dialysed away when cell suspensions were dialysed in a cellophan sausage skin in running tap water and in distilled water. Ultrasonic irradiation at 350 kcy./sec. under an atmosphere of air, carbon dioxide or nitrogen resulted in inactive suspensions. The decrease in activity apparently paralleled the rupturing of the cells. Allinson (1943) and Nichol & Michaelis (1947) were also unsuccessful in obtaining active cell-free enzyme preparations.

SUMMARY

1. Cell suspensions of *Pseudomonas fluorescens* oxidized nicotinic acid, consuming 3.9 molecules of oxygen per molecule of nicotinic acid attacked.

2. The respiratory quotient was low at the start of the oxidation but rose to 0.90 towards the end of the respiration.

3. The degradation of the nicotinic acid did not take place under anaerobic conditions. The enzyme involved in the initial attack on the molecule is therefore a true oxidase.

4. The evidence obtained very strongly supports the conclusion that the first step in the degradation of the nicotinic acid molecule is the opening of the pyridine ring.

5. The specificity of the enzyme has been studied. If the carboxyl group of the nicotinic acid

molecule is treated to form the amide, or replaced by a sulphonic acid group, an amino group, or a sulphanilamide group, or removed entirely, the enzyme is inactive on the resulting compound.

6. Indolylpropionic acid and L-tryptophan were not attacked.

7. Aromatic diketones and cysteine inhibited nicotinic acid oxidation. The effect of one could be neutralized by the other at specific concentration ratios.

8. The enzyme studied contains a non-dialysable metal.

9. Attempts to obtain active cell-free enzyme preparations were unsuccessful.

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o-Phenylenediamine and 1:2-Diamino-4-nitrobenzene as Reagents for α -Keto Acids

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It is possible to isolate and characterize as 2:4-dinitrophenylhydrazones many of the keto acids formed by *Penicillium chrysogenum* and thus to study the mould's intermediary metabolism. These hydrazones are, however, sometimes neither adequately stable nor chromatographically separable.

The condensation of *o*-phenylenediamine (OPD) with α -ketonic acids has been shown to yield stable

quinoxalinols (cf. Hinsberg, 1887), which were found to be separable by paper chromatography. These derivatives were most useful, but it was thought that the introduction of a chromogenic grouping might make chromatography easier. We chose 1:2-diamino-4-nitrobenzene (DANB) in preference to a diazo derivative, as this might involve an increase in molecular size such as to swamp the effect of differences in the original

keto acids. The products are compounds of the general formula I. The position of the nitro group (x , 6 or 7) has not yet been determined.

EXPERIMENTAL

Synthesis of nitroquinoxalinol derivatives of α -ketoacids

2-Hydroxy-3-methyl-x-nitroquinoxaline. To 110 mg. pyruvic acid (1.25 m-mole) in 10 ml. water was added a solution of 190 mg. (1.25 m-mole) DANB in 10 ml. 2N-HCl. After 5 min. a precipitate appeared, and after 2 hr. the resulting quinoxalinol was filtered off and dried (yield 160 mg.). The product was twice recrystallized from hot ethanol-water; m.p. 245°. (Found: C, 52.5; H, 3.5; N, 20.3. $C_9H_7N_3O_3$ requires C, 52.7; H, 3.4; N, 20.5%.)

2-Hydroxy-3-isopropyl-x-nitroquinoxaline. To 145 mg. dimethylpyruvic acid (1.25 m-mole) in 10 ml. water were added 10 ml. of a solution of 190 mg. DANB in 10 ml. 2N-HCl. After 5 min. the product began to precipitate. After 2 hr. it was filtered off and dried (yield 190 mg.). The product was twice recrystallized from hot ethanol; m.p. 257°. (Found: C, 57.1; H, 4.9; N, 17.8. $C_{11}H_{11}N_3O_3$ requires C, 56.7; H, 4.7; N, 18.0%.)

2-Hydroxy-3-phenyl-x-nitroquinoxaline. To 188 mg. benzoylformic acid (1.25 m-mole) in 10 ml. water were added 10 ml. of a solution of 190 mg. DANB in 10 ml. 2N-HCl. There was an immediate precipitate. After 2 hr., it was filtered off and dried (yield 231 mg.). The product was twice recrystallized from hot ethanol; m.p. 264°. (Found: C, 63.1; H, 3.6; N, 15.3. $C_{14}H_9N_3O_3$ requires: C, 62.9; H, 3.4; N, 15.7%.)

