Crystal Violet (CV) and Malachite Green (MG), as well as *p,p'*-methylenebis-(N,N-dimethylaniline) (MBis) all yield N,N,N',N'-tetramethyl-benzidine (TMB) and its corresponding diquinoid (TMBOx) upon oxidation at platinum and carbon electrodes in acidic, aqueous buffers. It can be shown that this reaction must take place *via* ejection of an integral unit composed of the central carbon atom attached to a phenyl group. In the case of the MBis, where the central group is merely —CH_2—, formaldehyde results. The ethylated dyes Ethyl Violet (EV) and Brilliant Green (BG) which are analogous to Crystal Violet and Malachite Green, respectively, yield the corresponding N,N,N',N'-tetraethylbenzidine. These results are especially interesting in view of the recent work of Eastman, Engelsma and Calvin, who showed CV was formed by oxidation of dimethylaniline with chloranil.[1] Here the central carbon of the CV cation must originate from a methyl carbon of dimethylaniline. Our results show the central carbon residue can be removed by the relatively mild process of electrochemical oxidation. Thus it would appear that the central carbon of triphenylmethane dyes (and related compounds) is an unusually facile portion of a rather complex molecule. These results appear to be of fundamental interest in complex organic oxidation–reduction processes.

The cyclic voltammetry and other electrochemical techniques used here were identical with those reported in the study of the anodic oxidation of N,N-dimethylaniline.[2]

In 1 *N* sulfuric acid–sodium sulfate medium, CV oxidizes at *ca.* + 0.8 v. *vs.* s.c.e. Using a 2 v./min. triangular wave sweep voltage, no evidence of any oxidation at less than + 0.8 v. is evident on the *first* anodic sweep. However, on the second and all subsequent sweeps, an almost reversible oxidation-reduction system is found at the lesser anodic potential of *ca.* + 0.55 v. The anodic and cathodic half-peak potentials of this system correspond *within 2 millivolts* with those of TMB-TMBOx in the same medium.

Chemical oxidation of triphenylmethane dyes to TMBOx is fairly well established.[3,4] The most recent work of Hanousek and Matrka on MG is probably most definitive.[5] To have further proof that the compound formed electrochemically was TMBOx, CV, MG and MBis were oxidized with lead peroxide in sulfuric acid and the corresponding oxidation products were isolated as perchlorates. Solutions of all these compounds showed cyclic voltammetry in complete agreement with the TMB-TMBOx oxidation-reduction system formed by electro-chemical means only.

Further, these oxidation products were dissolved in 50% acetone–50% aqueous buffer of pH 3.8. These solutions all showed electron paramagnetic resonance (e.p.r.) spectra identical with that of the cation radical of TMB. This spectrum recently has been interpreted in detail.[6]

Finally, the uncertainty exists that, (a) one molecule of TMB arises by oxidation of 2 molecules of dye each losing a N-substituted phenyl, followed by chemical coupling reactions, or, (b) that one dye molecule loses the central carbon phenyl unit and the remaining two N-substituted phenyl groups couple *intra* to give TMB. It can be shown that the latter is the case. The peak current due to TMB which arises from MG, CV or MBis is *ca.* 1.8 times that obtained from similar oxidation of dimethylaniline. Since it has been shown that 2 moles of dimethylaniline are oxidized to give 1 mole of TMB, these results can be interpreted to mean that 1 mole of MG, CV or MBis gives 1 mole of TMB.

Another interesting facet of the triphenylmethane dye oxidation is that it occurs in a 2-step wave. It can be shown that the two waves are due to the oxidation of the hydrated and non-hydrated forms of the dye proposed by Cigen.[7] It is only the hydrated form which gives rise to the TMB during anodic oxidation in strong acid medium. A detailed interpretation of the electrochemical results will be given soon.

Acknowledgment.—This work was supported by the Atomic Energy Commission through contract AT(11–1)–686 and this support is gratefully acknowledged.

(1) J. W. Eastman, G. Engelsma and M. Calvin, *J. Am. Chem. Soc.*, **84**, 1339 (1962).
(2) Z. Galus and R. N. Adams, *J. Am. Chem. Soc.*, **84**, 2061 (1962).
(3) J. Knop, *Z. anal. Chem.*, **85**, 253 (1931).
(4) F. Kehrmann, G. Roy M. and Ramm, *Helv. Chim. Acta*, **5**, 153 (1922).
(5) V. Hanousek and M. Matrka, *Coll. Czechoslov. Chem. Comm.*, **24**, 16 (1959).
(6) Z. Galus and R. N. Adams, *J. Chem. Phys.*, **36**, 2814 (1962).
(7) R. Cigen, *Acta Chem. Scand.*, **12**, 1456 (1958).

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ON THE MECHANISM OF THE ENZYMATIC DECARBOXYLATION OF ACETOACETATE. II[1]

Sir:

The decarboxylation of acetoacetate by the decarboxylase[2] purified from *Cl. acetobutylicum* previously had been shown to involve obligatory exchange of the carbonyl oxygen atom with the oxygen of the water used as solvent.[3] These findings suggest[3] that a Schiff base formed between the enzyme and its substrate may be an active intermediate in the decarboxylation. We have therefore tried to trap the postulated Schiff base intermediate by reduction with borohydride as has been done with the Schiff bases present in other enzymic[4–6] and similar systems.[7] The successful results of these experiments are reported below.

