The Photoreduction of Kynurenic Acid to Kynurenine Yellow and the Occurrence of 3-Hydroxy-L-kynurenine in Butterflies

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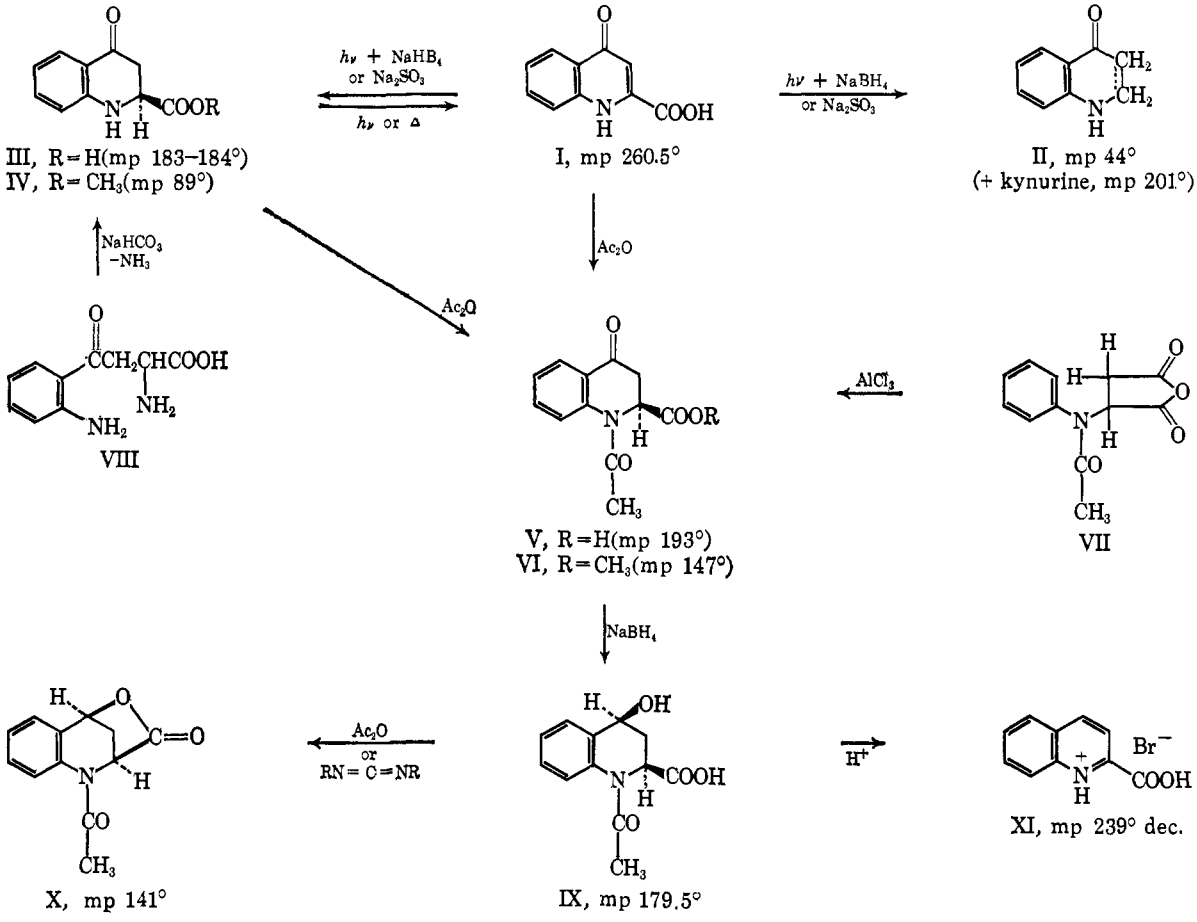
Abstract: A novel photoreduction takes place when the photoexcited state of kynurenic acid (I) reacts with excess aqueous sodium sulfite. The product, kynurenine yellow, DL-2-carboxy-2,3-dihydroquinolone-4 (III), reverts to kynurenic acid (I) by spontaneous dehydrogenation which is catalyzed by light or heat. N-Acetylkynurenine yellow (V) is reduced stereoselectively by sodium borohydride to the dihydro derivative IX which is easily dehydrated to the *cis*-γ-lactone X of N-acetyl-1,2,3,4-tetrahydro-4-hydroxyquinoline-2-carboxylic acid by the action of acetic anhydride or dicyclohexylcarbodiimide. The properties of the optically active amino acid isolated from the bodies and wings of South American butterflies (*Ithomiinae* and *Heliconius*) were compared with 3-hydroxy-L-kynurenine and found to be identical, whereas hydroxykynurenine yellow (2-carboxy-2,3-dihydro-8-hydroxyquinolone-4) was completely different.

