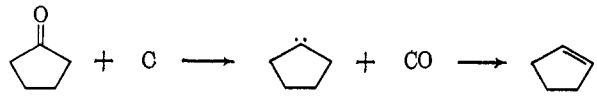
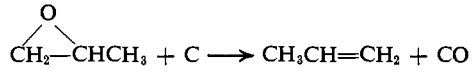
At present, we have no satisfactory explanation for the formation of doubly unsaturated products.

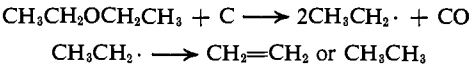


A side reaction to be considered in the deoxygenation of ketones by carbon vapor is photolysis by radiation from the arc. The extent of this side reaction has been estimated in the case of 2-butanone by measuring the relative amounts of methane and carbon monoxide: CH_4:CO = 1:25. Since methane and carbon monoxide are formed in similar amounts during photolysis,[8] the photolytic pathway does not appear significant in the 2-butanone reaction. The olefins observed in the deoxygenation reactions are not produced by photolysis.

The reaction of propylene oxide with carbon atoms leads to propylene in 160% yield. The yield value greater than 100% indicates that the C_2 and/or C_3 present in the carbon vapor is also participating in oxygen abstraction. This result is consistent with a deoxygenation by a carbon atom to form carbon monoxide with the simultaneous formation of propylene.



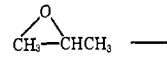
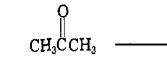
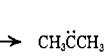
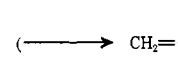
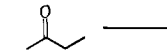
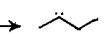
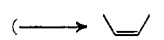
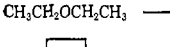
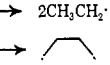
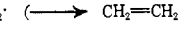
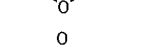
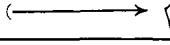
The reaction of ethyl ether with carbon atoms gives ethylene and ethane in 40 and 35% yields, respectively (yields calculated on the basis of 1 mole of ethylene and 1 mole of ethane/mole of C_1). This result may also be explained by a deoxygenation process to produce two ethyl radicals and a molecule of carbon monoxide. A small amount of *n*-butane, the radical coupling product, was also observed.



By analogy to the ethyl ether reaction, the reaction with tetrahydrofuran (THF) was expected to form a 1,4-diradical. The same 1,4-diradical has been examined in the vapor phase[9] and gives ethylene and cyclobutane as the major products. In the carbon atom reaction with THF, ethylene was formed in 27% yield (yield calculated on the basis of 2 moles of ethylene/mole of C_1) with a trace of cyclobutane. Also recovered from the THF reaction were 1-butene and traces of *n*-butane and *cis*-2-butene.[10] The failure to observe cyclobutane as a major product is surprising and may be an indication that a "hot" 1,4-diradical is formed in the highly exothermic deoxygenation process.

Deoxygenation is not observed with simple alcohols and water, even though it is thermodynamically favored, and other reactions dominate.[11] Table I summarizes the results to date with the calculated ΔH's required for the removal of the oxygen atom from each substrate.[12] In calculating the ΔH values, it was assumed C_1 abstracted the oxygen. While C_1 must be the major abstracting species, C_2 and C_3 might also function as deoxygenating agents.

Table I

Substrate	Intermediate	Products	ΔH(calcd) for O-abstrn step, kcal/mole
		CH_2=$CHCH_3$	135–145
			85–100
			85–100
			85–100
			85–100
			85–100

Although it seems probable that the metastable singlet states (^{1}S and ^{1}D) of carbon are the major reactants, experiments are in progress which will indicate the reactive species.

Acknowledgment. We acknowledge the financial support of the Air Force Office of Scientific Research.

(8) C. H. Bamford and R. G. W. Norrish, *J. Chem. Soc.*, 1504 (1935).

(9) S. W. Benson and G. B. Kistiakowsky, *J. Am. Chem. Soc.*, **64**, 80 (1942).

(10) The formation of C_4 olefins from 1,4-dibromobutane and alkali metal in solution has been reported. See W. S. Smith, *J. Org. Chem.*, **23**, 509 (1958).

(11) P. S. Skell and R. F. Harris, unpublished results.

(12) All required bond energies taken from R. L. Cottrell, "The Strengths of Chemical Bonds," 2nd ed, Butterworth & Co. (Publishers) Ltd., London, 1958.