2-Hydroxy-3-(2-carboxyethyl)-x-nitroquinoxaline. To 182 mg. α -ketoglutaric acid (1.25 m-mole) in 10 ml. water were added 10 ml. of a solution of 190 mg. DANB in 10 ml. 2N-HCl. After 10 min. a precipitate began to form slowly. After 2 hr. it was filtered off and dried (yield 280 mg.). The product was twice recrystallized from a 1:4 mixture of ethyl acetate and light petroleum (b.p. 60–80°); m.p. 204°. (Found: C, 50.2; H, 3.8; N, 15.9. $C_{11}H_9N_3O_5$ requires C, 50.2; H, 3.4; N, 16.0%.)

Separation of quinoxalinols by paper chromatography

Large sheets of Whatman no. 4 paper were cut into 30 mm. strips perpendicular to the 'grain'. About 50 μ g. of derivative (from OPD or DANB) were applied in about 20 μ l. of ethyl acetate or $CHCl_3$, about 60 mm. from the end of the paper. The papers were hung, by means of small (6 mm.) 'bull-dog' clips, from the rubber stoppers of 500 ml. graduated cylinders, so as to dip into 25 ml. of solvent therein. They were developed at room temperature until the solvent front had risen into the top tenth of the strip. The strips were removed and air-dried at room temperature.

Location of spots. After drying, the NDB derivatives were visible as pale-yellow spots in daylight or, more clearly, as dull-brown spots against a purplish background under ultraviolet light. The yellow colour could also be accentuated by spraying with a 2% ethanolic solution of KOH.

The OPD derivatives were colourless in daylight, but showed clearly as yellowish fluorescent patches under ultraviolet light. In addition, spraying with a 0.1% aqueous solution of 2-chloro-4-nitrophenyldiazonium naphthyl-2-sulphonate (Heinrich & Schuler, 1947, 1948) gave a pink spot with the derivative from pyruvic acid, although no coloured compound seemed to be formed by that from dimethylpyruvic, benzoylformic or α -ketoglutaric acids. This reaction was presumably due to coupling on the methyl group.

Chromatography of nitroquinoxalinols on alumina

The columns were made up by transferring a slurry of 30 g. of alumina (Peter Spence and Co., Grade H) in $CHCl_3$ into 16 mm. (internal diameter) tubes and allowing them to drain slowly, with occasional tapping to give a depth of about 130 mm.

About 60 mg. of the mixture of nitroquinoxalinols were applied in 2–3 ml. ethyl acetate. Development was carried out with the following succession of solvent mixtures: $CHCl_3$, $CHCl_3$ -ethanol mixtures (9:1, 4:1, 1:1, 1:4), ethanol, ethanol-water mixtures (9:1, 3:1, 1:1, 1:3) and ethanol-aqueous N-NaOH (1:3). If reasonably fast movement of the front band was obtained, the same solvent was continued until complete elution ('break-through') was obtained. The loading of these columns may be increased to about 50 mg. of each component, with some deterioration in sharpness of separation.

RESULTS

The nitroquinoxalinols from pyruvic acid, dimethylpyruvic acid, ketoglutaric acid and benzoylformic acid were synthesized as described in the experimental section.

Paper chromatographic separation of OPD derivatives of α -ketoacids

The quinoxalinols derived from pyruvic, dimethylpyruvic and α -ketoglutaric acids were found to be separable by filter paper chromatography (Table 1).

With the ammoniacal solvents the α -ketoglutarate spot was remarkably clear and compact.

Paper chromatographic separation of DANB derivatives of α -keto acids

The nitroquinoxalinols derived from pyruvic, dimethylpyruvic and α -ketoglutaric acids could be readily separated by paper-strip chromatography. The derivative from benzoylformic acid was found to streak badly with most solvents.

The best separations were achieved with dry or aqueous ethanol or methanol (Table 2). The following solvents were found to be poor or useless: *n*-butanol, *sec*.-butanol and their water mixtures, chloroform, benzene, ethyl acetate and their mixtures with various light petroleum, ether and wet ether. Benzene and toluene were ineffective when run on papers saturated with formamide, propylene glycol or 60% aqueous ethanol as the stationary phase.

