29. *The Synthesis of* N′-*Formyl*-DL-*kynurenine*, N$^{\alpha}$-*Acetyl*-DL-*kynurenine and Related Compounds, and Observations on the Synthesis of Kynurenine.*

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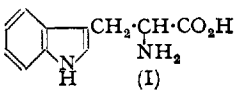
Exclusive formylation of the aromatic amino-group of kynurenine to give, in high yield, *N*′-formyl-DL-kynurenine (II) can be accomplished under suitable conditions. Syntheses of *N*$^{\alpha}$-acetyl-DL-kynurenine (III), *N*$^{\alpha}$-acetyl-*N*′-formyl-DL-kynurenine (IV; R = CHO, R′ = Ac), and related compounds are also reported, together with observations on the formylation of amino-groups and on the synthesis of DL-kynurenine

WORK in progress in these laboratories on the intermediary metabolism of tryptophan (I) (cf., *e.g.*, Dalgliesh, Knox, and Neuberger, *Nature*, 1951, **168**, 20) has made it desirable to prepare various derivatives of kynurenine which occur in, or are related to, the tryptophan metabolic chain (Dalgliesh, *Quart. Reviews*, 1951, **5**, 227). Recent work (Knox and Mehler, *J. Biol. Chem.*, 1950, **187**, 419, 431) has shown that *N*′-formylkynurenine (II) is the first identifiable intermediate in the normal biological degradation of tryptophan. Formylkynurenine has not been isolated from biological systems, in which it is normally split by the enzyme formylase to kynurenine and formic acid, but the evidence for its participation in these systems is weighty. In the laboratory it has hitherto only been prepared with some difficulty, and in low yields, by the ozonisation of tryptophan (Witkop and Graser, *Annalen*, 1944, **556**, 103; Amano, Torii, and Irritani, *Med. J. Osaka Univ.*, 1950, **2**, 45; Knox and Mehler, *loc. cit.*) and a more convenient synthesis was desirable to facilitate further investigation of its biological behaviour. Two other derivatives of kynurenine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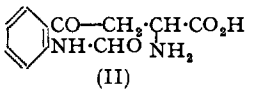
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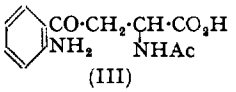
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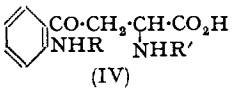
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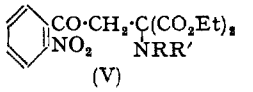
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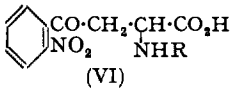
(VI)

are also of possible importance in tryptophan metabolism. *N*$^{\alpha}$-Acetyl-L-kynurenine (III) was isolated from cultures of a *Neurospora* mutant in which there existed a genetic block preventing further metabolism of kynurenine by the normal pathway (Yanofsky and Bonner, *Proc. Nat. Acad. Sci.*, 1950, **36**, 167). It has also been detected in the urine of pyridoxine-deficient rats fed on tryptophan (Dalgliesh, Knox, and Neuberger, *loc. cit.*), and it is possible (cf. Dalgliesh, *loc. cit.*) that *N*$^{\alpha}$-acetylkynurenine may lie on the direct tryptophan metabolic pathway in animals. If such were the case, then *N*$^{\alpha}$-acetyl-*N*′-formylkynurenine (IV; R = CHO, R′ = Ac) might be an intermediate lying between (II) and (III). This paper describes, first, improvements in the synthesis of DL-kynurenine (IV; R = R′ = H), and, secondly, the synthesis of *N*′-formyl-DL-kynurenine (II), *N*$^{\alpha}$-acetyl-DL-kynurenine (III), *N*$^{\alpha}$-acetyl-*N*′-formyl-DL-kynurenine (IV; R = CHO, R′ = Ac), and of various related compounds. The preparation of optical isomers will be described in a later communication.

