The Preparation of α -Oxo Acid Derivatives Suitable for Specific-Radioactivity Determination

By J. MOWBRAY* AND J. H. OTTAWAY

Department of Biochemistry, University of Edinburgh Medical School,
Teviot Place, Edinburgh EH8 9AG, U.K.

(Received 18 May 1970)

1. Conditions favouring the condensation of *o*-phenylenediamine with α -oxo acids to form substituted quinoxalines have been investigated. The best yields (85–100%) are obtained by incubation of the reagents in 2M-hydrochloric acid. Substitution of acetic acid for hydrochloric acid, or of halogen-substituted 1,2-diaminobenzenes for phenylenediamine, increases the initial rate of reaction, but not the yield of quinoxalines. 2. The quinoxaline derivatives are insoluble in water and can be readily purified. They are very suitable for determination of the specific radioactivities of radioactively labelled α -oxo acids because they are colourless, soluble in dioxan-based scintillators, and their strong absorption of u.v. light facilitates their chemical determination. As little as 1 nmol can be accurately determined by means of the blue fluorescence produced by absorption of light at 340–360 nm. 3. Optimum conditions for preparation, purification and separation by paper and thin-layer chromatography are described.

Several α -oxo acids are important intermediates in metabolism and it is therefore essential to be able to determine accurately their specific radioactivities in studies of tracer kinetics. The acids are usually present in low concentrations in tissues, since the redox potentials of the oxo acid–hydroxy acid couples are usually well above those of NAD⁺–NADH. Moreover, pyruvic acid and oxaloacetic acid, in particular, are remarkably unstable except in acid solution (von Korff, 1969; Kesner & Muntwyler, 1969).

Several methods for the separation of α -oxo acids by paper chromatography exist (Neish, 1957). These are not very satisfactory in that even enzymic methods are at the limit of their sensitivity in measuring the small amounts of the acids present in the separated chromatographic spots. It is also necessary to ensure that no α -amino acids are present, as transamination reactions occur on the paper (Giri, 1954) apparently catalysed by the paper itself (Neish, 1957).

Cavallini, Fontali & Toschi (1949) introduced the use of the 2,4-dinitrophenylhydrazine derivatives of α -oxo acids for separation purposes. These phenylhydrazones have been used for determining the specific radioactivities of α -oxo acids (Aronoff, 1956; Evans, Opie & Renold, 1963) but they have serious disadvantages. The hydrazones exist as

cis- and *trans*-isomers (Isherwood & Jones, 1955) which may run separately on chromatography (Isherwood & Cruikshank, 1954). Also an artifact is produced in the reaction that co-migrates with the slower-moving isomer of the 2,4-dinitrophenylhydrazone of pyruvic acid (Towers & Mortimer, 1954). In addition, the hydrazones are highly coloured, and hence quench in liquid-scintillation systems (Baillie, 1960; Bush, 1963).

α -Oxo acids undergo an acid-catalysed condensation with *o*-phenylenediamine to give very stable quinoxalinols (Hinsburg, 1887). Hockenhull & Floodgate (1952) introduced the reagent 1,2-diamino-4-nitrobenzene to form coloured 3-alkyl-quinoxalinols of the metabolically significant α -oxo acids. Taylor & Smith (1955) used this reagent to measure the concentrations of aliphatic α -oxo acids in blood and urine. Nielsen (1963) used quinoxalinols to measure aromatic α -oxo acids.

The present paper reports some investigations into the preparation, chromatographic separation and determination of the colourless quinoxalinols that are prepared by allowing α -oxo acids to react with *o*-phenylenediamine, 1,2-diamino-4-chlorobenzene and 1,2-diamino-4-fluorobenzene. Our interests have been confined almost entirely to the preparation of derivatives of pyruvic acid and α -oxoglutaric acid, which can be determined by their intense u.v. absorption, or by the blue fluorescence that they show on excitation with u.v. light.

A preliminary account of the radioassay of α -oxo

* Present address: Department of Chemical Biodynamics, University of California, Berkeley, Calif. 94720, U.S.A.

acids by this method was given by Mowbray & Ottaway (1967).

