PHOTOMETRIC DETERMINATION OF 2-OXOGLUTARIC ACID AND SOME OTHER 2-OXO ACIDS BY REACTION WITH 3-METHYL-2-BENZOTHIAZOLINONE HYDRAZONE

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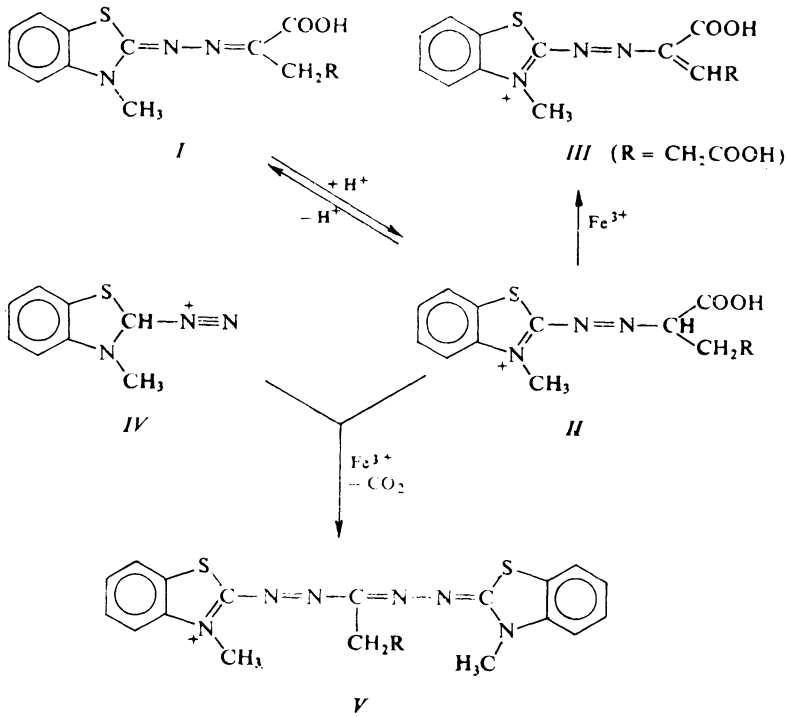
2-Oxoglutaric acid reacts with 3-methyl-2-benzothiazolinone hydrazone in acid solutions in the presence of $FeCl_3$ to give a steady yellow colour. The method enables the substance to be determined in concentrations as low as 15 μmol l^{-1} with an accuracy of about 10%. The analysis can be performed in the presence of some additional natural or synthetic 2-oxo acids. The applicability of the method was tested in the determination of the activity of some enzymes involved in the metabolism of 2-oxoglutaric acid. Glutamic acid can be determined after its prior non-enzymatic transamination. A number of 2-oxo acids including 2-oxoglutaric acid can be determined photometrically with a high sensitivity by reaction with 3-methyl-2-benzothiazolinone hydrazone in conditions favouring the formation of blue tetra-aza-pentamethinecyanine dyes. The molar absorptivities largely lay within the region of $(2-4{\cdot}5) \cdot 10^4$ l mol^{-1} cm^{-1}.

The glutamic acid - 2-oxoglutaric acid (OGA) transamination pair cooperates in more than thirty of the fifty six enzymatically defined transaminations[1]. The activities of the transamination enzymes, which often are of significance in the diagnostics of pathologic states, can be monitored based on the formation of OGA from glutamic acid, and therefore analytical methods making it possible for OGA to be selectively determined are attracting interest.

A highly selective photometric determination of OGA is based on enzymatic reductive amination[2]. The sensitivity and selectivity of chemical ways of determination[3,4] are poorer. The basis for a relatively selective method of determination of OGA is provided by its colour reaction with 3-methyl-2-benzothiazolinone hydrazone in oxidant solutions.

3-Methyl-2-benzothiazolinone hydrazone (MBH) combined with compounds containing a carbonyl group gives the corresponding azines. The dependence of 2-oxo acid azines spectral curves on pH reveals that the compounds can exist in several protolytic forms[5]. In neutral or weakly acid solutions the unconjugated form *I* (see the Scheme) predominates; the spectrum exhibits peaks at 210 and 310 nm (Fig. 1). The former band decreases and ultimately vanishes as the solution is made acidic and a new band appears at 355–360 nm. The conjugated form *II* is typically present in less polar media.