Treatment of kynurenine (VIII, Chart I) with sodium bicarbonate leads to intramolecular cyclization. The fluorescent yellow compound formed in this reaction was named *kynurenine yellow*[5] and formulated as 2-carboxy-2,3-dihydroquinolone-4 (III) on the basis of the following observations.[6] (i) The ultraviolet spectrum of kynurenine yellow III resembles the absorption of *o*-aminoacetophenone[7,8] and, more so, that of 1,2,3,4-tetrahydroquinolone-4 (II).[9] (ii) Kynurenine yellow III is readily converted to (colorless)

Chart I. Formulas Representing the L Forms of the Racemic Amino Acids and Their Derivatives



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(5) Y. Kotake and G. Shichiri, *Z. Physiol. Chem.*, **195**, 152 (1931).
(6) T. Tokuyama, S. Senoh, Y. Hirose, and T. Sakan, *J. Chem. Soc. Japan*, **79**, 752, 759 (1958); *Chem. Zentr.*, **130**, 7125 (1959).
(7) Typical example, aminoacetophenone, *cf.* P. Grammaticakis, *Bull. Chim. Soc. France*, [5] **20**, 94 (1953).
(8) W. D. Kumler, *J. Am. Chem. Soc.*, **68**, 1184 (1946).
(9) W. S. Johnson, E. L. Worsch, and B. G. Buell, *ibid.*, **71**, 1900 (1949).

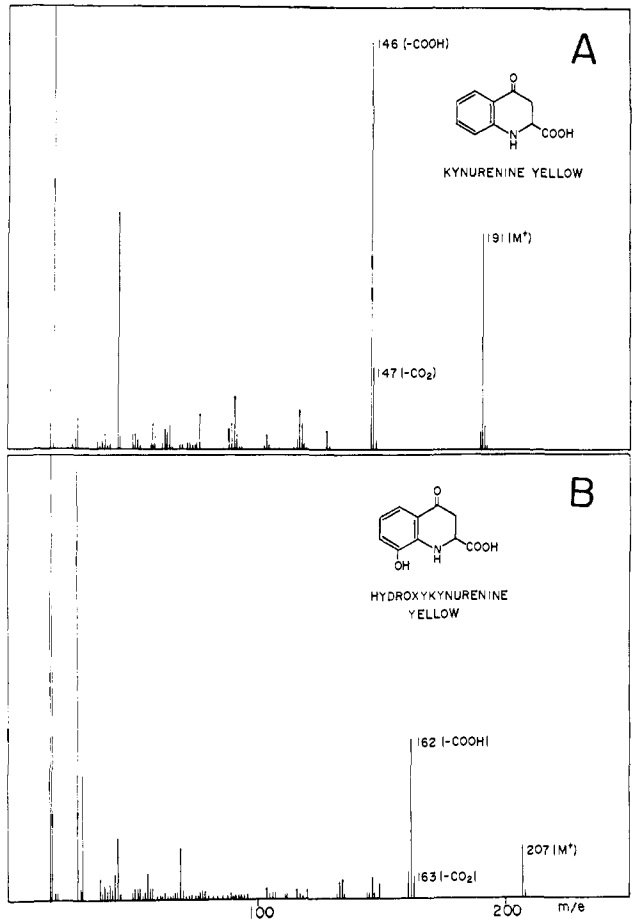
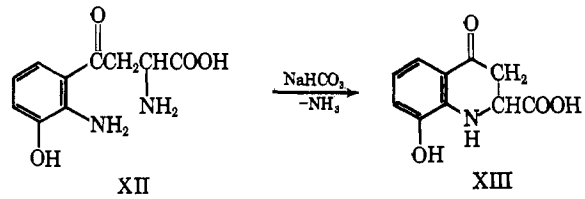