MATERIALS AND METHODS

Reagents. *o*-Phenylenediamine was obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. It was twice recrystallized from hot neutral ethylenediaminetetraacetate solution (1%, w/v) containing decolorizing carbon. The cream-coloured plates were kept in a desiccator under N_2 and stored away from strong light. 1,2-Diamino-4-chlorobenzene was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. It was recrystallized from light petroleum-benzene (2:1, v/v). 1,2-Diamino-4-fluorobenzene was prepared from 3-fluoronitrobenzene (Koch-Light Laboratories Ltd.) by nitration followed by catalytic reduction (Suschitzky, 1953). It was recrystallized from light petroleum-benzene (2:1, v/v). 2,3-Diaminophenazine was prepared by adding $FeCl_3$ to an aqueous solution of *o*-phenylenediamine (Ullmann & Mauthner, 1902). The brown crystals were recrystallized from aniline-xylene (1:1, v/v). *o*-Aminophenol was obtained from Koch-Light Laboratories Ltd. and was twice recrystallized from hot water. Sodium pyruvate was obtained from BDH Chemicals Ltd. (reagent grade). It was found to contain 87% pyruvate by enzymic analysis. Sodium oxaloacetate and sodium α -oxoglutarate were obtained from Koch-Light Laboratories Ltd. MN-Kieselgel-G was obtained from Macherey, Nagel G.m.b.H., Düren, Germany. Florisil (200-300 mesh) and *n*-butanol were obtained from BDH Chemicals Ltd. The latter compound was redistilled before use, and the fraction boiling at 117°C was retained. All other chemicals were obtained from BDH Chemicals Ltd. and were of analytical grade.

Preparation of reference compounds. Equimolar amounts of α -oxo acid and *o*-phenylenediamine (or the diamino-4-chloro- or diamino-4-fluoro-benzene) were dissolved in 2M-HCl and the precipitate of quinoxalinol was filtered off after allowing the mixture to stand for 2 h at room temperature. The final yield in all cases was about 15% of theoretical after recrystallization. The oxaloacetate derivative was used within a few days of preparation.

2-Hydroxy-3-methylquinoxaline was recrystallized from ethanol-water (1:1, v/v), m.p. 245°C (uncorr.) (Found: C, 67.4; H, 5.1; N, 17.6. $C_9H_8N_2O$ requires C, 67.5; H, 5.0; N, 17.5%). 3-Carboxymethyl-2-hydroxyquinoxaline was recrystallized from ethanol-water (1:10, v/v) m.p. 252°C (uncorr.) (Found: C, 59.9; H, 4.1; N, 14.0. $C_{10}H_8N_2O_3$ requires C, 58.8; H, 3.9; N, 13.7%). 3-Carboxyethyl-2-hydroxyquinoxaline was recrystallized from methanol-water (2:1, v/v) m.p. 273°C (uncorr.) (Found: C, 60.3; H, 4.7; N, 12.7. $C_{11}H_{10}N_2O_3$ requires C, 60.5; H, 4.6; N, 12.8%). α -Chloro-2-hydroxy-3-methylquinoxaline was recrystallized from ethanol-water (1:1, v/v) m.p. 252°C (uncorr.) (Found: C, 55.2; H, 4.0; N, 13.8; Cl, 18.2. $C_9H_7N_2ClO$ requires C, 55.5; H, 3.6; N, 14.4; Cl, 18.3%). 3-Carboxyethyl- α -chloro-2-hydroxyquinoxaline was recrystallized from ethanol-water (1:1, v/v) m.p. 245°C (decomp.) (uncorr.) (Found: C, 50.5; H, 4.1; N, 10.6; Cl, 14.3. $C_{11}H_9N_2ClO_3$ requires C, 52.3; H, 3.6; N, 11.1; Cl, 14.1%). α -Fluoro-2-hydroxy-3-methylquinoxaline was recrystallized from ethanol-water (1:1, v/v) m.p. 260°C (uncorr.) (Found: C, 60.5; H, 4.1; N, 15.4. $C_9H_7N_2FO$ requires C, 60.7; H, 3.9; N, 15.7%).

3-Carboxyethyl- α -fluoro-2-hydroxyquinoxaline was recrystallized from ethanol-water (1:1, v/v), m.p. 238-240°C (decomp.) (uncorr.) (Found: C, 54.4; H, 4.2; N, 11.4. $C_{11}H_9N_2FO_3$ requires C, 55.9; H, 3.8; N, 11.9%).