In the presence of $FeCl_3$ the conjugated form of the aldehyde azine can be oxidatively coupled with the diazonium ion *IV* formed from the excess MBH; blue tetra-aza-pentamethinecyanine dyes (TC) (*V*) are formed[6-9].



It is less common knowledge that oxidative coupling leading to TC also occurs with azines of 2-oxo acids and is of general nature, particularly in acid media (Ta-

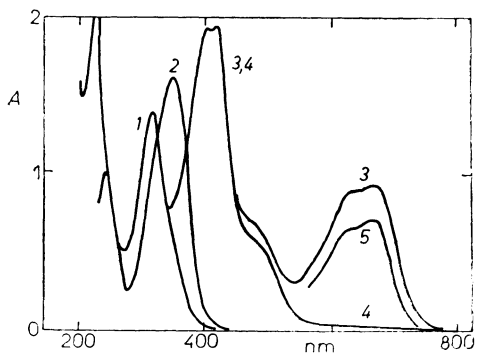


FIG. 1

Absorption spectra of OGA-MBH azine solution (0·1 mmol l^{-1}) in 0·01M phosphate buffer pH 7·4 *1*, OGA-MBH azine solution (0·1 mmol l^{-1}) in hexanol *2*, reaction product of OGA with MBH (0·6 mmol l^{-1}) in procedure *B* using 36% HCl in place of phosphoric acid *3* and in unmodified procedure *B* *4*, and a section of the spectrum of TC dye prepared from succinic semialdehyde solution (0·3 mmol l^{-1}) by procedure *B* using 36% HCl in place of H_3PO_4 *5*

ble I). For instance, it takes place also with OGA as evidenced by the separate absorption band with maxima at 630 and 668 nm (Fig 1, curve 3), which is analogous to the band in the 550–700 nm range formed on the reaction of succinic semialdehyde (Fig. 1, curve 5).

OGA differs from other 2-oxo acids in that in addition to TC dyes it gives preferentially yellow colour characterized by a rather sharp absorption band with maxima at 400–420 nm (Fig. 1, curves 3 and 4) if reacted with MBH and Fe^{3+} under fa-

TABLE I

Molar absorptivities ε for 2-oxo acids and related compounds after their reaction with 3-methyl-2-benzothiazolinone hydrazone. The calculations are based on the introduced amount of substance and the final volume of the dye solution. The standard deviations of ε calculated from 12 measurements are given in parentheses

Substance	$\varepsilon \cdot 10^{-3}$, 1 mol^{-1} cm^{-1}, in procedure		
	A (410 nm)	*B* (420 nm)	*C* (668 nm)
2-Oxoglutaric acid	20·2 (1·3)	19·9 (0·2)	28·3 (0·8)
Glyoxylic acid	0·1	0·1	42 (11)
Acetaldehyde	0	0	56 (2)
Pyruvic acid	0·2 (0·1)	0·4 (0·2)	39 (0·8)
Hydroxypyruvic acid	0·1	0·1	12·8 (0·8)
Oxomalonic acid	0·8 (0·1)	0·2	4·1 (2·1)
Propiolic acid	0·3 (0·1)	0	36[a] (1·3)
2-Oxobutyric acid	0·6 (0·2)	0·7 (0·4)	39·2 (1·8)
2-Oxosuccinic acid	1·8 (0·2)	2·6 (0·3)	36·6 (1·7)
Dihydroxytartaric acid	0·15 (0·1)	0·7 (0·1)	0
2-Oxosuccinamic acid (A form)	0	0·1	2·3 (0·3)
Succinic semialdehyde	0	0	54 (3)
Acetylenedicarboxylic acid	0	0	20·8[a] (2·5)
2-Oxovaleric acid	1·3 (0·1)	1·2 (0·2)	39·5 (3)
2-Oxo-3-methylbutyric acid	0	0	1 (0·4)
4-Methiol-2-oxobutyric acid	0·5 (0·2)	0·2	28·5 (1·7)
2-Oxoglutaramic acid (A form)	0·1	0	2·3 (0·6)
2-Oxo-3-methylvaleric acid	0	0	1·3 (0·2)
2-Oxo-4-methylvaleric acid	6·1 (0·6)	3·7 (0·7)	20·5[b] (2)
2-Oxocapronic acid	5·4 (0·4)	2·3 (0·4)	37·8[b] (1)
2-Oxoadipic acid	3·4 (0·2)	1·2 (0·3)	45·7 (1)
2-Oxocaprylic acid	4·2 (0·3)	2·1 (0·4)	30·6[b] (1·2)
Phenylpyruvic acid	2·6 (0·3)	1·5 (0·7)	37[b] (1·8)

