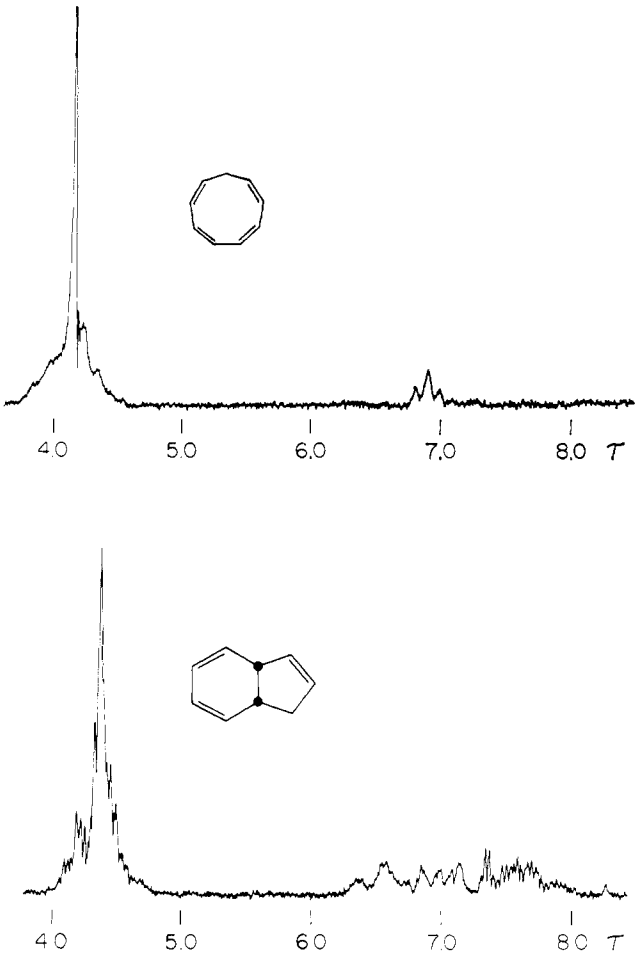

Figure 1. The 60-MHz nmr spectrum of **1** (top) and **4** (bottom) taken in CCl_4 at 38°.

The nmr spectrum of **1** is depicted in Figure 1 and shows the six vinyl protons at τ 4.15 and the two methylene protons at τ 6.88. Also shown in Figure 1 is the spectrum of the same sample taken after 5 hr at 40°. This spectrum is that of *cis*-8,9-dihydroindene (**4**). It is important to note here that no *trans*-8,9-dihydroindene is produced as a result of the thermal $6\pi \rightarrow 4\pi 2\sigma$ electrocyclic ring closure of **1**.[12] The nmr spectrum of **3** displayed a more complicated vinyl pattern for six protons at τ 4.22, a doublet for the methyl group at τ 8.88 (J = 7 Hz), and the tertiary proton at τ 8.70 (complex quartet). We have also taken infrared spectra of **1** and **3** in CCl_4 and find no absorption at 965 cm^{-1} characteristic of a *trans* double bond.

These data clearly indicate that we have isolated and observed directly **1** and **3**. The question now remains with regard to the degree of thermal stability of the *cis,cis,cis,cis*-1,3,5,7-cyclononatetraene skeleton. In qualitative experiments we have found that **1** has a half-life of *ca.* 14 min at 40°. A more quantitative result provided by Dr. Gary Petrowski[13] indicated that at 23° the rate constant for the thermal transformation of **1** → **4** is 6×10^{-5} sec^{-1}; the half-life for **1** at this temperature is 50 min.

Cyclononatetraene (**1**) now presents itself for further study as a remarkably stable compound. There are many questions which can now be raised and hopefully answered with respect to the properties and chemical reactivity of this very interesting olefin, and we hope to provide more information with regard to these points in the near future.

Acknowledgment. The authors gratefully acknowledge support from the Petroleum Research Fund of the American Chemical Society and partial support from the Intramural Research Fund of the University of California.

(12) One other compound is produced from **1** to the extent of about 7%; at present its structure is unknown.

(13) G. Petrowski, Ph.D. Dissertation, University of California, Los Angeles, 1969.

(14) Alfred P. Sloan Research Fellow.

(15) National Science Foundation Undergraduate Research Fellow.

