Hydroxamic Acid Production and Active-site Induced Bamberger Rearrangement from the Action of α-Ketoglutarate Dehydrogenase on 4-Chloronitrosobenzene

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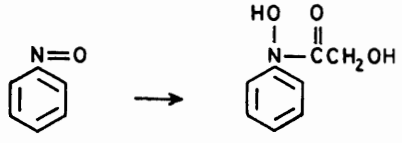
The α-ketoglutarate dehydrogenase complex obtained from *E. coli* has been found to convert 4-chloronitrosobenzene (3) into *N*-(4-chlorophenyl)succinohydroxamic acid (4) and *N*-(4-chloro-2-hydroxyphenyl)succinamic acid (5). The conversion of 4-chloronitrosobenzene (3) into these two products is not quantitative and attempts to identify other, significant low-molecular-weight metabolites have been unsuccessful. Partial enzyme-inactivation has been observed during the incubation of 4-chloronitrosobenzene (3) with α-ketoglutarate dehydrogenase. The direct enzymic conversion of the hydroxamic acid (4) into the isomeric product (5) did not occur. These results are interpreted on the basis of a mechanism in which N-(4-chlorophenyl)hydroxylamine (6) is generated at the enzyme active-site by a redox process. Condensation of the active-site bound products would give rise to the hydroxamic acid (4) directly, while a Bamberger-like rearrangement of the active-site bound hydroxylamine (6), followed by condensation of the resulting *o*-aminophenol, would explain the production of the succinamic acid (5).

THE ability of the thiamine-dependent enzymes pyruvate decarboxylase and transketolase to convert aromatic nitroso-compounds into hydroxamic acids has been well documented.[1,2] It has also been demonstrated that such enzymic reactions could be of considerable significance to the overall metabolic fate of nitroso-aromatic compounds in living organisms.[3,4] This significance was best illustrated by our finding that an intact unicellular algal species, *Chlorella pyrenoidosa*, converted a significant amount of nitrosobenzene (1) into the glycolyl-derived hydroxamic acid (2) (Scheme 1).[3] This metabolic conversion, which is most likely due to the action



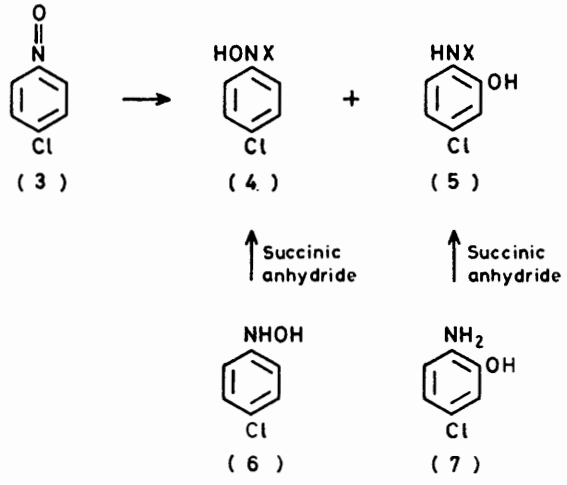
(1) (2)

SCHEME 1

of transketolase on the nitroso-functional group, has been found to be a rather general conversion among other photosynthetic organisms.[5]

On the basis of our previous work and the generally accepted mechanism of action of thiamine-dependent enzymes,[6] we expected the enzyme α-ketoglutarate dehydrogenase (α-KGD), to convert the model substrate 4-chloronitrosobenzene (3) into the succinyl-derived hydroxamic acid (4). We now report that this is the case; however, a totally unexpected reaction was also observed which reveals an interesting property of the active site of α-KGD. In addition to the production of the hydroxamic acid (4), α-KGD was also found to catalyse the conversion of the nitroso-substrate (3) into the *ortho*-hydroxylated product (5) (Scheme 2). The product (5) was strongly suggestive of a Bamberger-like rearrangement,[7] which occurred either with the hydroxamic acid (4) or its non-acylated analogue *N*-(4-chlorophenyl)hydroxylamine (6). Arylhydroxylamines such as compound (6) are potential products from thiamine-dependent enzymic reactions.[2]