Figure 1. Mass spectra of kynurenine yellow, A, and of hydroxykynurenine yellow, B.

kynurenic acid (I) and γ-quinolone (kynurine) by sunlight or by heating.[6] (iii) The dihydroquinolone structure of (N-acetyl)kynurenine yellow V was confirmed by synthesis *via* intramolecular Friedel–Crafts ring closure of N-acetyl-N-phenyl-DL-aspartic anhydride (VII).[6] Analogously, sodium bicarbonate treatment of DL-3-hydroxykynurenine (XII) gave a yellow fluores-



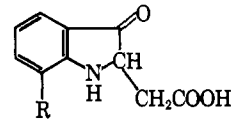
XII XIII

cent substance, $C_{10}H_9NO_4$, which was formulated as 2-carboxy-2,3-dihydro-8-hydroxyquinolone-4 (XIII) on the basis of its great resemblance to kynurenine yellow.[6]

An isomeric yellow pigment of similar properties has recently been isolated from Ithomid and Heliconian butterflies.[10] For this *optically active amino acid*, a structure was suggested identical with that of hydroxykynurenine yellow XIII. In this paper we show that the "new amino acid" from butterfly wings is in reality 3-hydroxy-L-kynurenine.

Discussion of Possible Alternate Structures. It was previously shown[6] that XIII in ethanol solution is easily converted to a mixture of xanthurenic acid and 4,8-dihydroxyquinoline. This transformation occurs spontaneously and is accelerated by sunlight.[6] Likewise, the natural compound from butterflies is also converted to xanthurenic acid by air oxidation in alkaline solution.[10] This observation together with the mass spectrogram originally led to the assignment of structure XIII to the butterfly pigment. However, the direct comparison of the butterfly pigment with XIII definitely excluded their identity (Table I).

The alternate possibility of an indoxylacetic acid formulation, XIV and XV, for (hydroxy)kynurenine yellow was ruled out as follows.



XIV, R = H
XV, R = OH

(i) In the mass spectra of kynurenine yellow (Figure 1A) and its hydroxy derivative (Figure 1B), we were unable to find any peaks corresponding to M − 59 (CH_2-COOH) expected from the fragmentation of indoxylacetic acids. (ii) In the nmr spectrum of the carboxylate anion of kynurenine yellow, the multiplet of the methine proton (2 H) is shifted upfield by comparison with the free acid III or its methyl ester IV (Figure 2).[11] This phenomenon is explicable only in terms of the structure of 2-carboxy-1,2-dihydroquinolone-4 (III).

Stereochemistry of Dihydrokynurenine Yellow. Kynurenine yellow III was further characterized by the following reactions. Its colorless N-acetyl derivative V[6] was readily reduced by sodium borohydride to an N-acetyldihydro derivative IX, mp 179.5°. The reduction is stereospecific, because a single dihydro product is obtained in good yield. The nmr spectrum of the dihydro compound (Table II) suggests a quasi-equatorial conformation of the hydroxy group. The steric relationship between this hydroxyl and the carboxyl group was established by the formation of an N-acetyl-γ-lactone X, mp 140–141°, by dehydration of the dihydro compound with acetic anhydride or by treatment with dicyclohexylcarbodiimide. The nmr spectrum of the γ-lactone supports the tricyclic structure X.

Hydrolysis of N-acetyldihydrokynurenine yellow IX with 2.0 *N* hydrochloric acid gave quinaldinic acid characterized as the hydrobromide XI. The aromatization, besides dehydration, involves probably oxidation by air, rather than extrusion of $CH_3\bar{C}O$, in the manner of decomposition of a Reissert compound.