Treatment of α -oxo acids in biological samples. A solution containing one or more oxo acids (10-200 nmol of each), in less than 10 ml, is placed in a glass-stoppered tube and made 2M with respect to HCl. A portion (0.2 ml) of a freshly prepared solution of *o*-phenylenediamine in 2M-HCl (10 mg/ml) is added, and the tube is incubated in the dark for 1-3 h at 37°C, or overnight at room temperature (20°C). The solution is then cooled to 4°C or below, and made approx. 0.5M with respect to KOH. It is immediately extracted three times with an equal volume of diethyl ether. It is then immediately neutralized with the appropriate amount of mineral acid. The ether is discarded. Then 2 ml of 0.2M-sodium formate buffer, pH 2.5, is added to the tube and the pH is adjusted to 2.5 by the addition of acid or alkali as necessary. The solution is then extracted twice with 10 ml of chloroform. The chloroform may be filtered through Whatman no. 1 paper to remove droplets of water before evaporation of the solution to a small volume by a stream of air. The concentrated solution is then chromatographed, and the quinoxalinols eluted and assayed as described below.

RESULTS AND DISCUSSION

Pyruvate. Hockenull & Floodgate (1952) used equimolar quantities of reagents to synthesize nitroquinoxalinols, but we found that with these proportions the yields were low. Nielsen (1963) observed that to obtain a maximum yield of quinoxalinol from aromatic oxo acids, 10^2 - 10^3 times the stoichiometric quantity of *o*-phenylenediamine was required. This is confirmed by the results shown in Fig. 1. Doubling the incubation time did not increase the yield. The use of a more than 100-fold excess of *o*-phenylenediamine gave rise to

Fig. 1. Effect of concentration of *o*-phenylenediamine on the yield of quinoxalinol from 1 μ mol of pyruvate. Conditions were as described in the Materials and Methods section.

lower yields. It was suspected that this might be due to the formation of phenazines, which Edson (1935) found to catalyse the decarboxylation of α -oxo acids.

With this in mind, two possible means of improving the yield were tried: (a) to increase the rate of reaction, and (b) to decrease, by steric hindrance, the probability of phenazine formation.

(a) Increase in temperature from 20°C to 37°C shortened the reaction time from 5–6 h to 1 h, but did not increase the yield. Further increase of reaction rate was achieved by carrying out the reaction in 2M-acetic acid, as Cram & Hammond (1959) suggested that the condensation is subject to general acid catalysis. Table 1 shows that the initial rate of reaction was increased approximately 10-fold, but the final yield was distinctly less than in hydrochloric acid. Moreover, the formation of yellow compounds with absorption spectra and chromatographic mobilities characteristic of diaminophenazines was increased. It was concluded that the use of acetic acid as a catalyst for the reaction offered no advantages over hydrochloric acid.

(b) It was thought that the presence of a third substituent on the benzene ring might hinder the formation of phenazines. As it was desired to restrict the investigation to colourless derivatives, the two halogen compounds 1,2-diamino-4-chlorobenzene and 1,2-diamino-4-fluorobenzene were tested. The fluoro derivative behaved very similarly to *o*-phenylenediamine itself, but the chloro derivative reacted more rapidly. The final yield was, however, no better than with *o*-phenylenediamine (Table 1). The time-course of the reaction was consistent with the hypothesis that a side-reaction occurred at a rate proportional to the amount of quinoxalinol that had been formed, i.e. that the latter slowly catalysed the decarboxylation of pyruvate.

In radioactive tracer studies it is important that

the derivative that is assayed cannot be formed from any other precursor. It was established that no quinoxalinol was formed when phosphoenolpyruvate was incubated with *o*-phenylenediamine in 2M-hydrochloric acid or 2M-acetic acid at 37°C for up to 2 h. Formation of methylquinoxalinol from oxaloacetate is discussed below.

α -Oxoglutarate. Quinoxalinol formation in hydrochloric acid was much slower with α -oxoglutarate than with pyruvate, but the reaction went to completion. In acetic acid, or with chlorophenylenediamine, the rates were slightly higher than with pyruvate, but the yield was not 100%. The values are given in Table 1.

Oxaloacetate. The formation of carboxymethylquinoxalinol (the oxaloacetate derivative) was always accompanied by up to 50% yields of methylquinoxalinol (the pyruvate derivative), no matter what reaction conditions were used. Moreover, although carboxymethylquinoxalinol can be recrystallized until it is chromatographically pure, it slowly but completely decarboxylates over a period of 3–4 weeks, even when stored in a desiccator in the dark. It is therefore not possible to assay pyruvate in a tissue extract that may contain oxaloacetate without taking precautions to eliminate contamination. As a routine we added malate dehydrogenase (EC 1.1.1.37) and 1 μ mol of NADH/ml to neutralized tissue-extract samples in which pyruvate was to be assayed, followed after 5 min by alcohol dehydrogenase (EC 1.1.1.1) and 1 μ mol of acetaldehyde/ml. The latter treatment removes excess of NADH, a breakdown product of which has an R_f value similar to that of methylquinoxalinol on t.l.c., and which interferes in the spectrophotometric determination of the quinoxalinol. Tests showed that this procedure eliminates completely any contamination by oxaloacetate in amounts up to 1 μ mol/sample, which is far more than would normally be expected. Oxaloacetate itself can be