[a] After 15 min heating on water bath with MBH in the presence of $HgCl_2$ (16 mmol l^{-1}); [b] $FeCl_3$ solution (150 mmol l^{-1}) in 70% (*V*/*V*) methanol used in the oxidative coupling.

vourable conditions. The absorption band of the blue TC formed from OGA or other 2-oxo acids or aldehydes practically does not encroach in the region of the yellow band. Interference between the absorption bands can be suppressed completely by complexation of Fe^{3+} with phosphoric acid leading to a quantitative decomposition of the TC (Fig. 1, curve *4*).

Attempts to elucidate the structure of the yellow product failed. Being not a complex with ferric ions, it probably is a dehydrogenated azine of MBH and OGA (*III*). The effect of pH on the colour reaction of OGA with MBH is similar to that for the formation of TC from 2-oxo acids, or for the formation of 2-oxo acids azines in general. Such reactions proceed fast in high yields in strongly acid solutions where the dissociation of the acids is suppressed (pH < 2) (Fig. 2). Curves *1* and *2* in Fig. 2 illustrate the typical differences in the formation of TC dyes from aliphatic aldehydes and 2-oxo acids due to the pH of the solution. The presence of Cu^{2+} ions in the oxidative stage of the coupling brings about a complete elimination of the formation of TC from 2-oxo acids (Fig. 2, curve *4*) and lowers the formation of TC aldehydes (curve *3*), so that at pH 3·5–4 aldehydes can be determined selectively and with a fair sensitivity in the presence of 2-oxo acids. For the determination of OGA it is important that the formation of TC be suppressed (Fig. 3, curve *2*) by oxidative coupling, which is a competitive reaction. This can be achieved by a suitable choice of the concentration of MBH. At low MBH concentrations in the reaction system

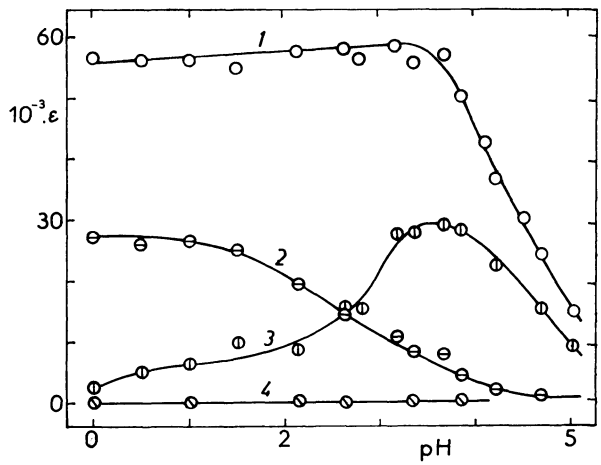


FIG. 2

Dependence of the molar absorptivity on pH of the reaction system for the formation of TC dyes from acetaldehyde (*1*, *3*) and OGA (*2*, *4*) in the absence (*1*, *2*) and in the presence (*3*, *4*) of Cu^{2+} ions. Procedure *C* with sodium trichloroacetate, chloroacetate, and formate buffers was used in the regions of pH 0–1·5, 2–3, and 3–5, respectively

the dye yields are reasonable and little dependent on the concentration of the reagent (Fig. 3, curve *1*).

Most 2-oxo acids interfere only slightly with the "yellow reaction" of OGA (Table I); only some 2-oxo acids with a longer chain give rise to appreciable interferences, probably due to the light absorption by their azines.

The method of determination of OGA has been applied in the determination of activity of L-glutamic acid dehydrogenase (E.C.1.4.1.3), determination of phenyl pyruvate: glutamate aminotransferase, and 2-oxoglutarate: 4-aminobutyrate aminotransferase (E.C.2.6.1.19).

Glutamic acid can be nonenzymatically transaminated to OGA in a good yield by reaction with glyoxylic acid (transaminations of this kind have been described[10]), and OGA then can be determined by reaction with MBH.

