments could not be due to reoxidation, but would prove that conjugation occurred without reduction and that the oxidized holoenzyme was an intermediate in the over-all activation process. The actual separation of these two reactions required not only anaerobiosis, but also incubation of a diluted mixture in which full activation would not occur spontaneously in the absence of tryptophan and ascorbate. Then, by two sequential incubations, the first with limited reduction and the second in the presence of globin to limit conjugation, it was possible to show the occurrence of conjugation as a first step, and reduction as a second (Table III). It was also possible to distinguish between the different requirements for the two steps of the activation; a-methyltryptophan and L-tryptophan promoted conjugation, but only L-tryptophan promoted reduction. Ascorbate promoted reduction but had no function in the conjugation.

Although all of the analogues of tryptophan tested were effective in permitting conjugation, the L isomers of tryptophan and α-methyltryptophan were effective in the lowest concentrations. Half-maximal conjugation occurred with 2.6 X
10^{-8} M of these compounds (Table IV), one-tenth of the apparent $K_m$ of the catalytic reaction and of the concentration for the prevention of oxidation of the reduced holoenzyme.

Schimke, Sweeney, and Berlin (8) earlier proposed that there was a second site on the enzyme with a greater affinity for L-tryptophan and for α-methyltryptophan than the catalytic site, because of the very low concentrations that protected the apoenzyme against heat and urea, relative to the (apparent) $K_m$ of L-tryptophan ($3.2 \times 10^{-4}$ M) and the $K_f$ of α-methyltryptophan ($1 \times 10^{-3}$ M) in the catalytic reaction. A different site can, in fact, be clearly distinguished on the apoenzyme as the one for conjugation. It has not only a relatively high affinity for L-tryptophan but an equally high affinity for α-methyltryptophan, although the affinity of the catalytic site of the holoenzyme for α-methyltryptophan is very low.

It is possible that combination of tryptophan and its analogues with the site for conjugation does not itself stabilize the enzyme, but that any stabilization results from the conjugation with hematin or by altering the configuration of the protein. Associated with conjugation with hematin changes the accessibility to the thiol group. It is possible that hematin covers a thiol group, perhaps conjugating through it, or that conjugation with hematin changes the accessibility to the thiol by altering the configuration of the protein. Associated with this change is the transformation of the reactive site for tryptophan from that characteristic of conjugation to that of catalysis.

The effects of the thiol reagents suggest that a thiol group on the protein is somehow involved in the activation and catalysis. Each of the reagents used reacts in a different way with thiols, but all three inhibited the conjugation step. Only p-mercuribenzoate also inhibited the reduction and catalysis, and only the inhibition of p-mercuribenzoate was reversed by GSH, immediately in the catalytic reaction in which the time course could be followed. Thus p-mercuribenzoate apparently caused no significant denaturation of the protein. Since it has often been noted that p-mercuribenzoate will react with certain thiols not accessible to N-ethylmaleimide (16, 17), it appears that conjugation may have made the thiol group inaccessible to N-ethylmaleimide and formamidine disulfide, while it remained accessible to p-mercuribenzoate. It is possible that hematin covers a thiol group, perhaps conjugating through it, or that conjugation with hematin changes the accessibility to the thiol by altering the configuration of the protein. Associated with this change is the transformation of the reactive site for tryptophan from that characteristic of conjugation to that of catalysis.

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