Photoreduction of Kynurenic Acid. Additional confirmation for the structure of kynurenine yellow is provided by the first direct preparation from kynurenic acid by photoreduction. The photooxidation and thermal aromatization of (hydroxy)kynurenine yellow to (hydroxy)kynurenic acid and their decarboxylation products, *viz.* 4-hydroxyquinoline and 4,8-dihydroxyquinoline, have their counterpart in the photoreduction of kynurenic acid to kynurenine yellow which has now become possible. Catalytic reduction (Pd–H_2) reduces the benzene rather than the quinolone part of the molecule. We have shown previously that heterocycles in their excited states are amenable to reduction in a selective fashion which is often not possible by

(10) K. S. Brown, Jr., *J. Am. Chem. Soc.*, 87, 4202 (1965).

(11) F. Taddei and J. Pratt, *J. Chem. Soc.*, 1553 (1964).

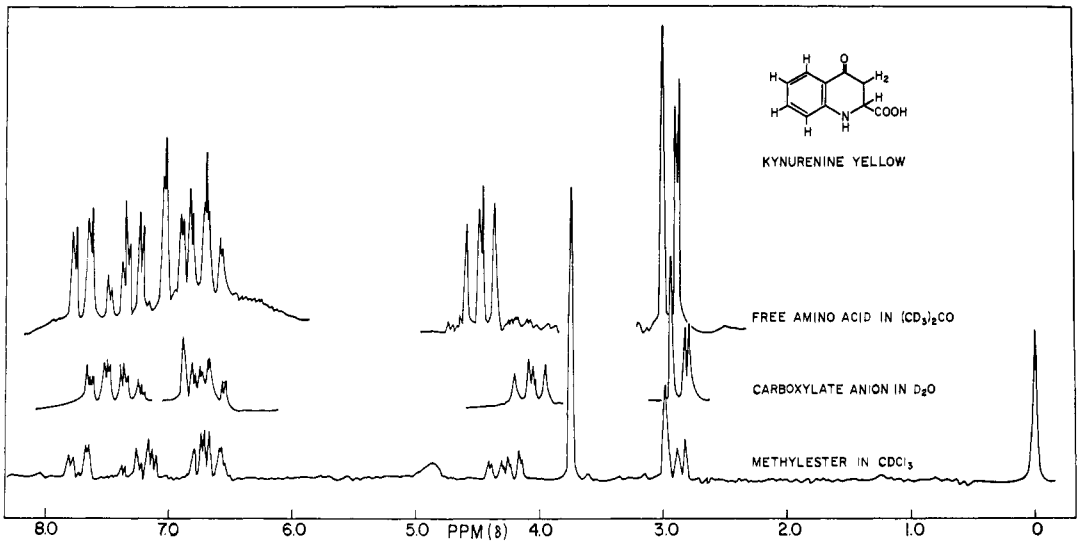


Figure 2. Nmr spectra of kynurenine yellow as the free amino acid, the carboxylate form, and the methyl ester. In the carboxylate form the multiplet for the 2 H proton is shifted markedly upfield.

catalytic hydrogenation.[12-14] We first tried to effect this reduction with hydride ions.

In the presence of 1 equiv of sodium borohydride and after a short period of irradiation, the conversion of a small amount of kynurenic acid to kynurenine after 5 min. With a 0.1 *M* solution, the yield was increased to 30% after 15 min. Finally, with a 1.0 *M* solution of Na_2SO_3, a yield of 40% was obtained in 25 min. In all cases the same light source and the same 0.001 *M* solution of kynurenic acid were used (Figure 3). Decarboxylation, commonly observed in photoreactions of amino acids, did not become competitive with photoreduction, until the formation of kynurenine yellow approached the maximum yield in each case. The decarboxylation of kynurenic acid was easily detected by the typical ultraviolet absorption of 4-hydroxyquinoline (λ_{max} 315 and 327 mμ). On a preparative scale we were able to obtain kynurenine yellow in 53% yield taking into account recovered kynurenic acid.