At higher acidities (pH 0–2) and higher MBH concentrations, 2-oxo acids give TC dyes in good yields. The reaction can serve for a sensitive photometric determination of 2-oxo acids with a reasonable selectivity provided that defined conditions are ensured as to the pH, ionic strength, temperatures, and procedure (procedure *C*). The data are given in Table I, from which 2-oxo-3,3-dimethylbutyric, tetrolic, and oxophenylacetic acids have been omitted because the two former acids exhibited a zero absorbance in all tests and the last acid showed a zero absorbance in test *C*. From 2-oxosuccinamic and 2-oxoglutaramic acids (both in their A[11] forms), dihydrotartaric acid, and from oxo acids branched at the carbon atom nearest to the carbonyl group the TC dyes were obtained in low yields.

If $HgCl_2$ is also present in the condensation steps with MBH, procedure *C* can also be employed for the determination of some alkyne acids.

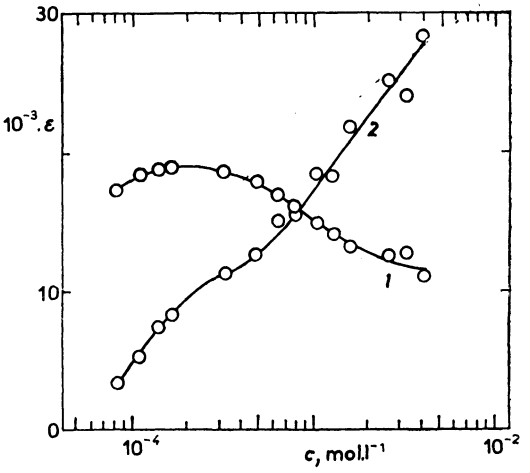


FIG. 3

Dependence of the molar absorptivity at 410 nm *1* and 678 nm *2* of the reaction products of OGA with MBH on the concentration of MBH in the system before the addition of acetone in procedure *A* (modified by replacing phosphoric acid by the same volume of 36% HCl)

The very intense "blue reaction" of 2-oxo acids causes difficulties if aliphatic aldehydes are to be determined in the presence of 2-oxo acids. This is the case, *e.g.*, with the conversion catalyzed by 4-aminobutyrate: 2-oxoglutarate aminotransferase. The formation of TC from OGA and other oxo acids can be avoided by a suitable choice of the reaction conditions making use of the fact that the TC yield from 2-oxo acids drops with increasing pH substantially faster than from aliphatic aldehydes (Fig. 2). In addition, the formation of TC from 2-oxo acids is suppressed by the presence of Cu^{2+} ions in the reaction system during the oxidative coupling.

EXPERIMENTAL

3-Methyl-2-benzothiazolinone hydrazone hydrochloride, m.p. 264–266°C (ref.[8], 240–260°C), was prepared from 2-mercaptobenzothiazole *via* 3-methylmercaptobenzothiazole[8].

2-Oxoglutaric acid (Lachema, Brno) was neutralized with the equivalent amount of sodium hydroxide, and the sodium hydrogen 2-oxoglutarate was crystallized four times from 80% (*V*/*V*) ethanol; the chemical used had the theoretical neutralization equivalent. Oxomalonic, 2-oxoadipic, 4-methiol-2-oxobutyric, and 2-oxocaprylic acids (free acids) and 2-oxovaleric, 2-oxocapronic, 2-oxo-3-methylbutyric, 2-oxobutyric, 2-oxo-4-methylvaleric, and 3-hydroxypyruvic and hydroxyphenylpyruvic acids (sodium salts) were chemicals of Sigma (St. Louis, USA) and were subject to no further purification treatment. Oxalacetic acid was prepared in the form of *cis*-hydroxymaleic acid (m.p. 149°C) from tartaric acid[12,13] (reported m.p. 154°C). Pyruvic acid was prepared by pyrolysis of tartaric acid, and prior to use, redistilled over a short column (b.p. 77–80°C/3·4 kPa). Sodium glyoxylate (monohydrate) was synthesized by oxidation of tartaric acid with periodic acid[10]. Disodium dihydroxytartrate was obtained from tartaric acid dinitrate and recrystallized by dissolving it in 0·1M-HCl and neutralizing with sodium carbonate solution[14]. 2-Oxosuccinamic acid (free) and 2-oxoglutaramic acid (sodium salt), both A[11] forms, and L-3 methyl-2-oxovaleric acid[15] were prepared from the corresponding amino acids by oxidation by elementary oxygen in the presence of L-amino acids oxidase[16] (*Crotallus viridis* toxin was employed for the two former amino acids, *Bothrops asper* for the last) and in the presence of crystalline human blood catalase[17]. 3,3-Dimethyl-2-oxobutyric acid was obtained synthetically[18]; m.p. of its 2,4-dinitrophenyl hydrazone was 170–171°C (decomposition), in accordance with published data[18]. Phenylpyruvic acid (m.p. 150–154°C) was prepared by azlactone synthesis[19], the sodium salt monohydrate was prepared by the method[20]. Propiolic acid was prepared by oxidation of 2-propin-1-ol[21] and purified to reported chemical constants. Tetrolic acid (m.p. 76°C) was obtained by hydrolysis of its methyl ester[22].

