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Short Communications

An Improved Enzymatic Method for the Preparation of Aromatic α-Keto Acids of Low Stability

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Besides being a useful tool for the preparation of many α-keto acids,[1] L-amino acid oxidase (snake venom) is also convenient for the quantitative determination of aromatic amino acids, since the corresponding α-keto acids are easily identified by spectrophotometric methods as enol-borate complexes.[2,3] Increasing pH favours the complex formation, but also renders the keto acids more liable to oxidation. When proper conditions are chosen, the α-keto acids are stabilized as borate complexes. This principle has been used in transaminase studies,[4] but for preparative purposes its usefulness seems to have been neglected. By introducing borate buffer we have prepared 3,4-dihydroxyphenylpyruvic acid (DHPPA) and indolylpyruvic acid (IPA) not previously accessible by the enzymatic method. The highly labile 5-hydroxyindolylpyruvic acid (HIPA) can be obtained in aqueous solution.

The lability of aromatic α-keto acids renders the isolation of highly pure substances difficult unless a nonoxidizing atmosphere is introduced.

Since oxygen is present in most biochemical experiments, the aromatic α-keto acids usually contain a certain amount of decomposition products. Judged from comparison of extinction coefficients our substances appeared to have a higher degree of purity than those commercially available.

Materials. Incubation buffer pH 7.2: 0.2 M borate, 0.1 M phosphate and 1 g of EDTA per l; air was bubbled through prior to use. Buffer for spectrophotometric analysis pH 7.2: 0.5 M borate, 0.5 M arsenate, and 1 g of EDTA per l. Enzymes: 16 mg of L-amino acid oxidase (Sigma) dissolved in 4 ml of distilled water, dialysed over night against running distilled water (about 5 l) at 5°. Catalase suspension in distilled water (Sigma C 10) dialysed in the same manner. 80 μl of the suspension contained 1.6 mg protein (48 000 Sigma units). Ether: Peroxides were removed by washing with $FeCl_2$, followed by 1 volume distilled water containing 1 g of EDTA per l, and 2 volumes of deionized water. Amino acids: Tracer amounts of ^{14}C labelled amino acids were added to 10 mg of the unlabelled compound. When the labelled amino acid was not available in the L form, the carrier was also racemic.

Method. The reaction mixture contained: 10 mg + tracer amounts of the amino acid, 600 μl of L-amino acid oxidase solution and 80 μl of catalase solution added to 50 ml of incubation buffer. After 3 h at 37°C the reaction mixture was saturated with solid NaCl and extracted by 3 portions of ether (25 ml) to which 6 N HCl (2 ml) had been added. An emulsion which easily forms in the separation funnel can be split by applying a light suction to the top for a few seconds. The combined ether extracts were washed with 1 volume of deionized water to remove traces of amino acids. The ether was removed under reduced pressure, and the residue dissolved in 70 % ethanol.

The spectra of α-keto acids were then examined in a Zeiss selfrecording spectrophotometer in the 250–400 mμ region, allowing 20 min for enol-borate-complex equilibration.

The yield was calculated from recovery of radioactivity in the ethanol solution. The radioactivity was measured in a Packard Tri-Carb scintillation spectrometer. A solution of PPO and POPOP in dioxane [5] was used and aliquots directly added to 15 ml of the

Table 1. The yields and extinction coefficients (at abs.max.) of aromatic-keto acids obtained by enzymatic synthesis.

Substrate	Counts/min × 10^3	α-Keto acid	Yield per extraction counts/min × 10^3			Total yield %	ε_M	abs.max. mμ
L-(3-^{14}C) phenylalanine	252	PPA	127.0	70.5	22.1	87	7 250	298
DL-(3-^{14}C) tyrosine	241	HPPA	32.4	22.4	14.2	29		
L-(3-^{14}C) tyrosine	692	HPPA	266.0	162.0	86.7	74	13 200	310
L-(1-^{14}C) tyrosine	623	HPPA	246.0	130.0	61.1	70	14 300	310
DL-(3-^{14}C) DOPA	880	DHPPA	63.4	34.7	13.8	15	20 300	335
DL-(3-C^{14}) tryptophan	274	IPA	85.6	27.8	5.3	43		
L-(3-^{14}C) tryptophan	1640	IPA				76	13 600	330
DL-(3-^{14}C) 5-OH-tryptophan	8690	HIPA	775.0	586.0	422.0	26	11 700	335

scintillation solvent. Quenching corrections were made by external standard.

Results and discussion. The yield of α-keto acids has been calculated from the recovery of radioactivity, Table 1.