Table I. Comparison of the Properties of the Natural Pigment from South American Butterflies with Synthetic Hydroxykynurenine and (Hydroxy)kynurenine Yellow

	Natural amino acid from *Heliconius eratophyllis*	3-Hydroxy-L-kynurenine	Hydroxy-kynurenine yellow	Kynurenine yellow
Paper chromatography[a]				
Fluorescence	Bright yellow	Bright yellow	Blue	Greenish yellow
Ninhydrin test	(+) Purple	(+) Purple	(−)	(−)
R_f	0.34 (0.49)[b]	0.35 (0.49)	0.82	0.87
Infrared spectrum, λ_{max} (Nujol), μ	6.14, 6.48	6.14, 6.48	5.85, 6.12	5.93, 6.05
Ultraviolet spectrum, λ_{max} (mμ) at pH 6.8 (0.15 *N* phosphate buffer)	370[c]	371	387	378
Specific rotation, deg	$[\alpha]^{20}D$ −45 (MeOH) $[\alpha]^{20}D$ +18 (MeOH + HCl)[e]	$[\alpha]^{27}D$ −34 (H_2O) $[\alpha]^{27}D$ + 8.5 (HCl)[d]	...	...

[a] Butanol–acetic acid–water (5:1:4). [b] These R_f values are only of diagnostic value when carried out simultaneously on the same strip of paper. Figures in parentheses are those obtained in the laboratory of K. S. Brown. [c] Previously reported as 378 mμ in neutral solution. [d] R. R. Brown, *J. Biol. Chem.*, **227**, 649 (1957). [e] *Cf.* ref 10.

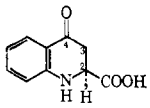
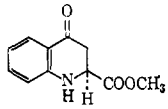
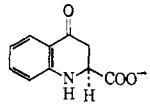
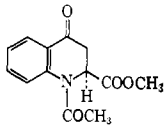
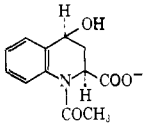
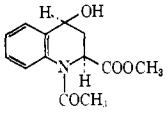
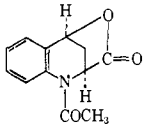
yellow and 1,2,3,4-tetrahydroquinolone-4 (II) was detected by thin layer chromatography. With a large excess of sodium borohydride at pH 10, photoreduction occurred immediately, to give a very unstable amino acid [λ_{max} 293 mμ (H_2O) ($\epsilon \sim 1800$)]. A large excess of $NaBH_4$ on methyl 8-methoxykynurenate without light reduced the ester function to the hydroxymethyl derivatives (mp 220–225°; no C=O in infrared, M^+ peak 205). The photoreduction was then carried out with *sodium sulfite as a reducing reagent.* The optimal yield of kynurenine yellow formed in this novel photoreduction depended solely on the concentration of sodium sulfite and not of kynurenic acid. With a 0.01 *M* solution of sodium sulfite the optimal yield was 15%

The Effect of Sodium Sulfite is Selective. Sodium bisulfite ($NaHSO_3$), sodium thiosulfate ($Na_2S_2O_3$), and sodium hydrosulfite ($Na_2S_2O_4$) were completely inactive as reducing reagents in the photoreduction of kynurenic acid.

So far, attempts to effect the photoreduction of xanthurenic acid or of its 8-O-acetate have not been suc-

(12) P. Cerutti, K. Ikeda, and B. Witkop, *J. Am. Chem. Soc.*, 87, 2505 (1965).
(13) G. Ballé, P. Cerutti, and B. Witkop, *ibid.*, **88**, 3946 (1966).
(14) O. Yonemitsu, P. Cerutti, and B. Witkop, *ibid.*, **88**, 3941 (1966).