Potassium hydrogen acetylenedicarbonate was prepared conventionally[23]. Succinic semialdehyde was prepared by modification of the published method[24]; m.p. of its 2,4-dinitrophenyl hydrazone 199°C (ref.[25], 199°C). The aldehyde content was standardized by the bisulphite method[26].

Oxophenylacetic acid was obtained by oxidation of mandelic acid[33] and recrystallized from carbon disulphide; m.p. 61°C.

Before use, the sodium salts were dried for several days over P_2O_5 at 0–5°C and 3·3 Pa.

OGA: MBH azine was prepared by reacting 3-methyl-2-benzothiazolinone hydrazone hydrochloride monohydrate (233 mg, 1 mmol) with sodium hydrogen 2-oxoglutarate (168 mg) in water (5 ml). The precipitate was collected and recrystallized from boiling water (150 ml). A yellow substance, m.p. 215–216°C, was obtained in a yield of 281·5 mg (quant.). For $C_{13}H_{13}N_3SO_4$ (307·3) calculated: 50·80% C, 4·26% H, 13·48% N; found: 50·68% C, 4·55% H, 13·48% N.

Sodium trichloroacetate buffers were prepared according to calculations using $pK_a = 0{\cdot}7$. The remaining buffers were made up from chemicals of reagent grade purity and their pH values were measured on an OP-205 pH-meter (Radelkis, Budapest) fitted with a glass electrode.

The photometric measurements were carried out on an SP-8-100 instrument (Pye-Unicam, Cambridge) in 1 cm cells at a spectral bandwidth of 0·5 nm.

RESULTS

Determination of 2-Oxoglutaric Acid

The reaction of OGA with MBH in the presence of $FeCl_3$, leading to yellow colour, is affected by the concentration of MBH. Fig. 3 shows that the yield varies only slightly over the region of MBH concentrations of $0{\cdot}1-0{\cdot}5$ mmol l^{-1}. A strongly acid solution is necessary for the reaction. The complexation of Fe^{3+} by the pH buffer system should be minimal; the trichloroacetate buffer pH $0-1$ suits well for this purpose. This buffer is strongly chaotropic, and the enzymatic reaction can be terminated by mixing with the reaction mixture sample. Conventional buffer substances such as tris(hydroxymethyl)aminomethane, 2-morpholinoethanesulphoacid, acetate, borate or ethanolamine, present in the enzymological samples in concentrations up to 160 mmol l^{-1} do not interfere with the colour reaction. N-(Tris(hydroxymethyl)methyl)glycine and 2-amino-2-methyl-1,3-propanediol in a concentration of 160 mmol l^{-1} interfere slightly (the absorbance changes by -10% and -8%, respectively), phosphate in concentrations of 40, 80, and 160 mmol l^{-1} interferes to a considerable extent (-12%, -48%, -90%, respectively).

The concentration of $FeCl_3$ should not be raised above the level indicated in the typical procedure because the absorbance of the blank solution (in procedure *A*) then is too high.

The colour reaction is accelerated by elevated temperatures: at 20°C the reaction is complete in $60-90$ min, at 37°C, in 10 min, and at 55°C, in 4 min. The yield at 55°C, however, is about 6% lower, and even at 37°C the dye formation is less reproducible.

The steadiness of the dye is excellent, no decomposition being observed in seven days for procedures *A* or *B*.

Lowering the oxidation-reduction potential of the system, phosphoric acid causes a breakdown of the TC dye. In the presence of OGA as the only oxo acid the complexation and breakdown is complete in 10 min. In the presence of other oxo acids or aldehydes the time of action of phosphoric acid before the dilution with acetone (in procedure *A*) or extraction (in procedure *B*) must be adjusted because the sensitivity to H_3PO_4 of TC derived from other substances is different.