The α-KGD obtained from *E. coli* is a macromolecular enzyme complex with a molecular weight of *ca.* 2.4 million.[8] Like the pyruvate dehydrogenase complex, it



X = CO·CH2CH2CO2H

SCHEME 2

is also composed of three, distinct enzymic groups, which are α-ketoglutarate decarboxylase, lipoyl reductase-transacylase, and dihydrolipoyl dehydrogenase.[8] The overall function of α-KGD is to convert α-ketoglutaric acid into CO_2 and succinyl coenzyme A, with production of NADH. Our interest was only in the thiamine-dependent action of the decarboxylase subunit of the enzyme complex, so the co-factors required for the reductase-transacylase (coenzyme A) and dehydrogenase (NAD^+) functions were usually omitted from our enzyme incubations. This modification increased the yields for the products of interest since coenzyme A was found to react chemically with the nitroso-substrate, which is expected for sulph-hydryl-

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containing compounds.[9] Although the presence of NAD^+ had no significant effect on the reaction under investigation, it was routinely omitted from enzyme incubations. Such manipulation of reaction conditions is commonly employed to investigate sub-unit reactions of these enzyme complexes.[10,11] The results described in this study were obtained by the incubation of the nitroso-substrate (3) with α-KGD in the presence of α-ketoglutaric acid and the co-factors Mg^{2+} and thiamine pyrophosphate in aqueous buffer.

In anticipation that α-KGD would convert the nitroso-substrate (3) into the hydroxamic acid (4), we first synthesized (4) by acylation of the hydroxylamine (6) with succinic anhydride. Upon analysis of incubates of compound (3) with α-KGD by high-pressure liquid chromatography (h.p.l.c.), we did observe a major product peak that behaved in a manner identical with the synthetic compound (4) in our h.p.l.c. system. In addition, we also observed a major peak in h.p.l.c. chromatograms that eluted before the peak corresponding to compound (4). Our initial thought was that this peak was due to the simultaneous production of the hydroxylamine (6), as previously observed in the case of pyruvate decarboxylase.[2] However, authentic compound (6) was found to chromatograph slightly faster than this second major peak in our h.p.l.c. system, thus demonstrating that compound (6) is not a major, detectable product in the case of the α-KGD action on substrate (3). At this point, we decided to synthesize the acid (5) and, indeed, found that the unknown product peak in h.p.l.c. chromatograms behaved in a manner identical with that of the synthetic acid (5). No other significant products were observed by h.p.l.c. analysis of the reaction between compound (3) and α-ketoglutaric acid in the presence of α-KGD. The solvent employed in our reversed-phase h.p.l.c. system contained desferal mesylate to facilitate the analysis of hydroxamic acids, for reasons previously reported.[12]

To prove that the major products in the title reaction were the acids (4) and (5), a large-scale incubation of the substrate (3) in the α-KGD system was conducted. From this incubate, several mg each of the acids (4) and (5) were isolated in sufficiently pure form such that u.v. and i.r. spectral data could be obtained, which were superimposable with the u.v. and i.r. spectra of the authentic acids (4) and (5).

The conversion of the nitroso-substrate (3) into the acids (4) and (5) was dependent upon the presence of both α-KGD and α-ketoglutaric acid, since controls lacking either gave no production of compounds (4) or (5). The concentration of α-ketoglutaric acid needed to obtain the maximum, initial rate of reaction was 2.0mM, which is the optimum concentration of this substrate for the normal enzymic reaction of α-KGD.[11] Likewise, the optimum pH for the conversion of compound (3) into the acids (4) and (5) together was at the known pH optimum of 8.0 for α-KGD.[11] The ratio of the acids [(5)] to [(4)] produced varied slightly with the concentration of α-ketoglutaric acid employed. At 1.0mM of α-ketoglutaric acid the ratio of the acids (5) to (4) was found to be 0.46, and this ratio decreased to 0.34 when 8.0mM of α-ketoglutaric acid was employed. The effect of buffer pH on the ratio of production of the acids (5) to (4) was much more dramatic since this ratio varied from 0.17 at pH 6.0 to 0.58 at pH 8.5. The initial rate of production of the acids (4) and (5) was linearly dependent upon the concentration of the nitroso-substrate (3), over the range of concentrations 0.05—0.40mM. The ratio of production of the acids (5) to (4) was independent of the concentration of compound (3). The pseudo-first-order rate constant for the conversion of compound (3) into the acids (4) and (5) together was calculated from data obtained after 10 min incubation and found to be 0.023 min^{-1} for a concentration of 1.0 unit per ml of α-KGD at 30 °C and pH 7.5, with α-ketoglutaric acid at a concentration of 2.0mM.