Phillip Radlick,[14] Gary Alford[15]
Department of Chemistry, University of California
Riverside, California 92502

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α-Iminoglutarate Formation by Beef Liver L-Glutamate Dehydrogenase. Detection by Borohydride or Dithionite Reduction to Glutamate[1]

Sir:

The enzyme L-glutamate dehydrogenase catalyzes the reversible oxidative deamination of glutamate to α-ketoglutarate, with DPN or TPN as coenzyme.[2-4] The over-all reaction, in analogy with other oxidations of amines,[5-9] most likely proceeds through an intermediate α-iminoglutarate in at least two steps. Reaction 2

$$\text{glutamate} + \text{DPN} \rightleftharpoons \alpha\text{-iminoglutarate} + \text{DPNH} \quad (1)$$

$$\alpha\text{-iminoglutarate} + H_2O \rightleftharpoons \alpha\text{-ketoglutarate} + NH_3 \quad (2)$$

was at first thought to occur spontaneously,[2] but later studies have not shown evidence for the spontaneous formation of the postulated α-iminoglutarate.[3,4]

We wish to present here direct evidence that the reverse direction of reaction 2 is enzyme catalyzed. We have trapped the presumed α-iminoglutarate-^{14}C, formed from α-ketoglutarate-^{14}C and ammonia in the presence of glutamate dehydrogenase and absence of coenzyme, by reduction to glutamate-^{14}C with sodium borohydride or sodium dithionite. Representative results are shown in Table I.

The formation of glutamate was dependent on enzyme and α-ketoglutarate concentration. Controls, not shown, with omission of enzyme, α-ketoglutarate, or borohydride, or with enzyme inactivated by heat, 10^{-3} M $AgNO_3$, or 30% ethanol, or substitution of yeast alcohol dehydrogenase or bovine serum albumin for the enzyme, did not result in significant glutamate formation. The borohydride appeared to reduce the α-iminoglutarate bound to enzyme, since glutamate was not formed if borohydride was added before α-ketoglutarate or if nonlabeled α-ketoglutarate was added before

(1) Supported by the National Institutes of Health through Research Grant GM-11799 and Training Grant GM-00184-11.

(2) H. von Euler, E. Adler, G. Günther, and N. B. Das, *Z. Physiol. Chem.*, **254**, 61 (1938).
(3) J. A. Olson and C. B. Anfinsen, *J. Biol. Chem.*, **202**, 841 (1953).
(4) H. J. Strecker, *Arch. Biochem. Biophys.*, **46**, 128 (1953).
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(9) D. S. Goldman, *ibid.*, **34**, 527 (1959).

Table I[a]

$NaBH_4$, M	Changes in expt	Glutamate formed— nmoles	Moles/50,000 g of enzyme
0.02[b]	None	132	1.65 ± 0.17[c]
0.02	2 mg of enzyme	82	2.05
0.02	0.5 mg of enzyme	14	1.40
0.55[d]	None	102	1.27 ± 0.42[c]
0.55	0.005 M α-ketoglutarate	48	0.60
0.55	0.001 M α-ketoglutarate	13	0.17
0.55	0.0005 M α-ketoglutarate	4	0.05
0.55	0.0001 M α-ketoglutarate	1.6	0.020
0.55	$NaBH_4$ added before α-ketoglutarate	0	0.00
0.55	Nonlabeled α-ketoglutarate added, then $NaBH_4$, then labeled α-ketoglutarate	0	0.00
0.02[b]	4×10^{-5} M added DPN	96	1.20
0.02	4×10^{-5} M added DPNH	332	4.15
None	20 mg of $Na_2S_2O_4$ in place of $NaBH_4$	64	0.90

[a] Experiments were conducted at 0°, with reagents added in the following order: glutamate dehydrogenase (Boehringer), 4 mg; ammonium acetate, 0.2 M, pH 9.3; α-ketoglutarate-5-^{14}C, 5×10^7 dpm/mmole, 0.02 M; $NaBH_4$, as noted below, or $Na_2S_2O_4$. The final volume was 1 ml. The mixture was incubated 10 min, and then protein was precipitated with 1 M $HClO_4$ and centrifuged. Glutamate was separated by ion-exchange chromatography on Dowex 50 and was assayed for ^{14}C by liquid scintillation. [b] $NaBH_4$ in 10^{-3} M NaOH, added in 20 portions over 10 min. [c] Average ± standard error of mean. [d] $NaBH_4$, 5 mg, added as solid.

the borohydride. The reaction was unaffected by addition of 0.02 M DL-glutamate or 0.02 M DL-α-hydroxyglutarate, or variation of pH from 8.1 to 10.3. Several experiments indicated that sodium dithionite could replace borohydride as reducing agent.