Substances containing SH groups interfere strongly; $HgCl_2$ must be added in a fourfold stoichiometric excess.

Procedure A. A mixture of 0·25 ml of MBH solution (2 mmol l^{-1}) and 0·15 ml of sodium trichloroacetate buffer pH 0·5 (1 mol l^{-1}) is prepared in a test tube, 0·25 ml of sample containing

5—150 nmol OGA is added, the whole is allowed to stand at room temperature for a minimum of 4 min, and 0·25 ml of $FeCl_3$ solution (45 mmol l^{-1}) is added. After 60 min standing, 0·05 ml of 85% (m/m) H_3PO_4 is added, and the solution is diluted to 1·5 ml with acetone. In 10 min the absorbance is measured at 410 nm against a blank solution. The absorbance is linearly dependent on concentration up to absorbance 2.

Procedure B. The absorbance of the blank can be lowered significantly by extracting the coloured product into hexanol. The reaction mixture prepared as *sub A* and allowed to stand for 60 min with $FeCl_3$ is combined with 0·35 ml of a 6 : 1 (*V*/*V*) mixture of dimethyl sulphoxide with 85% (m/m) H_3PO_4, and in 10 min, extracted into 1·5 ml of hexanol, cooled shortly in a refrigerator, and centrifuged shortly to assist the phase separation. The absorbance is measured at 419 nm against a blank solution.

The absorption band of the dye in the aqueous–acetone solution exhibits a single peak at 410 nm, whereas with the hexanol solutions two peaks are observed (Fig. 1). The distribution constant of the yellow reaction product in the hexanol–aqueous phase system is higher than 20. By employing microcells in procedure *B*, smaller volumes of hexanol can be used, thereby lowering the limit of determination of OGA below 1 nmol.

Determination of Glutamic Acid

Nonenzymatic transamination of glutamic acid with sodium glyoxylate can be performed in buffered systems at pH 7·4 in the presence of mercury dichloride, by heating on boiling water bath. Suitable concentrations of sodium phosphate in the reaction system are 20—50 mmol l^{-1}. Increasing concentration of $HgCl_2$ has a favourable effect on the yield and kinetics of the reaction; above 32 mmol l^{-1}, however, the differences in the yield are low. Suitable concentrations of glyoxylate are 3—8 mmol l^{-1}. The time of heating the reaction mixture on boiling water bath (8—32 min) has a minor effect.

Procedure. 3—300 nmol of glutamic acid is added to a system involving sodium phosphate buffer pH 7·4 (20 mmol l^{-1}), $HgCl_2$ (32 mmol l^{-1}), and sodium glyoxylate (6 mmol l^{-1}) in a total volume of 0·5 ml. The test tube is closed with a glass cap and heated on boiling water bath for 15 min. After cooling the system, 0·8 ml of MBH solution (3 mmol l^{-1}) in TCA buffer pH 0·5 (1 mol l^{-1}) and 0·5 ml of $FeCl_3$ solution (135 mmol l^{-1}) are added. The colour reaction is terminated in 60 min by adding 1·2 ml of a 10 : 1 (*V*/*V*) mixture of acetone with 85% (*m*/*m*) phosphoric acid, and after 10 min standing, the absorbance is measured at 410 nm against a blank solution.

The absorbance is linearly dependent on the glutamic acid concentration up to absorbance 2·5 with an accuracy better than 1%. The molar absorptivity with respect to glutamic acid was $(16{\cdot}5 \pm 1) \cdot 10^3$ l mol^{-1} cm^{-1}. The transamination yield, using 100 nmol of glutamic acid, was about 80%.

The effect of other amino acids can be expressed in terms of the values of $\varepsilon \cdot 10^3$ (l mol^{-1} cm^{-1}): aspartic acid 0, cysteine 0, isoleucine 0, valine 0·04, serine 0·05, threonine 0·09, histidine 0·09, tyrosine 0·09, alanine 0·15, cysteic acid 0·22, arginine 0·35, hence less than 2·5% of the value of glutamic acid; 3-O-phosphoserine (0·5 ± ± 0·1), asparagine (0·5 ± 0·1), glutamine (0·56 ± 0·06), and homocysteic acid

(0·74 ± 0·01) have their ε values within the range of 2·5–5·5% ε for glutamic acid. Higher interferences arise from tryptophan (1·2 ± 0·08), lysine (1·9 ± 0·01), and methionine (3·2 ± 0·06), exhibiting 8·5–23% ε of glutamic acid.