Table 1. Rates of reaction of α -oxo acids with *o*-phenylenediamines under various conditions, and yields of quinoxalinols

The values are of the second-order rate constant, derived from the initial rate of reaction, $v = k[\text{diamine}][\text{oxo acid}]$, at 37°C. The dimensions of k are $\text{mol}^{-1} \text{min}^{-1}$. The yields were determined spectrophotometrically in separate experiments in which the reaction was allowed to go to completion (60 or 150 min).

Oxo acid	Conditions	Phenylene-diamine		Chlorophenyl-enediamine	
		k	yield (%)	k	yield (%)
Pyruvate	2M-HCl	1.7	90	4.6	73
	0.5M-Acetic acid	13	77	—	—
	2M-Acetic acid	25	75	38	84
	5M-Acetic acid	28	74	—	—
α -Oxoglutarate	2M-HCl	0.4	100	3.2	80
	2M-Acetic acid	44	82	28	88

assayed in a separate sample after removal of pyruvate by lactate dehydrogenase (EC 1.1.1.27) and NADH. Methylquinoxalinol is isolated; if preferred, the oxaloacetate may be catalytically decarboxylated before addition of *o*-phenylenediamine and hydrochloric acid.

Purification and chromatography. The condensation products of pyruvate and α -oxoglutarate are very stable compounds. Like all quinoxalinols (see also Nielsen, 1963), they are very insoluble in most common neutral solvents, although they dissolve readily in acid, alkali, pyridine and 1,4-dioxan. They are somewhat soluble (<1g/100ml) in chloroform and ethyl acetate. The solubility of methylquinoxalinol in acid and alkali, and the differences in the absorption spectra in various solvents (Fig. 2), indicate that protonation of one of the nitrogen atoms in the ring, and dissociation of the hydroxyl group, occur in fairly strong acid and alkali respectively. Advantage of this is taken in separating the quinoxalinols from excess of phenylenediamine and its decomposition products. Phenylenediamine itself does not interfere with the determination of quinoxalinols, but it runs near methylquinoxalinol on chromatograms and oxidizes on the paper or plate to a substance that co-migrates with *o*-aminophenol, and that absorbs strongly at 340–360 nm. Diaminophenazine, another decomposition product of phenylenediamine, can distort the carboxyethylquinoxalinol spot if present in too high a concentration.

Excess of phenylenediamine can be removed by making the solution 0.5 M with respect to potassium hydroxide, and extracting the unchanged diamine with ether. Since *o*-aminophenol is formed more

readily at high pH values, the extraction should be carried out quickly, and it is preferable to cool the solution beforehand. If the pH of the solution is then adjusted to between 2 and 3, the quinoxalinols may be extracted into chloroform, leaving the aminophenazines in the aqueous phase. If the pH is lower than 2, the quinoxaline nucleus begins to be protonated, and the compounds are no longer extractable into ether. If the pH approaches 3, derivatives of dicarboxylic acids that retain a carboxyl group begin to ionize, and remain in the aqueous phase. More precise recommendations are given in the Materials and Methods section.

Chromatography is best carried out in a slightly alkaline solvent. A selection of solvent and support systems is given in Table 2. The quinoxalinols may be detected by their fluorescence in u.v. light (preferably at a wavelength around 360 nm).

Determinations. Fig. 2 shows the absorption spectra of methylquinoxalinol in various solvents. We found it most convenient to elute the substances with 0.5–1 M-ammonia, and to measure their concentrations by their absorption at the peak around 345 nm (343 nm for the pyruvate derivative and 347 nm for the α -oxoglutarate derivative). The solutions obey Beer's law. The molar extinction coefficient at this wavelength is 8.3×10^3 . The solution can then be incorporated in a dioxan-based solvent for liquid-scintillation counting. Alkali, unlike acids, does not itself quench the scintillations.

Greater sensitivity can be obtained by measuring the quinoxalinols fluorimetrically. Fig. 3(a) shows the uncorrected excitation spectrum of methylquinoxalinol in 0.5 M-ammonia for emission at 405 nm, and Fig. 3(b) the uncorrected emission

Fig. 2. Absorption spectra of carboxymethylquinoxalinol in various solvents. —, 1 M-NH₃; ---, 1 M-HCl; ·····, 1 M-acetic acid; - · - ·, chloroform.