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Received February 3, 1967

The Instability of Muscle Aldolase Subunits in Dilute Alkali[1]

Sir:

Previous reports from this laboratory showed that, upon exposure to mildly alkaline conditions, the molecular weights of native aldolase ($\bar{M}_w^0$ = 142,000) and succinyl aldolase subunits ($\bar{M}_w^0$ = 54,500) decreased to values of approximately 2.24 and 2.70 × 10^4, respectively.[2] This led to the conjecture that aldolase might be comprised of six fundamental subunits rather than three, as postulated by previous investigators.[3]

Further confusion regarding the quaternary structure of muscle aldolase was generated by the recent communication of Kawahara and Tanford which indicated

(1) This investigation was supported by U. S. Public Health Service Grant AM08130 from the National Institutes of Health.

(2) L. F. Hass and M. S. Lewis, *Biochemistry*, **2**, 1368 (1963); L. F. Hass, *ibid.*, **3**, 535 (1964).

(3) A. Kowalsky and P. D. Boyer, *J. Biol. Chem.*, **235**, 604 (1960); E. Stellwagen and H. K. Schachman, *Biochemistry*, **1**, 1056, (1962); W. C. Deal, W. J. Rutter, and K. E. Van Holde, *ibid.*, **2**, 246 (1963).

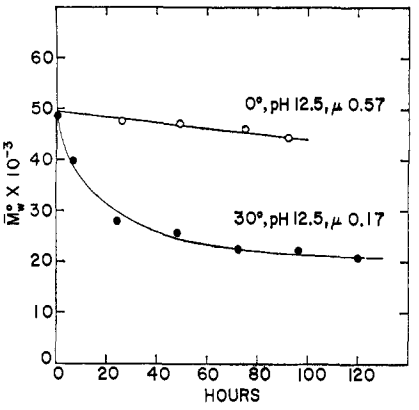

Figure 1. The kinetics of alkaline degradation of muscle aldolase subunits as measured by the decrease in $\bar{M}_w^0$. Approximately 0.6% aldolase was incubated in 0.1 *M* borate buffer under the conditions indicated in the figure. Ionic strength was adjusted by adding KCl. For the 30° study, aliquots were removed from the incubation medium at appropriate time intervals and dialyzed against KCl–borate buffer (pH 12.5, μ = 0.57) at 0° prior to centrifugation. All samples were centrifuged at 0° for a sufficient period of time to allow for complete protein equilibration under nondegrading conditions.[2]

that the native enzyme was dissociated into four subunits in the presence of 6 *M* guanidine hydrochloride.[4]

We now wish to report that, upon exposure of rabbit muscle aldolase[5] to mildly alkaline conditions (pH 12.5) at 0° and relatively high ionic strength (μ = 0.57), the enzyme spontaneously dissociates into three subunits. These subunits have apparently identical molecular weights of approximately 49,500, as indicated by the extrapolated, zero-time $\bar{M}_w^0$ value shown in Figure 1.[6] A very slight change in molecular weight with time is observed as long as the aforementioned conditions are maintained. If the temperature is elevated to 30° and the ionic strength[7] is lowered to 0.17, however, the enzyme subunits undergo an accelerated decrease in molecular weight which asymptotically approaches a value of 20,000 after 120 hr (Figure 1). Kinetic analysis of the data indicates that the rate of change of $\bar{M}_w^0$ can be described by the first-order rate law, with k_{app} = 0.041 hr^{-1} at 30°. Critical evaluation of these results suggests that aldolase subunits in dilute alkali undergo a degradation rather than a dissociation process. Similar results are obtained when succinyl aldolase subunits are exposed to alkali under equivalent conditions.

In order to ensure that the aforementioned kinetic studies reflected a simple rather than a complex process involving disulfide bridge formation, a control experiment was conducted in which the rate of alkaline degradation of fully amidomethylated aldolase was compared with that of unmodified aldolase. Prior to this study, amidomethylaldolase was prepared by first denaturing the native enzyme in 5 *M* guanidine hydrochloride–0.1 *M* tris(hydroxymethyl)aminomethane hydrochloride, pH 8.0. Mercaptoethanol was then added to give a mole ratio of thiol:aldolase of 54, and the reaction mixture was incubated at 23° for 1 hr to ensure the cleavage of any S–S bonds present. Finally, iodoacetamide was added in sufficient quantity to give a mole ratio of iodoacetamide:total SH of 2. After 2 hr at 23°, no free SH could be detected in the reaction medium with *p*-mercuribenzoate.[8] The sample was then exhaustively dialyzed against 0.1 *M* borate buffer, pH 12.5, at 0° and finally incubated at 30° for 24 hr along with a similar sample containing native aldolase. The results of sedimentation equilibrium experiments similar to those described in Figure 1 gave equivalent $\bar{M}_w^0$ values of 28,100 ± 400 and 28,600 ± 1000 for native and amidomethylaldolase, respectively, thereby suggesting that alkaline degradation proceeds by a simple hydrolytic process.

