Cell-free extracts of rat kidney catalyze the conversion of kynurenine to kynurenic acid by means of a transamination reaction (2). Such extracts lose kynurenine transaminase activity rapidly when added to phosphate buffer solutions with pH below 7 unless α-ketoglutarate is added (2). Further studies in this laboratory have shown that this inactivation is completely reversed by the addition of small amounts of pyridoxal phosphate or pyridoxamine phosphate. This observation suggests that inactivation results from dissociation of one or both of these coenzymes from the apo-transaminase and that α-ketoglutarate prevents this dissociation. The present report seeks to describe this association-dissociation phenomenon and to explain the protective action of α-ketoglutarate.

**Materials and Methods**

L-Kynurenine sulfate monohydrate was obtained by ozonolysis of N-acetyl-L-tryptophan.1 dl-Kynurenine, obtained commercially, was used in several experiments. dl-Kynurenine was less satisfactory as a substrate because the range of activity that could be measured spectrophotometrically was limited by absorption of the inert D isomer. Pyridoxal phosphate and pyridoxamine phosphate, each assayed as pure within the limits of accuracy of the spectrophotometric and chromatographic methods of assay, were purchased from the California Foundation for Biochemical Research. Ammonium pyridoxal phosphate, obtained earlier from the same source, underwent rapid decomposition during storage and failed to prevent the inactivation of kynurenine transaminase (2).

Conditions of incubation and assay were similar to those described earlier (2). The incubation periods were 1 hour or less as indicated; the total volume of incubation mixture was 1 ml. The concentration of kynurenine was 0.0035 mM and of keto acids, 0.006 mM, except when indicated otherwise.

* A report of this study was presented before the American Society of Biological Chemists, Atlantic City, April 18, 1956 (1).
1 Warnell, J. L., and Berg, C. P., unpublished. We are indebted to Dr. J. L. Warnell for the use of an ozonizer and an improved modification of a method for preparing kynurenine sulfate (3).
Transamination was measured by determining kynurenic acid formation and is expressed as the change (Δ333) of optical density at 333 nm after correction for the absorption due to kynurenic acid.

**Enzyme Purification**—The preparation of the kynurenine holotransaminase is handicapped, since the enzyme is readily inactivated in slightly acidic buffer solutions (2). In the present study, it was found to be completely inactivated by ammonium sulfate fractionation (see below) and almost completely inactivated by alcohol fractionation and by freezing followed by thawing. Activity was restored in each case by the addition of small amounts of pyridoxal phosphate, indicating that inactivation was caused by dissociation of the coenzyme.

The kynurenine apotransaminase, on the other hand, was fairly stable. Some purification of the apotransaminase was obtained as follows: 18.5 gm. of rat kidney were homogenized with 75 ml. of ice-cold water. The homogenate was centrifuged briefly to remove cell debris, and ammonium sulfate was added slowly to the supernatant fluid to bring it to 25 per cent of saturation. The precipitate was removed by centrifugation, and ammonium sulfate was added to the supernatant fluid to 75 per cent of saturation. The new precipitate was removed by centrifugation, dissolved in 30 ml. of water, and dialyzed against 0.001 M phosphate buffer, pH 7.0, for 20 hours. More protein precipitated upon freezing followed by thawing; this was removed by centrifuging. The supernatant fluid had approximately 6 times as much apotransaminase activity per mg. of dry weight as the original homogenate. It was inactive unless pyridoxal phosphate or pyridoxamine phosphate was added. The preparation was stored in the frozen state for 2 months without significant loss of kynurenine apotransaminase activity.

**Results**

As reported before (2), the kynurenine transaminase of rat kidney extracts became inactive within a few minutes at 37° in phosphate buffer, pH 6.3, unless α-ketoglutarate was present. At pH 7.4, on the other hand, inactivation was slow (Fig. 1). The rate of inactivation also depended upon the concentration of the phosphate buffer (Fig. 2). Subsequent addition of pyridoxal phosphate or pyridoxamine phosphate to the inactivated preparations restored the activity to levels which were higher than those that existed before preincubation. Purified preparations which were completely inactivated during ammonium sulfate fractionation were similarly activated as illustrated in Fig. 3. When such reconstituted enzyme preparations were dialyzed in 0.002 M phosphate buffer, pH 7.4, to remove the unbound coenzyme, much of the activity was retained (Table I). These

Preincubation is used here to designate a period of preliminary incubation during which the absence of one or more factors prevents transamination.
Fig. 1. Effect of pH on the rate of inactivation of kynurenine transaminase during preincubation in 0.05 M phosphate buffer. The kidney extract was added to buffer solutions at zero time. α-Ketoglutarate was added after the intervals indicated by the points on the curves. After 16 minutes of preincubation, kynurenine was added to start the reaction.