Determination of 2-Oxo Acids under Conditions Favouring the Formation of TC

The reaction of 2-oxo acids with MBH in conditions of oxidative coupling leading to blue dyes requires more acidic solutions as compared with aliphatic aldehydes (Fig. 2). The latter provide TC usually in higher yields than the corresponding 2-oxo acids, probably owing to the simpler reaction mechanism involved. The colour reaction of aldehyde could not be eliminated while maintaining that of the 2-oxo acid, by any of the proved variations. The reaction of 2-oxo acids, on the other hand, can be eliminated as will be shown later.

The absorption spectra of the blue dyes arising from 2-oxo acids were measured, in hexanol solutions, for all the oxo acids that gave a positive reaction. Over the 550–700 nm range the spectra resemble those of the TC of the corresponding aldehydes, and also resemble each other closely. Insensitive to the structure variations of the parent compound, the principal maxima lie at 668 ± 1 nm.

Procedure C. A mixture of 0·25 ml of MBH solution (25 mmol l^{-1}) and 0·15 ml of sodium trichloroacetate buffer pH 0·5 (1 mol l^{-1}) is prepared in a test tube, 0·25 ml of 2-oxo acid sample containing 0·6–60 nmol OGA (or the corresponding amount of other oxo acids, Table I) is added, and the closed test tube is heated on boiling water bath for 4·5 min and cooled down to room temperature. 0·25 ml of $FeCl_3$ solution (150 mmol l^{-1}) is added and in 25 min the system is diluted to 1·5 ml with a 10 : 1 (*V*/*V*) mixture of acetone with concentrated hydrochloric acid. The colour is steady for 45 min. The absorbance is measured at 668 nm.

Determination of Simple Aliphatic Aldehydes in the Presence of 2-Oxo Acids

In the determination of the activity of aldehyde-producing transaminases based on the reaction with MBH (*e.g.*, ref.[27]), interferences arise from the colour reaction of the excess 2-oxo acids, usually 2-oxoglutaric acid. An improvement in the selecitity of the "blue reaction" in favour of aldehydes can be achieved by pH adjustement in the condensation and coupling stages.

Copper(II) ions in mixture with Fe^{3+} favour the suppression of the colour reaction of OGA (Fig. 3) and other 2-oxo acids with MBH, probably on account of the Cu^{2+} complex formation with the 2-oxo acid azines. The presence of complexes with the metal-to-ligand ratios 1 : 2 and 1 : 1 for MBH and OGA azine can be proved rather easily by means of the Job's method[28] (pH 3·5, c_{total} = 1 mmol l^{-1}, λ_{max} = 390 nm).

The yield of the TC from aldehydes decreases to approximately a half if Cu^{2+} is added, the remaining factors, however, are positive.

The formation of TC from aliphatic aldehydes by oxidative coupling is accelerated approximately ten times and the colour is stabilized by Cu^{2+} ions. The use of buffers in the analytical oxidative coupling in MBH–$FeCl_3$ system adds to the reproducibility, but only buffers that do not form strong complexes with Fe^{3+} can be employed. Trichloroacetate, monochloroacetate, and formate buffers[29] satisfy well this requirement.

Procedure. A mixture of 0·25 ml of MBH solution (10 mmol l^{-1}) and 0·15 ml of formate buffer pH 3·75 (450 mmol l^{-1}) is prepared in a test tube, and 0·25 ml of sample containing typically 1—75 nmol of aliphatic aldehyde (MBH reactive[8]) is added. The sample may contain 0·6 μmol OGA, pyruvic acid, or oxosuccinic acid. The mixture is heated on boiling water bath for 4·5 min and cooled to 20—25°C, and 0·25 ml of oxidative solution containing $FeCl_3$ and $CuCl_2$ in concentrations of 45 and 80 mmol l^{-1}, respectively, is added. With acetaldehyde or succinic semialdehyde the formation of TC is complete in 1·5 min and the colour is steady (in this stage) for 40 min. The system is diluted to 1·5 ml with a fresh 10 : 1 (*V*/*V*) mixture of acetone with concentrated HCl. The absorbance is measured at 668 nm.