DL-Kynurenine was first synthesised by Butenandt and his co-workers (*Naturwiss.*, 1942, **30**, 51; *Z. physiol. Chem.*, 1943, **279**, 27) by condensing *o*-nitrophenacyl bromide with sodiophthalimidomalonic ester to give [V; RR′ = *o*-$C_6H_4(CO)_2$], which was hydrolysed to the hydrochloride of *o*-nitrophenacylglycine (α-amino-β-*o*-nitrobenzoylpropionic acid) (VI; R = H), the latter being then reduced Subsequently the synthesis was improved by the use of benzamidomalonic ester (Butenandt, Weidel, and Neckel, *ibid.*, 1944, **281**, 120) but in this case the intermediate (V; R = H, R′ = Bz), obtained as a thick red oil, was not purified. Although other synthetic routes to kynurenine are available (Sakan, *J. Chem. Soc. Japan*, 1942, **63**, 1545; Butenandt and Hellmann, *Z. Naturforsch.*, 1950,

5b, 445) the acylamidomalonate route was felt to be preferable in that it combined relative simplicity with formation of an intermediate (VI; R = H) in which the aliphatic amino-group could be modified before the aromatic amino-group was introduced. Acetamidomalonic ester is more readily available than the phthalimido- or the benzamido-analogue, and its use would avoid subsequent troublesome separations of phthalic or benzoic acid. It was found essential in using the acetamido-compound to effect the condensation of the sodio-derivative with the bromide at room temperature to avoid formation of dark by-products. Under these conditions the ester (V; R = H, R' = Ac) was readily obtained. Although the yield of recrystallised ester was only 40%, use of this pure intermediate gave yields in the subsequent hydrolysis and hydrogenation exceeding 90% in each step, and eliminated wasteful purifications required in the previous methods. The same procedure was satisfactory with phenacyl bromide, giving, ultimately, phenacylglycine (as VI).

Conditions have been found under which it is possible to formylate the aromatic amino-group of kynurenine without affecting the aliphatic amino-group. In the first experiment kynurenine was dissolved in formic acid (98—100%), one equivalent of acetic anhydride added, and after two hours the mixture poured into ether, precipitating a cream-coloured solid. The ultra-violet spectra of *N'*-acylated kynurenines show two maxima, at about 260 and 320 mμ, whereas kynurenine and N^{α}-acylated kynurenines show maxima at about 260 and 360 mμ (cf. Knox and Mehler, *loc. cit.*). Spectroscopic examination of the reaction product showed that acylation had occurred solely on the aromatic amino-group, the low absorption at 360 mμ and absence of any sign of a peak at this point showing the absence from the product of kynurenine or a N^{α}-acylkynurenine. The product contained a free α-amino-group (ninhydrin) and was markedly water-soluble. The pH of the solution (~3·5) made it likely that the material was a salt, and addition of one equivalent of pyridine to its aqueous solution precipitated the amino-acid which analysis showed, rather surprisingly, to be *N'*-acetyl-DL-kynurenine, and not the formyl compound. The salt was the formate thereof. The free acetyl compound showed a marked tendency to form gels, but could be obtained with difficulty as crystals of hydrated material. On removal of the water at elevated temperatures the product became very hygroscopic.

When two equivalents of acetic anhydride were added to kynurenine in formic acid, diacylation occurred smoothly, and the product contained one acetyl and one formyl group. As the aromatic amino-group was found above to have been acetylated under similar conditions, the product was *N'*-acetyl-N^{α}-formyl-DL-kynurenine (IV; R = Ac, R' = CHO). It seemed, therefore, that under the conditions used aromatic amino-groups were acetylated and aliphatic amino-groups were formylated. This was confirmed by the finding that N^{α}-acetylkynurenine (III; see below) was acetylated to give *N'*N^{α}-diacetyl-DL-kynurenine (IV; R = R' = Ac), whereas phenacylglycine, *o*-nitrophenacylglycine, tryptophan, and phenylalanine were all formylated.

Under the same conditions anthranilic acid underwent mainly acetylation. The active agent in formylations using formic acid and acetic anhydride is presumably acetic formic anhydride, which in reactions with alcohols and aromatic amines is known to give solely formyl derivatives (Béhal, *Compt. rend.*, 1900, **128**, 1460; Hurd *et al.*, *J. Amer. Chem. Soc.*, 1939, **61**, 3355; 1946, **68**, 789). The occurrence of acetylation in the above reactions must therefore have been due to the more rapid reaction of the acetic anhydride with the aromatic amino-groups than with formic acid. In confirmation of this it was found that, if the acetic anhydride was added to the formic acid (heat is evolved), and after 30 minutes anthranilic acid was added, solely formylation occurred.

