mal epithelium to carcinoma changes in chromosome number and morphology in the dividing cells first appear.

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Alpha-Ketoglutaric Semialdehyde: A Metabolic Intermediate

Abstract. *α-Ketoglutaric semialdehyde has been obtained as a product of hydroxyproline through reactions catalyzed by purified enzymes from* Pseudomonas. *It has been characterized both by chemical and enzymatic derivatives and by comparison with the chemically synthesized compound. This reactive compound, not previously known as a product of biological reactions, may participate in other metabolic pathways.*

α-Ketoglutaric semialdehyde (2,5-dioxovalerate) was earlier proposed (*1*) as an intermediate in the inducible degradation of hydroxyproline to α-ketoglutarate by extracts of *Pseudomonas striata.* This compound has now been isolated as an enzymatic product of hydroxyproline.

Hydroxyproline is degraded by extracts of *Pseudomonas* through the reactions shown in Fig. 1. The immediate precursor of α-ketoglutaric semialdehyde, Δ^1-pyrroline-4-hydroxy-2-carboxylate, has been obtained as an enzymatic product of reaction 2; the enzymes catalyzing reactions 3 and 4 have been purified from soluble extracts of *P. striata* grown on hydroxyproline (*2*). α-Ketoglutaric semialdehyde (KGSA) was made enzymatically on a millimolar scale and purified by chromatography through Dowex-1-Cl. Eluates in 0.1*N* HCl were concentrated and assayed by the use of enzyme 4 (Fig. 1). The following observations support the identification of the semialdehyde.

1) The reaction catalyzed by partly purified enzyme 4 leads to the formation of TPNH (reduced triphosphopyridine nucleotide) and α-ketoglutarate in quantities equivalent to the KGSA utilized. α-Ketoglutarate was measured by oxidation of DPNH (reduced diphosphopyridine nucleotide) with crystalline glutamic dehydrogenase and was identified as the 2,4-dinitrophenylhydrazone by paper chromatography.

2) On treatment with H_2O_2, KGSA releases a molar equivalent of CO_2. The residual product appeared to be succinic semialdehyde by paper-chromatographic comparison of the 2,4-dinitrophenylhydrazone with that derived from chemically synthesized succinic semialdehyde (*3*). The succinic semialdehyde-2,4-dinitrophenylhydrazone was also reduced (PtO_2, H_2, at 1.4 atm), yielding γ-aminobutyrate as the only ninhydrin-positive product. This reduction product migrated identically with authentic γ-aminobutyrate on paper chromatography.

3) Enzymatically formed KGSA could not be distinguished from chemically synthesized KGSA (*4*) by paper chromatography of the free KGSA (Table 1) or of the 2, 4-dinitrophenylhydrazones, or by elementary analyses and melting points. The 2,4-dinitrophenylhydrazine derivative of chemically or enzymatically synthesized KGSA is relatively insoluble in ethyl acetate and other common organic solvents and was recrystallized from nitrobenzene after Murakami *et al.* (*6*). Enzymatically derived KGSA-2,4-dinitrophenylhydrazone was reduced to ornithine (the only ninhydrin-positive product) with PtO_2 and H_2 at 1.4 atm. Analysis (percentage by weight) of the recrystallized phenylhydrazones gave the following composition: Chemically made: C, 41.5; H, 3.24; N, 22.3. Enzymatically made: C, 41.8; H, 2.76; N, 22.5. Calculated (for bis-2,4-dinitrophenylhydrazone, $C_{17}H_{14}N_8O_{10}$): C, 41.6; H, 2.83; N, 22.9. The melting point for the chemically made derivative of KGSA was 231° to 233°C; for the enzymatically made derivative it was 227° to 229°C. The melting point of the mixture was 229° to 233°C.

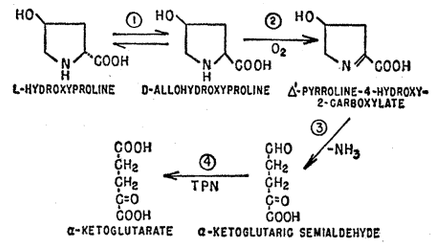

Fig. 1. Individual enzymatic steps in the inducible degradation of hydroxyproline by extracts of *P. striata.*

Little attention appears to have been paid to the chemical characterization or reactivity of free KGSA. The 2,4-dinitrophenylhydrazone of KGSA methyl ester has been synthesized by Clauson-Kaas and Limborg (*5*) from methyl-2-furoate, by way of 2,5-methoxylation and reduction. The 2,4-dinitrophenylhydrazone of KGSA itself has been obtained by Murakami *et al.* (*6*). Reductive amination of KGSA to proline has also been noted (*7*).

The semialdehyde appears to be formed from Δ^1-pyrroline-4-hydroxy-2-carboxylate (reaction 3, Fig. 1) in an irreversible reaction catalyzed by a single enzyme. No evidence has been obtained for the participation of a cobamide coenzyme or of pyridoxal phosphate. 2-Keto-3-deoxy-L-arabonate (*8*) has been ruled out as a free intermediate in the reaction.

In addition to its position as an intermediate in the bacterial degradation of hydroxyproline, KGSA has been provisionally identified by Dr. R. H. Abeles (*9*) as an intermediate in the oxidation

Tabel 1. Chromatographic behavior of KGSA made by chemical or enzymatic synthesis. Ascending chromatograms were run on Whatman No. 1 paper at room temperature. Spots were made visible by spraying with alkaline $AgNO_3$ (*11*), 2,3,5-triphenyltetrazolium (*11*), or *o*-phenylenediamine (*12*).