Kinetic studies with catalytic amounts of enzyme in a similar system, with 1.6×10^{-4} M DPNH and without borohydride, have shown that the rate of over-all enzyme reaction depended on the concentration of α-ketoglutarate in almost exactly the same manner as did the formation of α-iminoglutarate.

The enzyme was specifically assayed for bound DPN or DPNH by fluorometry of DPNH.[10] No bound coenzyme was detected, and the sensitivity of the method enabled the detection of 0.03 molecule per enzyme subunit. The nonmediation by coenzyme was corroborated by the effects on borohydride reduction of the addition of much larger amounts of DPN or DPNH (Table I).

Curiously, about one or two molecules of glutamate was formed per enzyme subunit of mol wt 50,000, regardless of reaction conditions, suggestive of a stoichiometric reaction. The reason for this is not immediately clear; however, we have found that the enzyme retained full activity under these conditions.

The product from borohydride reductions was identified as glutamate-^{14}C by gradient ion-exchange chromatography, recrystallization with carrier, and paper chromatography in three different solvent systems. In the latter, the radioactivity coincided with ninhydrin color of added glutamate. The glutamate-^{14}C was recrystallized with D- and L-glutamate·HCl after Graff, Rittenberg, and Foster,[11] and the results, shown in

(10) T. L. Chan and K. A. Schellenberg, *J. Biol. Chem.*, **243**, 6284 (1968).

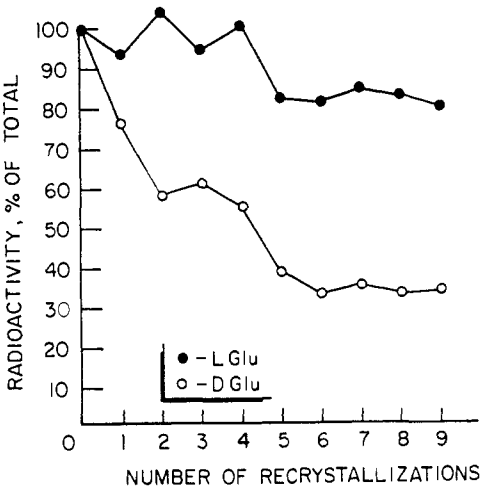

Figure 1. Carrier L- or D-glutamic acid hydrochloride was added to portions of the labeled glutamate produced as described in Table I, row 1. The carrier plus label was recrystallized from water–acetone, and samples from each recrystallization were assayed.

Figure 1, indicate that the glutamate formed from the presumed enzyme-bound α-iminoglutarate was predominantly in the L configuration. The dithionite product was identified as glutamate by ion exchange and paper chromatography; the stereochemistry has not yet been determined.

The stereochemistry of the glutamate from borohydride reduction, the dependence on active enzyme, and the correlation of over-all enzyme rate and extent of α-iminoglutarate formation with the concentration of α-ketoglutarate all suggest that the α-iminoglutarate formation by the enzyme is a part of the enzyme reaction rather than a side reaction or nonspecific reaction. Further studies are in progress. In particular the effect of ammonia concentration, analogs of ammonia, and other reductive trapping agents will be investigated, and attempts will be made to determine the reason for the near-stoichiometric amount of α-iminoglutarate that is produced.

Acknowledgment. We gratefully acknowledge the technical assistance of Mrs. Evelyn Connor.

(11) S. Graff, D. Rittenberg, and G. L. Foster, *ibid.*, 133, 745 (1940).
(12) Predoctoral Fellow of The Johns Hopkins University.
(13) John and Mary R. Markle Scholar in Academic Medicine.

Margaret C. Hochreiter,[12] Karl A. Schellenberg[13]
Department of Physiological Chemistry
The Johns Hopkins University School of Medicine
Baltimore, Maryland 21205
Received July 31, 1969

Spectral Evidence for the Existence of the Superoxide Ion in Molten LiF–NaF–KF

Sir:

We wish to report the existence of an unusual oxidant in molten LiF–NaF–KF (46.5–11.5–42.0 mole %) at 500°. We believe this oxidant to be the superoxide ion, O_2^-. This solute species can be added directly to the molten solvent (as NaO_2) or can be generated by the selective oxidation of oxide ion in the melt.