The conversion of the nitroso-substrate (3) into the enzymic reaction products (4) and (5) as a function of time is illustrated in the Figure. The most notable feature

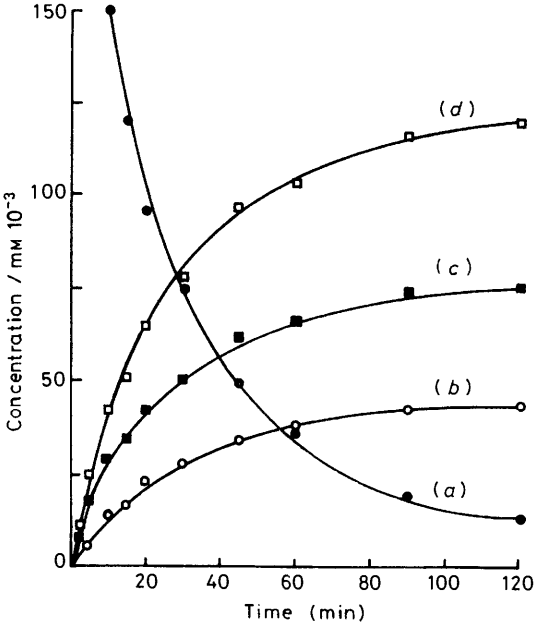
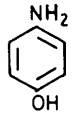


FIGURE The conversion of the nitroso-substrate (3) into the major enzymic products as a function of time. The conditions of the reaction are described in the Experimental section. The initial concentration of compound (3) was 0.25mM and the enzyme activity present was 0.24 units per ml. (*a*), Compound (3); (*b*), compound (5); (*c*), compound (4); and (*d*), molar sum of compounds (4) and (5)

is the lack of a material balance, with only 54% of compound (3) being accounted for at the end of a 2 h incubation period. This was also noted in numerous additional studies, and suggested that other reactions must be occurring to give products which were undetectable by our standard h.p.l.c. technique. A careful re-examination of concentrated organic extracts of enzyme incubates did indicate the presence of trace amounts of compound (7), of *p*-aminophenol (8), and of its succinic acid amide (9). The latter two metabolites are sugges-

tive of a Bamberger-like rearrangement, just as are compounds (5) and (7). However, the total amount of the products (7), (8), and (9) was insignificant compared with the amount of the substrate (3) unaccounted for in material balance studies. Extensive binding to protein by a reactive metabolite of compound (3) was considered as a possible cause of the poor material balance;



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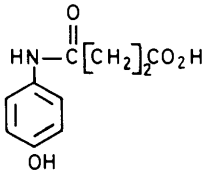


(9)

however, such an explanation would require the covalent binding of a large number of such metabolites to each α-KGD molecule. A more plausible explanation of the low material balance is that a highly polar metabolite has not yet been detected.

That covalent protein binding probably occurs during the action of α-KGD on compound (3) was suggested from the results of a study to determine whether enzyme inactivation accompanied the reaction of the nitroso-substrate (3) with α-KGD. Two simultaneous incubates of α-KGD with α-ketoglutaric acid were conducted, only one of which initially contained the nitroso-substrate (3) (0.18mM). After 60 min incubation time both incubates were treated with equal amounts of α-ketoglutaric acid and compound (3), such that the minimal concentration of compound (3) in both incubates was 0.18mM after 60 min. The reaction was then allowed to proceed for an additional 15 min. Throughout the entire incubation period aliquots were taken at appropriate intervals so that enzyme activity, as indicated by the rate of conversion of compound (3) into the acids (4) and (5) together, could be determined. The incubate that did not receive an initial treatment with compound (3) was used to correct for enzyme inactivation due to all processes other than reaction with the substrate (3), and showed that α-KGD maintained 94% of its original activity after the initial 1 h incubation period. The incubate that had contained compound (3) throughout the incubation period showed that only 43% of the original enzyme activity remained after 1 h. This clearly demonstrated that either compound (3) or a product of its reaction with α-KGD led to extensive inactivation of the enzyme. The failure to observe significant amounts of compound (6) tends to rule out enzyme inactivation as the result of the oxidation of sulph-hydryl groups by compound (3). Although the results of this study and the observed lack of a material balance during the enzyme reaction is suggestive of rather extensive protein binding, this point can only be proven following studies with radiolabelled compound (3), which is not currently available.