On applying these modified conditions, kynurenine gave *N'*-formyl-DL-kynurenine (II), in a yield of over 90%. As in the case of the corresponding acetyl compound, a tendency to gel formation was observed, and the compound was very hygroscopic. The ultra-violet absorption spectrum agreed with that found by Knox and Mehler (*loc. cit.*), and identity was confirmed by examination of the behaviour towards the enzyme formylase, kindly carried out by Dr. W. E. Knox: the synthetic material was hydrolysed by the enzyme at approximately seven times the rate found for the reference compound, formyl-

anthranilic acid, this ratio agreeing within experimental error with that found for formylkynurenine formed during tryptophan degradation; the rate of hydrolysis was linear with time, also a characteristic of natural formylkynurenine; *N'*-acetyl-DL-kynurenine was hydrolysed by the formylase preparation at about one-tenth of the rate of the *N'*-formyl compound, also agreeing with expectation. Further formylation of the *N'*-formyl compound gave $N'N^{\alpha}$-diformyl-DL-kynurenine.

N^{α}-Acetyl-DL-kynurenine (III) was prepared by hydrogenation of *N*-acetyl-*o*-nitrophenacylglycine (VI; R = Ac). Attempted acetylation of *o*-nitrophenacylglycine with acetic anhydride alone, or with sodium acetate, pyridine, or sulphuric acid, gave only dark intractable products, and similar results were obtained on the attempted hydrolysis of the ester (V; R = H, R' = Ac) by carbonate (cf. Albertson, *J. Amer. Chem. Soc.*, 1950, **72**, 1396). However, acetylation proceeded satisfactorily under Schotten–Baumann conditions. Also prepared under these conditions was *N*-carbethoxy-*o*-nitrophenacylglycine (VI; R = CO_2Et), which was designed as an intermediate for an alternative synthesis of *N'*-formylkynurenine. Hydrogenation gave N^{α}-carbethoxy-DL-kynurenine which when heated in a sealed tube with ethyl formate (Human and Mills, *J.*, 1948, 1457) gave, probably, N^{α}-carbethoxy-*N'*-formyl-DL-kynurenine. In view of the success attending direct formylation of kynurenine removal of the carbethoxy-group was not attempted.

N^{α}-Acetylkynurenine (III) was submitted to the modified formylation conditions described above, and the required N^{α}-acetyl-*N'*-formyl-DL-kynurenine (IV; R = CHO, R' = Ac) was obtained.

The monoacyl derivatives of DL-kynurenine show great resistance to crystallisation. In spite of many attempts N^{α}-acetyl-DL-kynurenine (III) could only be obtained as a somewhat hygroscopic glass, although analysis and paper-chromatographic examination demonstrated its homogeneity. The gel-forming tendency of *N'*-acetyl- and *N'*-formyl-DL-kynurenines has already been noted. The diacyl derivatives of kynurenine, and the nitro-compounds corresponding to kynurenine, crystallise readily.

In view of recent reports on the resolution by means of paper-chromatography of amines (Bonino and Carassiti, *Nature*, 1951, **167**, 569) and amino-acids (Kotake *et al.*, *J. Amer. Chem. Soc.*, 1951, **73**, 2973) it is interesting that DL-kynurenine is readily resolved on paper (Whatman No. 4) by use of butanol–acetic acid–water (4 : 1 : 5) (Partridge, *Nature*, 1946, **158**, 270). The D-isomer travels under these conditions at about 0·9 times the rate of the L-isomer.

Experimental

Formic acid used was 98—100% (B.D.H.). M. p.s are uncorrected.

Preparation of DL-*Kynurenine.*—Condensation of *o*-nitrophenacyl bromide and sodioacetamidomalonic ester in dry benzene under reflux resulted in a thick red-brown oil similar to that described by Butenandt *et al.* for the analogous benzamido-compound. No crystalline material could be obtained from the product, and the *o*-nitrophenacylglycine hydrochloride obtained on hydrolysis was contaminated with much tar, the removal of which was very wasteful. Hydrogenation to kynurenine did not proceed satisfactorily unless pure nitro-compound was used. The difficulties obviously proceeded from side-reactions in the initial condensation, and many other media were therefore tried. At higher temperatures results were worse, *e.g.*, in boiling xylene the whole mixture charred. The product (V; R = H, R' = Ac) was obtained crystalline in low yield after condensation in boiling alcohol, and it was then found that condensation in ethanol at room temperature occurred smoothly, the product separating from solution. Condensation at room temperature in dimethylformamide (Sheehan and Bolhoffer, *J. Amer. Chem. Soc.*, 1950, **72**, 2786) was so vigorous that a brown by-product was formed in considerable quantity, and the same occurred to a lesser extent in methanol. The following procedure has been found satisfactory.