Table II. Nmr Data of Kynurenine Yellow and Its Transformation Products

Structure[b]	Solvent	δ, ppm[a] (J, cps) 2 H	3 H_a	3 H_a and 3 H_b	3 H_b	4 H
	$(CD_3)_2CO$	4.45, q (6, 7.5)		2.90, (6, 7.5)		
	$CDCl_3$	4.31 q (6, 9.5)		2.93 (6, 9.5)		
	D_2O, $NaHCO_3$	4.06 q (6.5, 8.5)		2.85 (6.5, 8.5)		
	$CDCl_3$	4.46, t (4.5, 4.5)		3.13, d (4.5)		
	D_2O, $NaHCO_3$	4.73, q (8.5, 10)	2.83 (4.5, 8.5, 12)		1.63 (10, 11, 12)	4.58, q (4.5, 11)
	$CDCl_3$	5.20, t (8.5, 8.5)	2.56 (5, 8.5, 13)		2.20 (8.5, 8.5, 13)	4.83, q (5, 8.5)
	$CDCl_3$	5.23, (0.5, 9)	2.58 (9, 9, 12.5)		2.20 (0.5, 0.5, 12.5)	4.85 (0.5, 9)

[a] Abbreviations used are d, doublet; t, triplets; q, quadruplets. [b] Formulas represent L forms of the racemic amino acids and their derivatives.

cessful. The free phenolic hydroxyl group in the 8 position interferes with photoreduction. Its protection by acetyl is insufficient, because deacetylation is observed under the conditions of photoreduction.

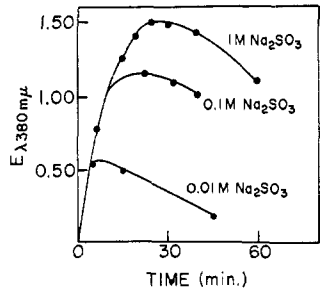


Figure 3. Dependence of the yield of kynurenine yellow III from kynurenic acid (I) on the concentration of sodium sulfite present during photoreduction. The concentration of the kynurenic acid was 0.001 *M*.

Identification of Yellow Pigment from Butterflies. The paucity of the pigment from butterfly wings necessitated mass spectrometric determination of the empirical formula. This led to the erroneous composition $C_{10}H_9NO_4$. We have now found that in the mass spectrometer 3-hydroxykynurenine undergoes loss of ammonia and cyclization to hydroxykynurenine yellow so easily that only a minor peak for the intact molecule, $C_{10}H_{12}N_2O_4$, is observed. This led us to compare the butterfly pigment with synthetic 3-hydroxy-DL-kynurenine and natural 3-hydroxy-L-kynurenine[15-17] (Table I). For a sample of the latter we are indebted to Professor P. da S. Lacaz. The comparison established identity with respect to ultraviolet, infrared, nmr, and mass spectral data as well as R_f values. 3-Hydroxykynurenine is a natural product which has been isolated before, *viz.* from pupae of *Calliphora*,[18] silkworms (*Bombyx mori*),[19] and as a urinary metabolite from patients with severe tuberculosis[20] and hemoblastosis.[21]

Experimental Section

cis-**N-Acetyl-1,2,3,4-tetrahydro-DL-kynurenic Acid (N-Acetyldihydrokynurenine Yellow, IX).** A solution of 4 mg of sodium borohydride in 0.5 ml of water was added to 46 mg of acetylkynurenine yellow V dissolved in 2 ml of water containing a small amount of sodium bicarbonate. The mixture was allowed to stand

(15) T. Sakan and S. Senoh, *J. Inst. Polytech., Osaka City Univ., Ser. C*, 1 35 (1950); M. Kotake, T. Sakan, and S. Senoh, *J. Am. Chem. Soc.*, 73, 1832 (1951).
(16) A. Butenandt, G. Hallmann, and R. Beckmann, *Chem. Ber.*, **90**, 1120 (1957); *cf.* A. Butenandt and G. Hallmann, *Z. Naturforsch.*, **5b**, 444 (1950).
(17) Y. Hirata and K. Nakanishi, *Bull. Chem. Soc. Japan*, **23**, 134 (1950).
(18) A. Butenandt, W. Weidel, and H. Schlossberger, *Z. Naturforsch.*, 4, 242 (1949).
(19) Y. Hirata, K. Nakanishi, and H. Kikkawa, *Japan. J. Genetics*, **24**, 188 (1949); *Science*, **112**, 307 (1950).
(20) K. Makino, K. Satoh, T. Fujiki, and K. Kawaguchi, *Nature*, **170**, 977 (1952).
(21) L. Musajo, C. A. Benassi, and A. Parpajola, *ibid.*, **175**, 855 (1955).