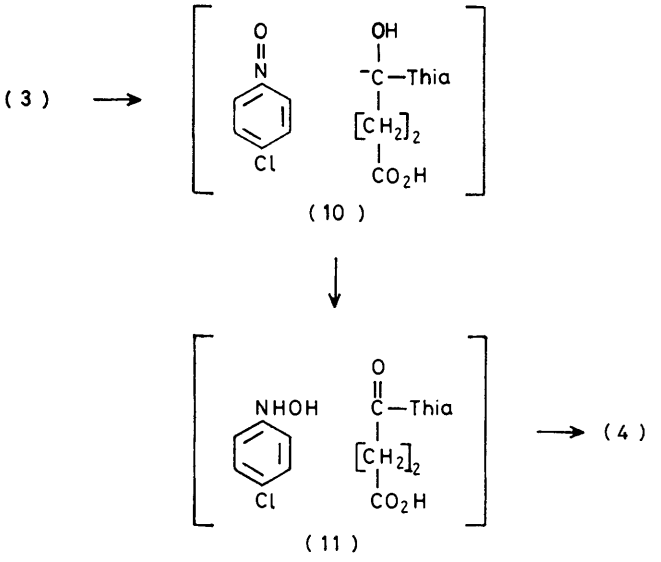
We also attempted to determine the mechanism by which compound (5) was produced from the substrate (3). The hydroxamic acid (4) was found to be quite stable upon incubation with α-KGD and α-ketoglutaric acid under the usual conditions. The enzymic co-incubation of the hydroxamic acid (4) (0.10mM) in the presence of compound (3) (0.25mM) did not affect the amount of compound (5) that was observed, as compared with a control incubate lacking (4). These results established that either the acid (5) is not produced from the acid (4), or that compound (4) is not accessible to an active-site of α-KGD due to its much greater polarity in comparison with the relatively non-polar nitroso-substrate (3).

In a parallel study, the incubation of the hydroxylamine (6) with α-KGD both in the presence and absence of compound (3) was investigated. Both the relative and absolute amounts of the acids (4) and (5) produced from compound (3) were not significantly affected by the presence of 0.10mM of compound (6). Small amounts of the acids (4) and (5) were produced when compound (6) was incubated with α-KGD alone, but this was accounted for by the oxidation of a portion of compound (6) to (3), which is a known reaction of arylhydroxylamines in aqueous solution.[13]

Finally, a study was conducted on the effect of 4-chloro-2-hydroxyaniline (7) on the relative amounts of the acids (4) and (5) produced by the action of α-KGD on compound (3). The ratio of the acids (5) to (4) was found to be only 17% greater when compound (7) (0.5mM) was added to the incubate with (3) (initially 0.25mM). This result implies that the enzymic product (5) arises from the acylation of compound (7) by ' active succinate.' [8,10] However, the fact that an excessive concentration of compound (7) added to the enzyme incubates did not greatly increase the ratio of the acids (5) to (4) suggests that compound (7) in solution does not compete well for the ' active succinate.' This observation is consistent with the fact that compounds (6) and (7) are only minor products of the α-KGD reaction, for which a probable partial mechanism can now be proposed.