Table 2. R_F values for substituted quinoxalinalols

The systems used were: (1) *n*-butanol-ethanol-0.5M-NH₃ (7:1:2, by vol.), Whatman no. 1 paper; (2) *n*-butanol-*n*-propanol-0.5M-NH₃ (9:7:4, by vol.), Kieselgel G (layer thickness, 250 μ m) activated at 110°C for 10 min; (3) chloroform-acetone-ethanol-6M-NH₃ (7:8:7:3, by vol.), Florisil (layer thickness 250 μ m) activated at 110°C for 10 min. The R_F values of some likely contaminants are also given. The 'aminophenol' spot probably contains considerable quantities of benzoquinone before it is examined under u.v. light. Authentic diaminophenazine gives two spots with R_F values identical with 'phenazine A' and 'phenazine B' in these solvent systems.

Compound Solvent/support system . . .	$R_F \times 100$			Fluorescence colour
	1	2	3	
Methylquinoxalinalol	83	63	95	Light blue
Carboxymethylquinoxalinalol	18	—	45	Light blue
Carboxyethylquinoxalinalol	23	32	33	Light blue
<i>o</i> -Phenylenediamine	68	71	—	Purple
Aminophenol	96	—	—	Green
Phenazine A	65	43	—	Yellow
Phenazine B	54	36	—	Yellow

Fig. 3. (a) Excitation spectrum of carboxymethylquinoxalinalol in 0.5M-NH₃. Slit width 3nm; emission wavelength setting 405nm. (b) Emission spectrum of carboxymethylquinoxalinalol in 0.5M-NH₃. Slit width 3nm; excitation wavelength 343nm.

spectrum for excitation at 343nm. The emission maximum is at 405nm. The instrument used was a Perkin-Elmer MPF-2A (The Perkin-Elmer Corp., Norwalk, Conn. U.S.A.). Very similar curves were obtained from both carboxymethylquinoxalinalol and carboxyethylquinoxalinalol; emission maxima were at 407nm and 409nm respectively. The range over which measurement can be made is 0-70 nmol/ml.

This work was supported by a grant from the Medical Research Council to J.H.O.

REFERENCES

- Aronoff, S. (1956). *Techniques of Radiobiology*, p. 133. Ames: Iowa State College Press.
- Baillie, L. A. (1960). *Int. J. appl. Radiat. Isotopes*, **8**, 1.
- Bush, E. T. (1963). *Analyt. Chem.* **35**, 1024.
- Cavallini, D., Fontali, N. & Toschi, G. (1949). *Nature, Lond.*, **163**, 568.
- Cram, D. J. & Hammond, G. S. (1959). *Organic Chemistry*, p. 316. New York: McGraw-Hill.
- Edson, N. L. (1935). *Biochem. J.* **29**, 2082.
- Evans, J. R., Opie, L. H. & Renold, A. E. (1963). *Am. J. Physiol.* **205**, 971.
- Giri, K. V. (1954). *Naturwissenschaften*, **41**, 14.
- Hinsburg, O. (1887). *Justus Liebigs Annln Chem.* **237**, 340.
- Hockenhull, D. J. D. & Floodgate, G. D. (1952). *Biochem. J.* **52**, 38.
- Isherwood, F. A. & Cruikshank, D. H. (1954). *Nature, Lond.*, **173**, 121.
- Isherwood, F. A. & Jones, R. L. (1955). *Nature, Lond.*, **175**, 419.
- Kesner, L. & Muntwyler, E. (1969). In *Methods in Enzymology*, vol. 13, p. 415. Ed. by Lowenstein, J. M. New York: Academic Press Inc.
- Mowbray, J. & Ottaway, J. H. (1967). *Abstr. FEBS 4th Meet., Oslo.*, p. 68.
- Neish, W. J. P. (1957). In *Methods of Biochemical Analysis*, vol. 5, p. 107. Ed. by Glick, D. New York: Interscience Publishers Inc.
- Nielsen, K. H. (1963). *J. Chromat.* **10**, 463.
- Suschitzky, H. (1953). *J. chem. Soc.* p. 3042.
- Taylor, K. W. & Smith, M. J. H. (1955). *Analyst, Lond.*, **80**, 607.
- Towers, G. H. N. & Mortimer, D. C. (1954). *Nature, Lond.*, **174**, 1189.
- Ullmann, F. & Mauthner, F. (1902). *Chem. Ber.* **35**, 4302.
- von Korff, R. W. (1969). In *Methods in Enzymology*, vol. 13, p. 425. Ed. by Lowenstein, J. M. New York: Academic Press Inc.