The more water in the solvent, the faster was the travel of the materials but the greater the streaking.

Chromatography on alumina of DANB derivatives of keto acids

A mixture of about 20 mg. each of methyl-, *iso*-propyl- and carboxyethyl-nitroquinoxalinols in about 2 ml. ethyl acetate were applied to an alumina column as described earlier. *iso*Propyl-nitroquinoxalinol moved slowly with chloroform alone, but 100 ml. of chloroform-ethanol (4:1)

Only in one category are the hydrazones more useful, namely in qualitative examination, where paper chromatography on aqueous ammoniacal alcoholic mixtures (*n*-butanol, *sec*.-butanol, amyl alcohol, *n*-hexanol, *sec*.-octanol (after Cavallini, Frontali & Toschi, 1949)) gives a very clear distinction between derivatives. Even here, the DANB derivatives are useful in corroboration, or where speed is an urgent consideration.

Table 1. *Paper chromatography (ascending) of OPD derivatives of ketoacids*

Solvent	Methanol	Ethanol	<i>n</i> -Butanol saturated with 1.5N-NH ₄ OH	<i>sec</i> .-Butanol-1.5N-NaOH (4:1)	Amyl alcohol saturated with 1.5N-NH ₄ OH
Duration of run (hr.)	2.5	4	20	20	20
Distance traversed by solvent (cm.)	21.2	21.6	27.4	24.5	25.2
<i>R_f</i> values of quinoxalinols from:					
α -Ketoglutaric acid	0.39	0.10	0.22	0.33	0.02
Pyruvic acid	0.62	0.72	>0.90	>0.90	>0.90
Dimethylpyruvic acid	0.78	0.87	>0.90	>0.90	>0.90

Table 2. *Paper chromatography of NDB derivatives of keto acids (ascending)*

Solvent	Methanol	Methanol-water (9:1 v/v)	Methanol-water (7:3 v/v)	Ethanol	Ethanol-water (9:1 v/v)	Ethanol-water (7:3 v/v)
Duration of run (hr.)	4	4	4	4	4	4
Distance traversed by solvent (cm.)	27	26.5	26.0	27.2	27.0	26.3
<i>R_f</i> values of nitroquinoxalinols from:						
α -Ketoglutaric acid	0.43	0.55	0.75	0.32	0.55	0.72
Pyruvic acid	0.59	0.60	0.75	0.63	0.77	0.80
Dimethylpyruvic acid	0.73	—	—	0.80	—	—

mixture washed the band right off the column. Methylnitroquinoxalinol only began to move down slightly with ethanol and was completely washed off by 110 ml. of ethanol-water (9:1). Only the alkaline solution moved the carboxyethyl derivative which came right through with 40 ml. The spread of the bands before leaving the column was about 20 mm.

DISCUSSION

The advantages of 1:2-diamino-4-nitrobenzene over 2:4-dinitrophenylhydrazine as a reagent for α -keto acids are the following: (1) the reaction is more specific for α -keto groups; (2) theoretically, the quinoxalinols, since they contain conjugated heterocyclic ring systems, should be much more stable than the hydrazones; (3) the chromatographic separation of characterizable quantities is easier than for the hydrazones, which still possess carboxylic acid groups, rendering alumina separations difficult; (4) the melting points of the compounds are reasonably sharp and have a range adequate for differentiation by this means.

The OPD derivatives are reasonably useful, but suffer from their lack of colour and failure generally to couple with diazo reagent. Their chromatographic separation is not so clear as that of DANB.

SUMMARY

1. A series of stable nitroquinoxalinols, new coloured derivatives of α -keto acids, has been prepared.

2. The quinoxalinols obtained by condensation of either *o*-phenylenediamine or 1:2-diamino-4-nitrobenzene with pyruvic, dimethylpyruvic and α -ketoglutaric acids, may be separated by paper chromatography.

3. The nitroquinoxalinols may also be separated on alumina.

4. 1:2-Diamino-4-nitrobenzene is superior to 2:4-dinitrophenylhydrazine as a reagent for α -keto acids, in specificity, stability, separability and characterizability of the products.

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