We have previously described two mechanisms by which a hydroxamic acid functional group can result from the action of an enzyme bound ' active aldehyde ' on an aromatic *C*-nitroso-group.[2] One of these mechanisms involves the nucleophilic addition of the carbanion form of the ' active aldehyde ' to the nitroso-nitrogen.[1,2] This mechanism closely parallels both the mechanism proposed by Breslow on the chemistry of thiamine catalysis [14] and the reductive-acylation mechanism proposed by White and Ingraham for the interaction of ' active aldehydes ' with lipoic acid in α-keto-acid dehydrogenases.[15] An alternative mechanism proposes a redox reaction between the nitroso-group and the ' active aldehyde,' which results in an ' active acyl ' group and an arylhydroxylamine. Direct condensation of the two products would give a hydroxamic acid. At least for the case of pyruvate decarboxylase, the redox mechanism appears to be most likely.[2] This conclusion was made on the basis of our observation that pyruvate decarboxylase converts a major portion of nitroso-

substrates into the arylhydroxylamines, in addition to producing the *N*-acetylhydroxamic acids.[2] Although we observed only trace amounts of the hydroxylamine (6) produced from α-KGD and compound (3), we feel that the results in this current study favour the redox mechanism over the nucleophilic mechanism; however, the latter mechanism cannot be conclusively excluded. The sequence in Scheme 3 illustrates the redox reaction between the nitroso-substrate (3) and ' active succinic semialdehyde ' to give the hydroxamic acid (4). The bracketed structure (10) is a representation of the reactants bound at an active site of the decarboxylase component of α-KGD. A $2e^-$ transfer and associated prototropic changes (not shown) would give the structure (11), which represents the hydroxylamine bound or in close association with the enzyme active-site. The thiamine at the active site is in the form of ' active

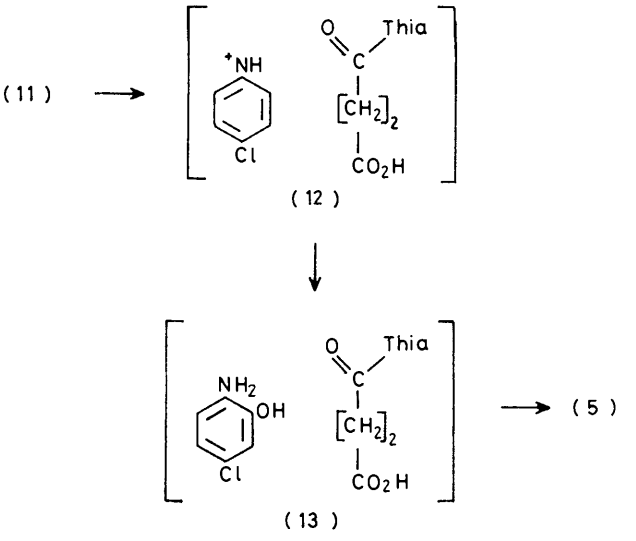


SCHEME 3 Thia = thiamine

succinate ' which is a reactive acylating agent.[10,16] Transfer of the succinyl group to the bound hydroxylamine before the latter can dissociate into solution explains both the formation of the acid (4) and the lack of significant free compound (6) in solution.

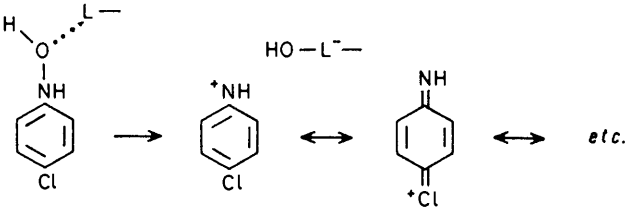
The production of the rearrangement product (5) is readily explained on the basis that the active-site associated hydroxylamine in structure (11) undergoes heterolytic N–O bond cleavage to produce an aryl nitrenium ion, also associated with the active site of α-KGD as illustrated in structure (12) of Scheme 4. Nucleophilic addition of the hydroxy-anion to an *ortho*-position would give the active-site associated *o*-hydroxyaniline (7), as shown in structure (13). As was the case for structure (11), succinyl transfer within structure (13) results in the product (5). The isomerization reaction from (11) to give (13) must be quite rapid relative to the dissociation of either compounds (6) or (7) from the enzyme active-site, since compounds (6) and (7) were only trace metabolites in the reaction. The intermediate (12) would also explain the trace amounts of compounds (8) and (9) that were observed. Most importantly, the structure (12) could readily account for the protein binding that the results of this study tend to suggest.