Sodium (1·8 g.) was dissolved in ethanol (100 ml.) and ethyl acetamidomalonate (19·2 g.) added. To the solution, cooled to room temperature, was added *o*-nitrophenacyl bromide (18·6 g.), and the whole shaken overnight. The mixture was set aside for a few hours at −3°, then filtered, and the residue washed well with water and dried. Recrystallisation from ethyl acetate (with addition of light petroleum if necessary) gave *ethyl acetamido-o-nitrophenacylmalonate* (V; R = H, R' = Ac) as pale cream-coloured crystals, m. p. 124° (14·0 g., 41%) (Found: C, 53·9; H, 5·5; N, 7·7. $C_{17}H_{20}O_8N_2$ requires C, 53·7; H, 5·3; N, 7·4%).

The ester (19 g.) was refluxed with acetic acid (40 ml.) and concentrated hydrochloric acid (40 ml.) for 5 hours, a further total of 40 ml. of hydrochloric acid being added at intervals. The mixture was cooled, poured into water, any slight precipitate being removed by filtration, and extracted three times with ether. The aqueous fraction was taken to dryness in a vacuum on the water-bath, the residue taken up in boiling alcohol, and filtered, and ether added to the cooled filtrate, to give *o*-nitrophenacylglycine hydrochloride as a voluminous colourless precipitate, isolated after a few hours at 0° (yield, 13 g., 94%).

On addition of one equivalent of pyridine to a hot aqueous solution of the hydrochloride DL-*o*-*nitrophenacylglycine* (VI; R = H), white needles (from water), decomp. at 195°, separated (Found: C, 51·0; H, 4·0; N, 11·6. $C_{10}H_{10}O_5N_2$ requires C, 50·4; H, 4·2; N, 11·7%).

The nitro-hydrochloride (10 g.) in N-sulphuric acid (73 ml.) was hydrogenated over palladium–charcoal. The catalyst was removed and washed with hot water, and pyridine (9 ml.) added to the filtrate. DL-Kynurenine (3·4 g.) separated on storage and the mother-liquors on concentration gave further material (3·6 g.).

Preparation of Phenacylglycine (α-*Amino*-β-*benzoylpropionic Acid*).—The condensation was repeated as above, with phenacyl bromide. The reaction mixture was poured into water, and the precipitated solid hydrolysed directly, to give the *amino-acid hydrochloride*, white silky needles (from ethanol), decomp. 205°, which on addition of one equivalent of pyridine to its aqueous solution gave DL-phenacylglycine, colourless crystals (from water), decomp. 200° (Found: C, 62·1; H, 6·0; N, 7·4. Calc. for $C_{10}H_{11}O_3N$: C, 62·2; H, 5·7; N, 7·3%). This acid has been prepared by an alternative route by Fraser and Raphael (*J.*, 1950, 2245).

N′-*Acetyl*-DL-*kynurenine Formate*.—To kynurenine (1·2 g.) in formic acid (4 ml.) was added acetic anhydride (0·55 ml., 1 mol.). After 2 hours at room temperature the mixture was poured, with shaking, into ether (500 ml.), precipitating N′-*acetyl*-DL-*kynurenine formate* as an almost colourless solid (1·33 g., 78%) The ultra-violet absorption spectrum, determined in M/5-phosphate buffer, pH 7·0, diluted 1 : 10, showed maxima at 259 (ε = 9740) and 320 mμ (ε = 3080), and no sign of a peak at 360 mμ. The absence of kynurenine or an N^{α}-acylkynurenine was confirmed by paper-chromatography, which however suggested the presence of a small amount of the *N*′-formyl compound. The substance showed a great tendency to form gels, and was purified by dissolution in cold formic acid and gradual precipitation by ether at 0°. It then decomposed at 136° (Found: C, 52·8, 52·3; H, 5·4, 5·5; N, 9·7. $C_{12}H_{14}O_4N_2,CH_2O_2$ requires C, 52·7; H, 5·4; N, 9·5%). The free hydrated acetyl compound was obtained on addition of pyridine to an aqueous solution of the formate. On being dried in a vacuum at elevated temperatures the product lost water and became very hygroscopic and difficult to analyse. The product obtained was approximately a *hemihydrate* (Found: C, 55·2; H, 5·5; N, 11·0. $C_{12}H_{14}O_4N_2,\frac{1}{2}H_2O$ requires C, 55·6; H, 5·8; N, 10·8%).