Fig. 2. Effect of phosphate buffer concentration on the rate of inactivation at pH 6.3. The experimental conditions were similar to those described in Fig. 1 except for the variation of concentration of the phosphate buffer. pH measurements before and after incubation showed that the lower concentrations of buffer were adequate to prevent significant pH changes.

Fig. 3. Relationship of activity to coenzyme concentration. Apoenzyme preparations were obtained by ammonium sulfate fractionation (see under "Materials and methods"). No preincubation. Solid lines, activation with pyridoxal phosphate; broken lines, activation with pyridoxamine phosphate. Curves 1 and 2, pH 6.3; Curves 3 and 4, pH 7.4.
dialyzed preparations, like the freshly prepared extracts, quickly lost their activity during preincubation in 0.1 M phosphate buffer, pH 6.3, unless α-ketoglutarate was present.

These observations strongly indicate that the inactivation resulted from dissociation of the holoenzyme and that the dissociation was slowed or prevented (although not measurably reversed) by α-ketoglutarate. The protective action of α-ketoglutarate may be a result of a greater affinity of the apoenzyme for pyridoxal phosphate than for pyridoxamine phosphate, as shown by the higher concentrations of the latter needed to saturate the apoenzyme (Fig. 3). The scheme presented in Fig. 4 shows how the holoenzyme may be rendered unstable in the absence of keto acid. The portion

| pH | Assay conditions, preincubation in 0.1 M buffer | Δα
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
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<tbody>
<tr>
<td>6.3</td>
<td>With α-ketoglutarate</td>
<td>0.441</td>
</tr>
<tr>
<td>7.4</td>
<td>Without α-ketoglutarate</td>
<td>0.343</td>
</tr>
</tbody>
</table>

* Purified preparations obtained by ammonium sulfate fractionation (see under "Materials and methods") were incubated in 0.02 M phosphate buffer, pH 7.4, with pyridoxamine phosphate (45 μg per ml.) for 2 hours at 37°, then dialyzed for 28 hours in the cold room in 400 volumes of 0.002 M phosphate buffer, pH 7.4. Kynurenine holotransaminase activity was assayed under the conditions and at the times indicated.

Consistent with the above interpretation, the abilities of the various keto acids in preventing inactivation (Fig. 5) paralleled their effectiveness in transaminating with kynurenine (Table II). α-Ketoglutarate and oxalacetate were considerably more effective in both the protective and sub-

To simplify illustration and discussion, a two-step transamination mechanism (4) is assumed. The suggestion that α-ketoglutarate prevents inactivation by converting the holoenzyme to a form which does not dissociate readily may be valid even if the two-step mechanism does not apply.
strate functions than any of the six monocarboxylic α-keto acids tested. Succinate had no protective action, indicating that the carbonyl group of oxalacetate is essential for its protective action.

Since α-ketoglutarate seemed to favor association, it was not surprising that rate-limiting amounts of the coenzymes reactivated apoenzyme preparations more completely when preincubated in mixtures containing α-ketoglutarate (Curve 1, Figs. 6 and 7) than when preincubated without it. Reaction mixtures which were preincubated without α-ketoglutarate (Curves 4 to 8) showed rates which were initially much lower but which were subsequently accelerated, suggesting that α-ketoglutarate promoted association during the transamination reaction.

In the absence of α-ketoglutarate, preincubation of the apoenzyme preparations with the coenzymes at pH 6.3 did not cause significantly more
### TABLE II

**Comparison of Various α-Keto Acids As Substrates for Kynurenine Transaminase Reaction***

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Keto acid</th>
<th>Δ333</th>
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<tbody>
<tr>
<td>1</td>
<td>α-Ketoglutarate</td>
<td>0.589</td>
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<tr>
<td></td>
<td>Oxalacetate</td>
<td>0.528</td>
</tr>
<tr>
<td>2</td>
<td>α-Ketoglutarate</td>
<td>0.978</td>
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<td></td>
<td>α-Ketoisovalerate</td>
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<td></td>
<td>α-Ketoisocaproate</td>
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</tr>
<tr>
<td></td>
<td>α-Keto-β-methylvalerate</td>
<td>0.063</td>
</tr>
<tr>
<td></td>
<td>Pyruvate</td>
<td>0.103</td>
</tr>
<tr>
<td>3</td>
<td>α-Ketoglutarate</td>
<td>0.900</td>
</tr>
<tr>
<td></td>
<td>α-Ketovalerate</td>
<td>0.318</td>
</tr>
<tr>
<td></td>
<td>α-Ketobutyrate</td>
<td>0.120</td>
</tr>
</tbody>
</table>

* Aliquots of a purified preparation obtained by ammonium sulfate fractionation (see under “Materials and methods”) were incubated with 30 γ of pyridoxal phosphate and standard concentrations of keto acids and kynurenine sulfate in m/15 phosphate buffer, pH 6.3, for 1 hour.