The colour is steady for a minimum of 3 h. The absorbance depends linearly on concentration up to absorbance 1·5; the molar absorptivities in the determination of acetaldehyde and succinic semialdehyde were identical $3{\cdot}3 \,.\, (\pm 0{\cdot}3) \,.\, 10^4$ l mol^{-1} . . cm^{-1}. The determination requires parallel calibration experiments.

The oxidation can be followed by extraction treatment, whereupon the sensitivity improves appreciably. The distribution constants in the hexanol–reaction phase systems are largely fairly high; TC derived from succinic semialdehyde exhibited a distribution constant of 9·36 ± 0·04. The TC in the hexanol phase are steady for about 30 min, the absorbance decrease is typically 1·5% in an hour.

Determination of L-*Glutamic Acid Dehydrogenase Activity*

L-Glutamic acid dehydrogenase from bovine liver (E.C.1.4.1.3, Sigma, USA, Type II) was used. The kinetics of the reaction was monitored photometrically for the mixture incubated at 25°C. The increase in the absorbance at 340 nm is due to the formation of the reduced form of nicotinamide adenine dinucleotide (NADH). The conventional technique[30] was modified by replacing phosphate by Tris buffer. Replicate samples were taken for kinetic measurements and OGA was determined in them by procedure *A*. In fifteen determinations in which the OGA and NADH concentrations lay in the region of 0·02—0·23 mmol l^{-1}, the concentrations (in mol l^{-1}) obeyed the relation

$$c_{OGA} = (1{\cdot}03 \pm 0{\cdot}036)\, c_{NADH} + (0{\cdot}75 \pm 0{\cdot}8) \,.\, 10^{-5} ,$$

with a correlation coefficient of $r = 0{\cdot}995$.

Determination of Phenylpyruvate: Glutamate Transferase Activity

The enzymatic activity was enriched from pre-isolated mitochondria of bovine kidneys to an approximately 35-fold increase in specific activity[31]. The reaction mixture composition corresponded to the original work. For the kinetic monitoring, replicate samples were taken to analysis of OGA by the enzymatic method[2,31] (c_{OGA-E}) and by procedure *A* (c_{OGA}). The OGA concentrations in 16 sample pairs lay in the 0·03–0·45 mmol l^{-1} range. The concentrations (in mol l^{-1}) determined by the two parallel methods were interrelated through

$$c_{OGA} = (1{\cdot}02 \pm 0{\cdot}035)\, c_{OGA-E} + (0{\cdot}94 \pm 1{\cdot}12) \,.\, 10^{-5} ,$$

$r = 0{\cdot}995$.

Determination of 4-Aminobutyrate: 2-Oxoglutamate Transferase Activity in the Reverse Reaction Direction

The enzyme (E.C.2.6.1.19) was enriched from pig brain to an approximately 25 specific activity increase[32]. This enzyme requires the presence of monothioglycol in the rather high concentration of 10 mmol l^{-1}. For SH dependent enzymes a modification must be adopted in the method so that typically 0·3 ml of the reaction mixture is added to 0·08 ml of $HgCl_2$ solution (160 mmol l^{-1}), heated shortly on boiling water bath and cooled, and the mercaptides and denatured proteins separated are centrifuged. Usually 0·25 ml of supernatant is taken to analysis by procedure *B*.

3 ml of the reaction mixture contained (in mmol l^{-1}): succinic semialdehyde (2·2), L-glutamic acid (10), pyridoxal-5-phosphate (0·11), monothioglycol (10), sodium tricine buffer pH 8·2 (100), and 0·08–0·4 mg of protein fraction. The reaction was accomplished in 0–60 min by incubation at 37°C. During the reaction, samples were taken for the determination of OGA by procedure *B* (c_{OGA}) and for the enzymatic determination[2] (c_{OGA-E}). The OGA concentrations in 16 sample pairs lay in the range of 0·03–0·50 mmol l^{-1}. The concentrations (in mol . l^{-1}) determined by the two methods satisfied the relation

$$c_{OGA} = (1{\cdot}05 \pm 0{\cdot}24)\, c_{OGA-E} + (0{\cdot}54 \pm 0{\cdot}98) \,.\, 10^{-5} ,$$

$r = 0{\cdot}996$.

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