The Bamberger rearrangement is catalysed by strong-acid conditions;[7] therefore, the conversion of compound (6) into (7) must be associated with the enzyme. Since the active site of the decarboxylase sub-unit of α-KGD



SCHEME 4 Thia = thiamine

has been proposed to be hydrophobic in order to favour the stabilization of carbanions,[8] it is difficult to envisage a protic acid catalysing the Bamberger rearrangement of the structure (11). Perhaps the cofactor Mg^{2+} can serve as a Lewis acid to facilitate heterolytic N–O bond cleavage, as illustrated in Scheme 5, where L is a Lewis acid such as Mg^{2+}. As pointed out by Hanzlik, the ability of Mg^{2+} to stabilize negatively-charged oxygen leaving groups is facilitated in a more anhydrous or hydrophobic environment.[17] If Mg^{2+} were properly oriented within the hydrophobic active-site of α-KGD, then it may well be the necessary catalyst to effect the conversion of structure (11) into (12) as illustrated in Scheme 5. A

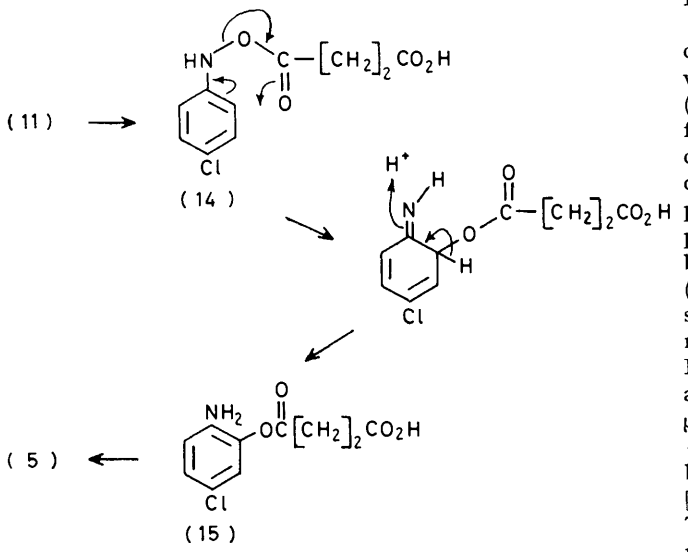


SCHEME 5

curious point is that other thiamine-dependent enzymes (including pyruvate dehydrogenase[5]) which require Mg^{2+} as a cofactor have not been found to catalyse an active-site, directed Bamberger-rearrangement.

A variation of the rearrangement part of the proposed mechanism can be seen to eliminate the necessity for an

acidic functional group at the active site of α-KGD. Acid catalysed N–O bond cleavage can be replaced by *O*-esterification. If transfer of the succinyl group in structure (11) were to be directed to the oxygen atom of the bound hydroxylamine in competition with or instead of to the nitrogen atom, then the reactive intermediate (14) would be produced. From the intermediate (14) both the acids (4) and (5) could be produced, the former by a favourable O→N acyl migration and the latter by a Claisen-like rearrangement to give compound (15) as illustrated in Scheme 6. Analogous rearrangements of



SCHEME 6

O-acylated aromatic hydroxylamines and hydroxamic acids to *o*-aminophenol derivatives have been reported to occur both by a cyclic concerted mechanism and by an ion-pair mechanism, depending upon the structure of the reactant.[18] Following such a rearrangement, an O→N acyl migration in compound (15) would result in the acid (5). Attempts to differentiate between these two possible rearrangement mechanisms of Scheme 5 and 6 are in progress with the appropriate ^{18}O-labelled reagents.

The ability of α-KGD to produce a product that must arise by a Bamberger-like rearrangement could be of toxicological significance to the degree that nitrenium cations or electrophilic species like (14) are produced. Sternson and Gammans have also reported an enzyme-catalysed Bamberger rearrangement and discussed the potential toxicological significance of such reactions.[19] The nature of the enzyme employed in their study was not characterized other than being present in the supernatant (105 000 g) of rat liver homogenates. α-KGD could not be responsible for that reported reaction since α-KGD can utilize only nitroso-substrates, from which it most likely generates hydroxylamines at the decarboxylase active site. The trapped hydroxylamine is then susceptible to the rearrangement reaction, in addition to being converted into a hydroxamic acid. Such a unique combination of reactions has not been observed during our investigations on other thiamine-dependent enzymes.