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**Figs. 6 and 7.** Effect of certain conditions during preincubation on the course of the subsequent kynurenine transaminase reaction. Fig. 6, activation with pyridoxamine phosphate, 7.5 γ per ml. Fig. 7, activation with pyridoxal phosphate, 7.5 γ per ml. Solid lines, pH 6.3; broken lines, pH 7.4. Preincubation condition, Curves 1 and 2, coenzyme plus α-ketoglutarate; Curves 3 and 4, coenzyme only; Curves 5 and 7, α-ketoglutarate only; Curves 6 and 8, neither. The curves for incubation mixtures which were not preincubated were like Curves 6 and 8 and are not shown. In all instances the reaction was started after 45 minutes by the addition of kynurenine and any other required factor not added earlier. Aliquots were removed and deproteinized after intervals indicated by the points on the curves.
activation. At pH 7.4, on the other hand, such preincubation resulted in substantial increases (Curve 3), although these were not as great as when α-ketoglutarate was present. Accordingly, there is both a lesser tendency to dissociate and a greater tendency to associate at pH 7.4 than at pH 6.3.

Although the kynurenine transaminase of rat kidney appears to dissociate more readily than most enzymes involving pyridoxal phosphate, it may be paralleled in this respect by a system reported by Blakley (5) which interconverts serine and glycine. This system became inactive at pH values above 9 and was reactivated by the addition of pyridoxal phosphate. Inactivation was thought to involve adsorption of the coenzyme by inert constituents of the enzyme preparation. Such an explanation has not been excluded in the present study, but it is believed to be of minor or secondary importance because little loss of kynurenine transaminase activity was evident after 45 minute preincubations of added coenzyme with apoenzyme preparations (Fig. 6). Cleavage of pyridoxal phosphate and pyridoxamine phosphate by a phosphatase in the kidney homogenates was demonstrated, however, by means of paper chromatography.4 This cleavage was discounted as a major factor in the inactivation phenomenon under study because increases in the concentration of phosphate buffer in the reaction mixtures resulted in decreases in the rate of the phosphatase reaction, whereas they increased the rate of inactivation of the kynurenine holotransaminase in kidney extracts at pH 6.3 (Fig. 2).

DISCUSSION

The transamination system under study is like the glutamate-aspartate transaminase system (6) in that an appreciable time interval is needed for maximal activation by pyridoxamine phosphate at pH 7.4 and that dialysis at pH 7.4 does not readily resolve the reconstituted holotransaminase. The kynurenine transaminase system, however, was maximally activated by pyridoxamine phosphate only after preincubation with α-ketoglutarate. Such an effect of keto acids in the activation of a transaminase seems not to have been reported previously, although superficially similar effects have been noted in the activation of bacterial tryptophanase (7) and in decarboxylase (8) systems. Judging from the nature of the reactions involved, pyridoxamine phosphate is probably not a coenzyme in the decarboxylase and tryptophanase systems, hence the necessity for its conversion to pyridoxal phosphate prior to activation. The activating effect of keto acids in the kynurenine transaminase system, on the other hand, seems to be a result of the conversion of the apoenzyme-pyridoxamine phosphate complex to a more stable apoenzyme-pyridoxal phosphate complex. Possibly the greater stability of the latter form arises from the anionic character of

4 Mason, M., unpublished data.
KYNURENINE TRANSAMINASE

Pyridoxal phosphate at pH 6.3 (by calculating from dissociation constants obtained by Williams and Neilands (9)) contrasted with the isoelectric character predominating for pyridoxamine phosphate at that pH. The more strongly anionic character of both coenzymes at higher pH reactions may similarly explain the greater stability of the holoenzyme at pH 7.4.

SUMMARY

The previously described inactivation of the kynurenine transaminase of rat kidney which occurs when the extracts are incubated with phosphate buffer, pH 6.3, was shown to be strongly dependent on the concentration of phosphate buffer and to be completely reversed by the addition of small amounts of pyridoxal phosphate or pyridoxamine phosphate.

Pyridoxamine phosphate was much less effective than pyridoxal phosphate in activating the kynurenine apotransaminase. Inactivation was therefore interpreted to be a result of rapid dissociation of the pyridoxamine phosphate from the apoenzyme. Both α-ketoglutarate and oxalacetate served adequately as substrates at concentrations of 0.006 M and prevented inactivation at concentrations as low as 0.002 M. Various monocarboxylic α-keto acids, at similar concentrations, were relatively ineffective both in the protective and substrate functions. It was concluded that the keto acids prevent dissociation during incubation at pH 6.3 by maintaining the holoenzyme in the more stable apoenzyme-pyridoxal phosphate form.

The author wishes to express his appreciation to Mrs. Eva McKenna for assistance in purifying the apoenzyme preparations and to Mr. Clare Johnston for preparing kynurenine sulfate.

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Merle Mason


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