R_F of KGSA	
Enzymatic	Chemical
n-*Butanol:pyridine:*H_2O *(6:4:3)*	
0.38	0.42
Ether:benzene:formic acid: H_2O *(70:30:14:10)*	
0.14	0.15
n-*Propanol:0.2*N NH_4OH *(3:1)*	
0.33	0.34
t-*Butanol:formic acid:*H_2O *(70:15:15)*	
0.52	0.52
n-*Butanol:ethylmethyl ketone:*H_2O*: triethylamine (40:40:20:4)*	
0.34	0.34

of 2-keto-3-deoxyarabonate to α-ketoglutarate by *P. saccharophila* (*10*).

Our preliminary observations indicate that KGSA in the presence of NH_3 is converted nonenzymatically to pyrrole-2-carboxylate and other compounds, including a probable Δ^1-pyrroline which is reducible to proline by sodium borohydride. KGSA appears also to be a substrate for reduction by DPNH with crystalline rabbit muscle lactic dehydrogenase, although the reduction product has not been characterized. We are investigating other possible involvement of this reactive dicarbonyl in both bacterial and mammalian metabolism.

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Spectra of Deoxygenated Hemoglobin in the Soret Region

Abstract. *Methemoglobin is easily formed during the deoxygenation of hemoglobin. It can be removed with methemoglobin reductase. The Soret spectrum of pure deoxyhemoglobin is reported. Comparison of these data with the published spectra shows that some of these are incorrect since they must represent mixtures of deoxy- and methemoglobin.*

The preparation of deoxygenated ferrohemoglobin (deoxyhemoglobin) from oxyhemoglobin presents certain difficulties. In general, three methods have been used for this purpose. The most commonly employed chemical reagent for the reduction of oxygen is sodium dithionite. Although this method removes oxygen rapidly and completely, there is little doubt that it produces chemical changes in the hemoglobin molecule, probably as a result of the action of hydrogen peroxide which is a product of the reduction of oxygen by sodium dithionite. Dalziel and O'Brien (*1*) have shown that this results in secondary changes in the extinction at 430 mμ within a very short time (less than 1 minute). They also found that the yield of deoxyhemoglobin decreases with decreasing hemoglobin concentration, decreasing concentrations of dithionite and increasing oxygen concentration. This method is therefore particularly unsuitable for preparing known concentrations of deoxyhemoglobin from exyhemoglobin for measurements in the Soret region.

The alternative method for converting oxy- to deoxyhemoglobin is the physical removal of oxygen, either by passage of an oxygen-free inert gas over the solution or by repeated evacuation in a high vacuum. Displacement with an inert gas involves relatively long exposures of the hemoglobin to low oxygen pressures. This is undesirable since, as Brooks has shown (*2*), the rate of oxidation of hemoglobin to methemoglobin is at a maximum at low oxygen pressures. This apparent paradox is due to the fact that deoxyhemoglobin is oxidized very much more rapidly than oxyhemoglobin. Such protection against oxidation by oxygen in oxygenated ferrous complexes is well-known and has been discussed by Williams (*3*). The spectra of deoxyhemoglobin prepared by slow deoxygenation are thus likely to contain contributions from methemoglobin.

Both the spectra published by Sidwell *et al.* (*4*) and those in Lemberg and Legge's textbook (*5*) show a shoulder between 410 and 415 mμ. The deoxyhemoglobin in both cases was prepared by slow deoxygenation. It thus appeared likely that this shoulder was due to methemoglobin.

In our experience a shoulder between 410 and 415 mμ was always observed in the spectra of deoxyhemoglobin prepared by slow deoxygenation. The magnitude of this effect (ε412/ε430) was quite variable and not reproducible. On the assumption that methemoglobin was responsible for the absorption in this region, the influence of methemoglobin reductase was investigated. This enzyme catalyzes the reduction of ferri- to ferrohemoglobin by DPNH (reduced diphosphopyridine nucleotide) in the presence of trace amounts of methylene blue (*6*). Three samples of identical hemoglobin concentration (0.0085 percent) were deoxygenated by passage of oxygen-free nitrogen over the solution for 2 hours, and the spectra were recorded. In each case the spectrum was again recorded after reoxygenation of the solution. The results are shown in Fig. 1. It can be seen that sample A, which initially contained only oxyhemoglobin, shows, after deoxygenation, a shoulder 412 mμ and an extinction coefficient ε of 1.20 at 430 mμ. The absorption maximum after reoxygenation was at 412 mμ. Sample B differed from sample A only by the addition of methemoglobin reductase, DPNH, and methylene blue before deoxygenation. Under these conditions the shoulder is absent and the extinction coefficient at 430 mμ has increased to 1.42. Reoxygenation results in a spectrum which is indistinguishable from that of the original oxyhemoglobin sample with a maximum at 415 mμ. The third sample (C) which was identical with sample B except for the substitution of methemoglobin for oxyhemoglobin, gives spectra which are identical with those shown for sample B. The points shown on Fig. 1*A* are calculated for a mixture of 20 percent methemoglobin and 80 percent deoxyhemoglobin. This was done by using the extinction coefficients of the deoxyhemoglobin spectrum shown in Fig. 1*B* and of a spectrum of methemoglobin prepared as described here. The observed spectrum can thus be accounted for by an impurity of 20 percent methemoglobin that is formed during the deoxygenation and

Table 1. Absorption of hemoglobins.

Our study		Sidwell *et al.*	
λ_{max} (mμ)	10^{-5} ϵ_{max}	λ_{max} (mμ)	10^{-5} ϵ_{max}
	Methemoglobin		
405	1.53		
	Oxyhemoglobin		
415	1.32	415	1.29
	Deoxyhemoglobin		
430	1.40	430	1.18