In support of this hypothesis, three new N-terminal amino acids (serine, threonine, and glycine) were found by comparison with known standards after incubating aldolase in 0.1 *M* borate buffer, pH 12.5, for 24 hr at 30°. Quantitative values for these amino acids, along with values for native N-terminal proline, are shown in Table I. These results were obtained by Edman degradation,[9] as modified by Fraenkel-Conrat.[10]

Table I. Aldolase N-Terminal Amino Acids

	N-Terminal amino acids found,[a] moles/mole of aldolase			
	Proline	Serine	Threonine	Glycine
Aldolase				
Expt 1	2.52			
Expt 2	2.58			
Alkali-treated aldolase				
Expt 1	3.48	1.00	0.37	0.32
Expt 2	2.85		0.26	0.30

[a] Corrected values based on a molecular weight of 142,000 for native aldolase.

The N-terminal analyses indicate that a limited number of apparently specific bonds are cleaved in dilute alkali. This finding is indeed surprising since the peptide linkage in proteins has been reported to be relatively alkali stable.[11] The specificity of the hydrolysis is borne out by the fact that muscle aldolase,

(4) K. Kawahara and C. Tanford, *Biochemistry*, **5**, 1578 (1966).

(5) Aldolase was five times crystallized and had a specific activity of 30–36 units/mg per ml at 23° as determined by the hydrazine method of V. Jagannathan, K. Singh, and M. Damodaran, *Biochem. J.*, **63**, 94 (1956).

(6) Apparent weight-average molecular weight values were obtained at three different protein concentrations (range, 0.15–0.6%) by employing the multiplace Spinco An-J rotor and the short column (1.6–1.7 mm) sedimentation equilibrium technique of E. G. Richards and H. K. Schachman, *J. Phys. Chem.*, **63**, 1578 (1959). Final rotor speed was adjusted so that the initial protein concentration was located near the center of the liquid column at equilibrium. For relatively high molecular weight material (>25,000) the speed was adjusted to 9945 rpm; for low molecular weight material (<25,000) the speed was increased to 15,220 rpm.

Molecular weights were calculated using a protein partial specific volume of 0.74 (J. F. Taylor and C. Lowry, *Biochim. Biophys. Acta*, 20, 109 (1956)) and were extrapolated to infinite protein dilution to obtain $\bar{M}_w^0$. No allowance was made for the possible decrease in $\bar{v}$ due to protein denaturation and temperature variation. If $\bar{v}$ decreased to 0.72, however, the extrapolated $\bar{M}_w^0$ value would be 48,000, which is still compatible with dissociation of the native enzyme into three subunits.

The homogeneity of the subunits was assessed from plots of ln *c* *vs.* x^2 (*c* = protein concentration; *x* = distance from rotational axis) which were virtually rectilinear after exposing aldolase to pH 12.5 buffer at 0° for 26 hr. With prolonged exposure to alkali, the tendency toward polydispersity increased, especially at 30°.

(7) Preliminary investigation indicates that at high ionic strength the rate of change in $\bar{M}_w^0$ in dilute alkali is significantly retarded.

(8) P. D. Boyer, *J. Am. Chem. Soc.*, **76**, 4331 (1954).

(9) P. Edman, *Acta Chem. Scand.*, **4**, 277, 283 (1950).

(10) H. Fraenkel-Conrat, *J. Am. Chem. Soc.*, **76**, 3606 (1954). Amino acid phenylthiohydantoins were chromatographically separated using the solvent systems of P. Edman and J. Sjoquist, *Acta Chem. Scand.*, 10, 1507 (1956).

(11) R. C. Warner, *J. Biol. Chem.*, **142**, 741 (1942).

which contains a large number of serine, threonine, and glycine residues,[12] does not randomly degrade under the conditions used; moreover, no evidence has been found for the liberation of N-terminal residues other than those mentioned. The possibility of the existence of alkali-labile ester bonds involving serine and threonine hydroxyl groups is ruled out by the fact that succinyl aldolase subunits yield new rather than no N-terminal residues after incubation in alkali. In fact, prior blocking of all free amino groups by succinylation provides a unique method for the detection of newly formed N-terminal amino acids resulting from moderate chemical action.

Acknowledgment. The skillful technical assistance of Mr. Joseph Morganti is gratefully acknowledged.

(12) S. F. Velick and E. Ronzoni, *J. Biol. Chem.*, **173**, 627 (1948).

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Received December 1, 1966

Electron Spin Resonance Spectra of *cis*- and *trans*-3,4,5-Trimethoxyphenylglyoxal Semidione Radical Anions

Sir:

Recently the preparation of a number of semidione radical anions containing a single aryl substituent has been reported.[1] In contrast to the case of the aliphatic compounds,[2] the esr spectra of monoaryl semidiones did not show evidence of the presence of *cis* and *trans* isomers. This fact was attributed to the complexity of the spectra which makes the detection of lines of low intensity difficult.