N′-*Acetyl*-N^{α}-*formyl*-DL-*kynurenine* (IV; R = Ac, R′ = CHO).—To kynurenine (1·2 g.) in formic acid (4 ml.) was added acetic anhydride (1·5 ml.). Crystals soon separated, and after 2 hours a little water was added, to give the N′-*acetyl*-N^{α}-*formyl* compound in almost theoretical yield, as cream-coloured crystals from ethanol, decomposing at 224° on fairly rapid heating (Found: C, 55·8, 55·9; H, 5·0, 5·1; N, 9·9, 10·1. $C_{13}H_{14}O_5N_2$ requires C, 56·1; H, 5·0; N, 10·1%).

N′N^{α}-*Diacetyl*-DL-*kynurenine* (IV; R = R′ = Ac).—To N^{α}-acetylkynurenine (0·8 g.; see below) in formic acid (2 ml.) was added acetic anhydride (0·32 ml.). Crystals soon separated, and after 2 hours addition of a little water completed precipitation. The diacetyl compound formed cream-coloured crystals from ethanol, decomposing at 185° (Found: C, 57·3, 57·6; H, 5·7, 5·9; N, 9·8. Calc. for $C_{14}H_{16}O_5N_2$: C, 57·5; H, 5·5; N, 9·6%). The ultra-violet absorption spectrum determined in 20% alcohol showed maxima at 260 (ε = 9760) and 320 mμ (ε = 2980). Diacetylkynurenine has been made by Butenandt *et al.* (*Z. physiol. Chem.*, 1943, **279**, 27) by treatment of kynurenine with keten.

Formylation of Phenacylglycine, o-Nitrophenacylglycine, and Tryptophan.—To the amino-acid dissolved in formic acid was added one equivalent of acetic anhydride. After 2 hours separation of the product, obtained in all cases in high yield, was completed by the addition of water. *Formyl*-DL-*phenacylglycine*, colourless prisms (from methanol), decomposed at 199° (Found: C, 59·9; H, 5·3; N, 6·6. $C_{11}H_{11}O_4N$ requires C, 59·7; H, 5·0; N, 6·3%). *Formyl*-DL-*o*-*nitrophenacylglycine* (VI; R = CHO), silky white needles from methanol, decomposed at 178° (Found: C 49·9; H, 3·9; N, 10·7. $C_{11}H_{10}O_6N_2$ requires C, 49·6; H, 3·8; N, 10·5%). *Formyl*-DL-*tryptophan*, colourless crystals (from water), had m. p. 167—168° (slight decomp.) (Found: C, 61·7; H, 5·2. $C_{12}H_{12}O_3N_2$ requires C, 62·1; H, 5·2%).

N′-*Formyl*-DL-*kynurenine* (II).—A cooled mixture of acetic anhydride (1 ml.) in formic acid (2 ml.) was set aside for 30 minutes and then added to kynurenine (2·08 g.) in formic acid (4·5 ml.). After 2 hours the mixture was poured, with shaking, into ether, and after a few hours at 0° the product was isolated (2·55 g., almost 100%). The product contained approx. 0·3 mol. of formic acid, as determined on a sample by electrometric titration. The material was taken up in hot water, the calculated amount of N-sodium hydroxide added, and the whole cooled with shaking. Cream-coloured needles separated which were obviously highly hydrated, as judged by their shrinkage on drying. The material when recrystallised from water and dried in a vacuum-desiccator over phosphoric oxide overnight still contained some water, which was removed in a vacuum at an elevated temperature. The dried compound decomposed at 162° (Found : C, 55·8; H, 5·5; N, 12·1. $C_{11}H_{12}O_4N_2$ requires C, 55·9; H, 5·1; N, 11·9%). The ultra-violet absorption spectrum determined in M/5-phosphate buffer, pH 7·0, diluted 1 : 10, showed maxima at 260 (ε = 10,980) and 321 mμ (ε = 3750).