EXPERIMENTAL

High-pressure liquid chromatography (h.p.l.c.) was conducted as for a previously described system.[12] U.v. spectra were obtained with a Beckman Model 35 spectrophotometer and i.r. spectra were obtained with a Perkin-Elmer Model 180 spectrophotometer. N.m.r. spectra were obtained on a Jeol 100 MHz spectrometer. M.p.s were obtained with a calibrated Thomas-Hoover m.p. apparatus and elemental analyses were performed by Galbraith Laboratories.

Preparation of α-Ketoglutarate Dehydrogenase from E. coli.—The growth of *E. coli* (Crookes strain, ATCC 8739) was achieved in a manner previously described.[11] Batches (*ca.* 5 l) each gave wet-cell yields of *ca.* 35 g. The sequence for the purification of the enzyme was the standard procedure [11] as modified by Eley *et al.*[20] It was necessary to decrease the amount of protamine sulphate employed in each precipitation step relative to that suggested in the standard procedure.[11] The α-ketoglutarate dehydrogenase obtained by polyethylene glycol precipitation [20] was of high purity [21] (3.1 units/mg protein) and was employed as such in this study. Determination of enzyme activity was by NAD^+ reduction assay [11] and protein was determined by the Bradford method.[22] One unit of enzyme activity is that amount which catalyses an initial rate of formation of 1 μmol of NADH per min. The enzyme was stored at −20 °C as solutions of *ca.* 2 units per ml in 0.02M KH_2PO_4 buffer (pH 7.0) or in 0.02M TRIS·HCl buffer (pH 7.0) [TRIS = tris(hydroxymethyl)methylamine], although the TRIS solutions were considerably less stable than were phosphate solutions of the enzyme.

Synthesis of N-(4-*Chlorophenyl*)*succinohydroxamic Acid* (4).—Freshly prepared 4-chlorophenylhydroxylamine [23] (7.2 g, 50 mmol) was dissolved in $CHCl_3$ (50 ml) and combined with a solution of succinic anhydride (5.5 g, 55 mmol) in $CHCl_3$ (100 ml). To the solution was added pyridine (1 ml) and the reaction mixture was left at room temperature for 30 min, after which it was stored overnight at −20 °C. The crystalline solid was filtered off and recrystallized from 20% EtOH, following treatment with Norit (neutral), and then recrystallized from acetone–diethyl ether (3 : 5) to give white crystals of the *acid* (4) (3.6 g, 30%), m.p. 121.5—122 °C (Found: C, 49.4; H, 4.3; N, 5.75. $C_{10}H_{10}ClNO_4$ requires C, 49.28; H, 4.14; N, 5.75%); $\lambda_{max.}$ EtOH 259 nm (ε 14 600); $\nu_{max.}$ (KBr) 3 200, 1 700, and 1 630 cm^{-1}; δ_H (D_2O) 7.38 (4 H, sym. m) and 2.62 (4 H, m).

Synthesis of N-(4-*Chloro*-2-*hydroxyphenyl*)*succinamic Acid.* (5).—To 4-chloro-2-hydroxyaniline [24] (2.2 g, 15 mmol) suspended in anhydrous Et_2O (30 ml) was added succinic anhydride (1.7 g, 17 mmol) in $CHCl_3$ (50 ml). The reaction mixture was stirred for 2 h and then stored overnight at −20 °C. The solid was filtered off, recrystallized from 20% EtOH and then from acetone–diethyl ether (1 : 3) to give the *acid* (5) (1.4 g, 38%) as gold needles, m.p. 164—165 °C (Found: C, 49.55; H, 4.2; N, 5.75. $C_{10}H_{10}ClNO_4$ requires C, 49.28; H, 4.14; N, 5.75%); $\lambda_{max.}$ (EtOH) 249 nm (ε 12 200); $\nu_{max.}$ (KBr) 3 380, 3 250, 1 705, and 1 665 cm^{-1}; δ_H (D_2O) 7.34—6.58 (3 H, m) and 2.65 (4 H, m).