at room temperature. Within 0.5 hr, the ultraviolet absorption of the solution no longer showed the characteristic peak of acetylkynurenine yellow [λ_{max}^{EtOH} 323 mμ (ε 2300)]. The reaction mixture was acidified with hydrochloric acid and extracted with ethyl acetate. The residue of the ethyl acetate extract (44 mg) crystallized immediately. After recrystallization from ethyl acetate, acetyldihydrokynurenine yellow IX (32 mg) formed a colorless microcrystalline powder, mp 179.5°.

Anal. Calcd for $C_{12}H_{13}NO_4$: C, 61.27; H, 5.57; N, 5.91. Found: C, 61.03; H, 5.68; N, 6.02.

The infrared spectrum showed peaks at 2.85, 5.79, 5.81, 6.20, and 6.35 μ.

cis-**N-Acetyl-1,2,3,4-tetrahydrokynurenic Acid Lactone (X). A. By Dehydration with Acetic Anhydride.** A mixture of 162 mg of N-acetyldihydrokynurenine yellow IX and 4 ml of acetic anhydride was warmed on a steam bath for 4 hr and then evaporated *in vacuo*. The residue was treated with ethyl acetate and the organic solution extracted with aqueous sodium bicarbonate until it was free of any acidic compound. The ethyl acetate layer was concentrated until colorless prisms (140 mg) started to form. The lactone X had mp 140–141° after recrystallization from methanol. The sample for analysis (mp 142°) was dried *in vacuo* at 50° for 3 hr.

Anal. Calcd for $C_{12}H_{11}NO_3$: C, 66.35; H, 5.10; N, 6.45. Found: C, 66.59; H, 4.85; N, 6.38.

The infrared spectrum showed peaks at 5.60 (lactone carbonyl) and 5.98 μ (amide carbonyl).

B. By Dehydration with Dicyclohexylcarbodiimide. N-Acetyldihydrokynurenine yellow IX (150 mg) and 150 mg of dicyclohexylcarbodiimide were dissolved in 3 ml of dry dimethylformamide and allowed to stand at room temperature overnight. The addition of 10 ml of benzene to the reaction mixture precipitated dicyclohexylurea which was removed by filtration. The filtrate was washed with sodium bicarbonate solution and evaporated to dryness. The residue, 96 mg, immediately became a crystalline solid which, after recrystallization from methanol, had mp 140.5–142°. This product was identical with the γ-lactone X described under A, in every respect.

Conversion of N-Acetyldihydrokynurenine Yellow IX to Quinaldinic Acid (XI). N-Acetyldihydrokynurenine yellow (180 mg) was warmed in 9 ml of 2.0 *N* hydrochloric acid on a steam bath for 1 hr. The clear solution was evaporated *in vacuo*. The residue was dissolved in a minimal volume of hydrobromic acid (48%) and allowed to stand in the cold room overnight. The deposited crystalline salt (44 mg) decomposed, after recrystallization from concentrated hydrobromic acid, at 239°.

Anal. Calcd for $C_{10}H_7NO_2 \cdot HBr \cdot 1.5H_2O$: C, 42.87; H, 3.96; N, 5.20. Found: C, 42.72; H, 3.68; N, 4.93.

This salt was identical with an authentic sample of the hydrobromide of quinaldinic acid.