Since extraction by ether is used for isolating the material, other substances *i.e.* amino acid, corresponding aldehyde and acetic acid may be suspected to be present.

The solubility of amino acids in ether is low. Extraction experiments with enzymes omitted from the incubation mixture showed that about 0.5 % of the tracer doses were extracted. One washing of the ether with deionized water reduced the amount of tracer to less than 0.3 %. These impurities were not only due to amino acids, but also other unidentified substances present in the tracer material, since paper chromatography of the ether extract (butanol-acetic acid) revealed several radioactive spots.

The aromatic α-keto acids in solution are oxidized by atmospheric oxygen, which renders analytical tests difficult. Even in acidic solution, where they are most stable, only phenylpyruvic acid (PPA) can be subjected to chromatography without decomposition.[6]

p-Hydroxyphenylpyruvic acid (HPPA) and IPA undergo an unusual reaction resulting in *p*-hydroxybenzaldehyde and indolylaldehyde,[6-8] and oxalic acid. Crosschecking extraction experiments with our own product and commercially available IPA (Fluka) showed that omission of borate resulted in destruction of IPA. The substance extracted gave in both cases an absorption spectrum identical with indolyl-3-aldehyde, which is clearly different from the closely related indolyl-3-acetaldehyde and also from indolylacetic acid (Fig. 1).

The amount of aldehydes in HPPA and IPA solutions can be evaluated by chromatography on Sephadex G 25 columns.[9] (Due to the low solubility of IPA thin layer chromatography on the same medium can

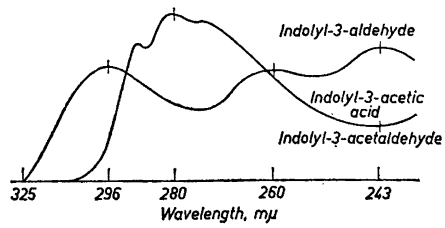

Fig. 1. UV-spectra of indol compounds in 70 % ethanol.

sometimes be more convenient). Similar experiment with DHPPA and HIPA have not been performed since these substances are even more labile than the preceding ones. Billek who has synthesized DHPPA used SO_2 to retard decomposition.[10]

Unless kept in a borate containing solution HIPA turns pink on standing and gives a dark residue when the ether extract is evaporated.

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Studies on Sulfinic Acids

II b.* Supplementary Note on the Titrimetric Determination of Aromatic Sodium Sulfinates with Perchloric Acid

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In previous papers of this series the titration of aromatic sodium sulfinates with perchloric acid has been described.[1,2] When a series of substituted aromatic sodium sulfinates were titrated with 0.1 N perchloric acid in glacial acetic acid it was found that the half neutralization potentials could be satisfactorily correlated with the Hammett equation. The result is shown in Fig. 1. Since the absolute value of the half neutralization potential has no useful or direct significance, no measures were taken to establish it, and hence only the potentital shifts are given in the figure. The correlation also holds for *ortho* substituted members, if the polar substituent constants given by Taft [3] are used.

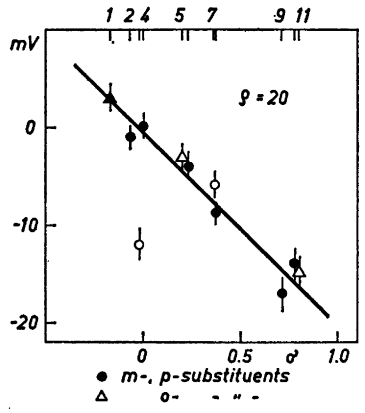

Fig. 1. Plot of half neutralization potentials against Hammett substituent constants for some aromatic sodium sulfinates titrated with perchloric acid.

Substituents: 1, *p*-CH_3, *o*-CH_3; 2, *m*-CH_3; 3, *p*-CH_3CONH; 4, none; 5, *o*-Cl; 6, *p*-Cl; 7, *m*-COOH; 8, *m*-Cl; 9, *m*-NO_2; 10, *p*-NO_2; 11, *o*-NO_2.

Ritchie *et al.* have previously shown that the pK's for some *p*-substituted sulfinic acids measured spectrophotometrically could be correlated with the Hammett equation.[10] The relationship between pK's and substituent constants can be expected to parallel that between the present half neutralization potentials and substituent constants. Thus it should be possible to predict the acid strengths of aromatic sulfinic acids knowing the acid strength of a few members. A summary of the dissociation constants of sulfinic acids found in the literature is given in Table 1. The large discrepancies found between the values reported by various authors may be partly due to the difficulty in determining disso-

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