Identification of the Acids (4) *and* (5) *in Enzymic Reactions.* —To the 0.05M KH_2PO_4 buffer (pH 7.5, 160 ml) was added $MgCl_2$ (19 mg, 200 μmol), thiamine pyrophosphate (18.4 mg,

40 μmol), sodium α-ketoglutarate (67 mg, 400 μmol), and 4-chloronitrosobenzene (7.1 mg, 50 μmol). The solution was placed in a water-bath at 30 °C and treated with a solution (40 ml) of α-ketoglutarate dehydrogenase (80 units of enzyme as 26 mg of protein). The reaction was agitated frequently for 2 h. At the end of the incubation period, solid NaCl (20 g) was added to the solution, the pH was adjusted to 3.5 with 1M HCl, and the solution then extracted twice with Et_2O (200 ml). The combined Et_2O extract was dried (Na_2SO_4) and evaporated under reduced pressure to give a pale yellow residue; thin layer chromatographic (t.l.c.) analysis indicated the acids (4) and (5) as the major components. The residue was chromatographed on an EM-silica-gel 60 (70—230 mesh) column (20 × 1.1 cm, internal diameter) and eluted with CH_2Cl_2–MeOH solutions with a sequentially increasing concentration of methanol (5—30%), which also contained 0.1% glacial acetic acid. The composition of the fractions obtained from this silica-gel separation was monitored by h.p.l.c., and fractions of similar composition were combined and evaporated under reduced pressure to give two major fractions, of which the first was enriched in the acid (5) and the second was primarily the acid (4). Final purification of the acid (5) from the first fraction and the acid (4) from the second fraction was achieved by preparative h.p.l.c. on μ-Bondapak C_{18} (3.8 mm × 30 cm) with 50% methanol, adjusted to pH 3.5 with glacial acetic acid as the eluant. H.p.l.c. fractions containing either the pure acids (4) or (5) were separately combined and the methanol evaporated off under reduced pressure, followed by lyophilization to give the acid (5) (2 mg) and the acid (4) (1.5 mg). Both the acids (4) and (5) gave u.v. (EtOH) and i.r. (KBr pellets) spectra nearly identical with the synthetic standards.

Analytical Determinations on the Enzymic Reaction.—The usual methodology for the incubation of 4-chloronitrosobenzene (3) with the enzyme consisted of combining all the reactants except the enzyme in the 0.05M KH_2PO_4 buffer (pH 7.5) and then adding the enzyme in 0.02M KH_2PO_4 (pH 7.0) (reactant buffer–enzyme buffer, 4 : 1) to initiate the reaction. The cofactors $MgCl_2$ and thiamine pyrophosphate were present at reaction concentrations of 1.0mM and 0.2mM, respectively, and 2.0mM of α-ketoglutaric acid was employed in most studies. The nitroso-substrate (3) was added just before the enzyme solution as a concentrated solution in 95% ethanol to give a reaction concentration of 0.05mM—0.50mM, with most studies being conducted with [(3)] 0.25mM. In certain, competitive studies an additional substrate, including *N*-(4-chlorophenyl)hydroxylamine (6), 4-chloro-2-hydroxyaniline (7), 4-bromoaniline, the hydroxamic acid (4), and the rearrangement product (5), was also added as a concentrated ethanol solution just prior to the addition of the nitroso-substrate (3) or an ethanol control. The amount of α-KGD activity was *ca.* 0.40 units per ml. In most cases the final incubate volume was 10.0 ml. Temperature control (generally at 30 °C) was maintained by a water-bath. At desired times following the start of the reaction, aliquots (1.0 ml) were taken from the incubate and combined with methanol (1.0 ml) which was pre-cooled to −20 °C. For h.p.l.c. analysis, 10 or 20 μl of the methanol-quenched aliquots were directly injected onto a μ-Bondapak C_{18} column and chromatographed with 50% methanol, buffered to pH 3.5 with 0.01M KH_2PO_4, which contained 0.01% (w/v) of desferal mesylate.[12] The solvent flow-rate was 1.5 ml/min and component detection was made at λ 254 nm. Quantitative calculations were made on the basis of component peak-heights compared with peak heights generated by known amounts of authentic standards.

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