N′N^{α}-*Diformyl*-DL-*kynurenine* (IV; R = R′ = CHO) was obtained on further formylation of *N′*-formylkynurenine. It formed cream-coloured crystals (from ethanol), decomposing at 213° (Found : C, 54·6; H, 4·7; N, 10·4. $C_{12}H_{12}O_5N_2$ requires C, 54·6; H, 4·5; N, 10·6%).

N-*Acetyl*-DL-*o*-*nitrophenacylglycine* (VI; R = Ac).—*o*-Nitrophenacylglycine hydrochloride (5·5 g.) in N-sodium hydroxide (80 ml.) was shaken with acetic anhydride (4 ml.). When the reaction was complete, dark material was removed from the alkaline solution by extraction with ethyl acetate. The aqueous fraction was acidified and the product allowed to separate at 0°. Recrystallisation from ethyl acetate gave the *acetyl* compound as cream-coloured crystals, m. p. 190° (Found : C, 51·2; H, 3·9; N, 9·9. $C_{12}H_{12}O_6N_2$ requires C, 51·4; H, 4·3; N, 10·0%).

N^{α}-*Acetyl*-DL-*kynurenine* (III).—The nitro-compound in ethanol solution was hydrogenated over palladium–charcoal at room temperature and atmospheric pressure, the theoretical amount of hydrogen being taken up smoothly. After removal of the catalyst and solvent the *product* was obtained as a rather hygroscopic yellow glass which could not be induced to crystallise (Found : C, 57·7; H, 5·5; N, 11·0. $C_{12}H_{14}O_2N_2$ requires C, 57·6; H, 5·6; N, 11·2%). It was shown to be homogeneous by paper-chromatography (cf. Dalgliesh, Knox, and Neuberger, *loc. cit.*) and on paper gave an immediate orange Ehrlich's reaction, no ninhydrin reaction, could be diazotised and coupled, and showed the same colour of fluorescence as kynurenine.

N^{α}-*Acetyl*-N′-*formyl*-DL-*kynurenine* (IV; R = CHO, R′ = Ac).—To a mixture of formic acid (2 ml.) and acetic anhydride (0·6 ml.) was added after 30 minutes a solution of N^{α}-acetylkynurenine (1·7 g.) in formic acid (4 ml.). After 2 hours separation of the product in high yield was completed by addition of water and setting the whole aside in the cold room. The N^{α}-*acetyl*-N′-*formyl* compound formed cream-coloured crystals (from ethanol), decomposing at 194° (Found : C, 55·9; H, 4·9. $C_{13}H_{14}O_5N_2$ requires C, 56·1; H, 5·0%).

N^{α}-*Carbethoxy*-DL-*o*-*nitrophenacylglycine* (VI; R = CO_2Et).—Carbethoxylation carried out under Schotten–Baumann conditions as for acetylation above, but using ethyl chloroformate, gave the *carbethoxy*-compound, stout almost colourless needles (from ethyl acetate–light petroleum), m. p. 154° (Found : C, 50·6; H, 4·6; N, 8·8. $C_{13}H_{14}O_7N_2$ requires C, 50·3; H, 4·5; H, 9·0%).

N^{α}-*Carbethoxy*-DL-*kynurenine*.—Hydrogenation as for the N^{α}-acetyl compound gave a gum which was induced to crystallise after some difficulty, recrystallisation from ethyl acetate–light petroleum giving pale yellow crystals of N^{α}-*carbethoxy*-DL-*kynurenine*, m. p. 120° (Found : N, 10·1. $C_{13}H_{16}O_5N_2$ requires N, 10·0%). The material behaved like kynurenine in the colour of its fluorescence and in its reaction to diazotisation and Ehrlich's reagent, but did not give a ninhydrin reaction.

Formylation of Carbethoxykynurenine.—Carbethoxykynurenine (0·2 g.) in ethyl formate (3 ml.) was heated in a sealed tube at 100° for 14 hours. Removal of excess of ethyl formate left a glass which appeared probably to be N^{α}-*carbethoxy*-N′-*formyl*-DL-*kynurenine* (Found : N, 8·8. $C_{14}H_{16}O_6N_2$ requires N, 9·1%), but its purification and hydrolysis were not investigated.

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