Photoreduction of Kynurenic Acid (I) to Kynurenine Yellow III. A. With Sodium Borohydride. The light source used in this experiment was a quartz mercury arc lamp, Hanovia 654 A-36, 200 w, with Pyrex filter 7740. The sample solution was irradiated in a prismatic quartz cuvet at a distance of about 2.5 cm from the light source. An aqueous solution (3 ml) of kynurenic acid (0.01 *M*) was adjusted to pH 10 with alkali. To this solution was added an equimolar amount of sodium borohydride (1.14 mg). A rapid stream of nitrogen was bubbled through the solution to remove dissolved oxygen. After 5 min of ultraviolet irradiation, the solution was acidified with hydrochloric acid and the deposited kynurenic acid removed by filtration. The filtrate was extracted three times with ethyl acetate. Thin layer chromatograms on silica gel of the extract showed three spots with R_f values of 0.35, 0.27, and 0.06, in the system ethyl formate–toluene–formic acid 4:5:1. The two spots of R_f 0.35 and 0.27 show a brilliant green fluorescence under ultraviolet light and are identical with spots of authentic 2,3-dihydro-4-quinolone (*II*) and kynurenine yellow III. The third spot, R_f 0.06, has a blue-white fluorescence under ultraviolet light and is identical with kynurenic acid (I).

B. With Sodium Sulfite. The light source used in this experiment was a quartz mercury arc lamp, Hanovia 679 A-36, 450 w, with Pyrex filter 7740. The sample solution was irradiated with the light source inside a cooling jacket surrounded by two semicircular, quartz irradiation chambers whose mean distance from the light source was about 4 cm. Kynurenic acid (414 mg, 2 mmoles) was dissolved in 200 ml of water containing 1.25 equiv of sodium hydroxide. To this solution, 32.3 g of anhydrous sodium sulfite was added. The solution was illuminated with the ultraviolet light for 8.5 hr while a steady stream of nitrogen was bubbled through it. The yellow solution was extracted with ethyl acetate in order to separate the nonacidic fraction (14 mg). The latter consisted mainly of kynurine (4-quinolone) and dihydro-4-quinolone in a ratio of 65:35 on the basis of ultraviolet absorption spectra and thin layer chromatography. When the aqueous layer was acidified, unreacted kynurenic acid (120 mg) deposited. The mother liquor was extracted three times with ethyl acetate. The combined extracts were concentrated *in vacuo* to yield 145 mg of yellow crystals. Thin layer chromatography shows that, apart from a very minor amount of kynurenic acid, the crystals are practically pure kynurenine yellow. Recrystallization from ethyl acetate–cyclohexane gave pure kynurenine yellow, mp 183–184°. The analytical sample was dried at 50° *in vacuo* for 2 hr.

Anal. Calcd for $C_{10}H_9NO_3$: C, 62.82; H, 4.75; N, 7.33. Found: C, 63.08; H, 4.80; N, 7.09.

1,2,3,4-Tetrahydro-4-keto-2-carbomethoxyquinoline (IV). To a methanolic solution of 20 mg of kynurenine yellow was added an excess of ethereal diazomethane. After the removal of excess diazomethane *in vacuo*, the ethereal layer was washed with sodium bicarbonate and condensed to yield 19 mg of kynurenine yellow methyl ester IV. Recrystallization from chloroform–cyclohexane gave yellow needles, mp 89°.

The infrared spectra showed peaks at 2.98 (NH), 5.75 (ester carbonyl), and 6.05 μ (quinolone carbonyl).

N-Acetyl-1,2,3,4-tetrahydro-4-keto-2-carbomethoxyquinoline. To a methanolic solution of 50 mg of acetylkynurenine yellow[6] was added an excess of ethereal diazomethane. After the removal of excess diazomethane *in vacuo*, the ethereal layer was washed with sodium bicarbonate and condensed to dryness. Recrystallization of the residue from benzene gave colorless prisms of the methyl ester of acetylkynurenine yellow, mp 146–147°.

The infrared spectra showed peaks at 5.78 (ester carbonyl), 5.85 (ketone carbonyl), and 6.00 μ (amido carbonyl).