We were stimulated to further investigations on similar molecules, and we chose 3,4,5-trimethoxyphenylglyoxal which was expected to yield a particularly simple spectrum.

The semidione radical may supposedly be prepared in two ways: by the method used by Fraenkel and coworkers,[3] which has been suggested to produce a semidione radical anion when one electrolyzes a solution of benzaldehyde in dimethylformamide (DMF),[1,4] and by the method described by Russell, *et al.*[2]

In one series of experiments we electrolyzed a DMF solution of 3,4,5-trimethoxybenzaldehyde, containing tetraethylammonium perchlorate as supporting electrolyte, with the experimental arrangement already reported.[5] The esr spectrum was recorded as soon as the electrolysis was stopped. In another series of experiments, the 3,4,5-trimethoxy-ω-bromoacetophenone was treated with potassium *t*-butoxide in a dimethyl sulfoxide (DMSO) solution and the esr spectrum of the solution recorded immediately after mixing.

Spectra were recorded with a Varian 4501 X band spectrometer equipped with 100-kc/sec field modulation. Field calibrations were performed with a Harvey–Wells precision gaussmeter whose frequency was measured with a Hewlett–Packard frequency counter.

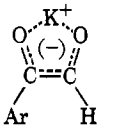
The esr signals consisted of the same number of lines with approximately the same frequency pattern in both experiments and decayed in about 1 hr to give other more complicated spectra which were not analyzed. In the DMF solution experiments, the signal was analyzed as the superposition of two six-line spectra with an intensity ratio of 1:3 and having their centers 180 mgauss apart. Each of the six-line spectra is composed of two equal 1:2:1 triplets.

In the DMSO experiments the relative intensity of the two spectra is very much dependent on the cation (K^+) concentration; if the latter is high enough only one six-line spectrum is observed and the species to which it refers is the one with the larger single proton coupling. The two species are labeled A and B in Table I, where the values of the coupling constants are summarized together with those obtained for the phenylglyoxal semidione radical anion[1,3] (C). It

Table I. Coupling Constants of Single (a_1) and of Two Equivalent (a_2) Protons (Gauss)

Species		DMF (NEt_4^+)	DMSO (K^+)
A	a_1	5.63	6.09
	a_2	1.38	1.43
B	a_1	6.46	7.00
	a_2	1.47	1.50
C	a_H^{CHO}	5.53	6.88
	a_H^{ortho}	1.36	1.50

is to be noted that species A is the one which is present at higher concentration in DMF (NEt_4^+) solution while species B is the one which is the major (or even the only) component in the DMSO (K^+) medium. Moreover, the *g* factor difference ($g_A - g_B$), which is 1×10^{-4} in the former solution, is almost three times as large ($g_A - g_B = 2.7 \times 10^{-4}$) in the latter solution. All these observations can be rationalized if we assume that species A corresponds to the *trans* isomer and species B to the *cis* isomer which is stabilized by the presence of K^+ through ion-pair formation.



If our interpretation is correct, then, to the best of our knowledge, this is the first time that different *g* values were measured for two stereoisomers. The large increase of the difference ($g^{trans} - g^{cis}$) in DMSO (K^+) may also be due to the presence of potassium cations which modify the $n-\pi^*$ excitation energies involved in the second-order mixing responsible for the deviation of the *g* factor from the free-electron value.[6] Theoretical calculations on this effect are in progress and will be discussed elsewhere.

Finally we wish to point out that our experiments confirm very nicely the interpretation of the spectra obtained from electrolyzed benzaldehyde solutions as given by Russell and co-workers.[1] We

(1) G. A. Russell, E. T. Strom, E. R. Talaty, and S. A. Weinor, *J. Am. Chem. Soc.*, **88**, 1998 (1966).
(2) G. A. Russell and R. D. Stevens, *J. Phys. Chem.*, 70, 1320 (1966), and references therein contained.
(3) P. H. Rieger and G. K. Fraenkel, *J. Chem. Phys.*, **37**, 2811 (1962).
(4) N. Steinberger and G. K. Fraenkel, *ibid.*, **40**, 723 (1964).
(5) C. Corvaja and G. Giacometti, *Ric. Sci. Rend.*, **35**, 1038 (1965).
(6) H. M. McConnell and R. E. Robertson, *J. Phys. Chem.*, **61**, 1018 (1957); see also A. J. Stone, *Mol. Phys.*, **6**, 509 (1963); **7**, 311 (1963).