(1) This work was supported, in part, by N. I. H. grant number RG-6687.
(2) G. Hamilton and F. H. Westheimer, *J. Am. Chem. Soc.*, **81**, 2277 (1959).
(3) G. Hamilton and F. H. Westheimer, *J. Am. Chem. Soc.*, **81**, 6332 (1959).
(4) E. H. Fischer, A. B. Kent, E. R. Snyder and E. G. Krebs, *J. Am. Chem. Soc.*, **80**, 2906 (1958).
(5) E. Grazi, T. Cheng and B. L. Horecker, *Biochem. and Biophys. Research Comm.*, **7**, 250 (1962).
(6) B. L. Horecker, S. Pontremoli, C. Ricci and T. Cheng, *Proc. Nat. Acad. Sci.*, **47**, 1949 (1961).
(7) W. B. Dempsey and H. N. Christensen, *J. Biol. Chem.*, **237**, 1113 (1962).

The enzyme was prepared from *Cl. acetobutylicum* (NRRL–B–527) obtained from the U. S. Department of Agriculture at Peoria, Illinois, and was purified according to Hamilton.[8] (This purified but uncrystallized enzyme is similar to that obtained earlier[2,3] from A.T.C.C. 862, which is unfortunately no longer available.) When the enzyme (100 μg./ml.) in 0.1 *M* potassium phosphate–citrate buffer at pH 7 was treated with 0.025 *M* sodium borohydride plus 0.025 *M* acetoacetate at 0° for 30 minutes, enzymatic activity was irreversibly lost. Enzymatic activity was assayed after exhaustive dialysis by observing the disappearance of the enol absorption band of 0.027 *M* acetoacetate at 270 mμ in 0.1 *M* potassium phosphate buffer, pH 5.9 at 30°. The data are recorded in Table I.

TABLE I

Sample, treatment prior to assay	% activity after dialysis
Enzyme	100
Enzyme + 0.025 *M* acetoacetate	100
Enzyme + 0.025 *M* BH_4^-	90 ± 5
Enzyme + 0.025 *M* acetoacetate + 0.025 *M* BH_4^-	25 ± 5
Same second treatment with acetoacetate plus BH_4^-	12
Same third treatment with acetoacetate plus BH_4^-	7
Enzyme + 0.025 *M* acetoacetate + 2.5×10^{-4} *M* HCN	84
Enzyme + 0.025 *M* acetoacetate + 0.025 *M* BH_4^- + 2.5×10^{-4} *M* HCN	69
Enzyme + 0.025 *M* acetoacetate + 0.025 *M* BH_4^- + 5×10^{-4} *M* acetopyruvate	68

Both HCN[9] and acetopyruvate[9,10] are strong, reversible inhibitors of the enzyme. The details of the inhibition by acetopyruvate remain unknown,[10] but HCN may be presumed to add to the C=N bond of a Schiff base intermediate, in the manner of the addition of HCN in the Strecker synthesis. This interpretation is supported by the observation that HCN inhibition develops with perceptible slowness (*e.g.*, 1 min. at pH 6) and that the time lag cannot be shortened by preincubation of HCN with either enzyme or acetoacetate; the inhibition reaction occurs only in the simultaneous presence of enzyme, substrate and HCN. Both HCN and acetopyruvate partially protect the enzyme against reduction and irreversible inhibition by borohydride plus acetoacetate.

The reduction has been performed with 3-C^{14}-acetoacetate (New England Nuclear Corporation). Enzyme (520 μg.) dissolved in 6 ml. of 0.10 *M* potassium phosphate–citrate buffer, pH 7, was treated with 0.00445 *M* 3-C^{14}-acetoacetate (1.68×10^6 disintegration/min. μmole) and 0.025 *M* BH_4^-. After 30 minutes at 0° the solutions were exhaustively dialyzed at 0° against 0.05 *M* potassium phosphate, pH 5.9, lyophilized and counted with a Packard Model 1314X Tri-Carb Scintillation Counter. The results are shown in Table II.

(8) G. Hamilton, Thesis, Harvard University, 1959.
(9) R. Davies, *Biochem. J.*, **36**, 582 (1942); **37**, 230 (1943).
(10) R. Coleman, Thesis, Radcliffe College, 1962.

TABLE II

Sample	D.P.M./200 μg. enzyme
Enzyme + labelled acetoacetate	345
Enzyme + labelled acetoacetate + BH_4^-	5450

On the assumption that the borohydride reduction was 75% complete, the equivalent weight of the enzyme is about 50,000.

A sample of the C^{14} labelled enzyme was hydrolyzed for 24 hours at 110° in 6 *N* hydrochloric acid in an evacuated, sealed tube and 200 μg. of the hydrolysate subjected to two dimensional paper chromatography.[11] A radioautograph showed a single spot, well removed from both the origin and the solvent fronts.

These results indicate that borohydride reduces a compound formed between the enzyme and acetoacetate, and that this reduction forms a bond stable to acid hydrolysis. The available evidence thus supports the validity of the hypothesis[3] that the enzymatic decarboxylation of acetoacetate, like the amine catalyzed non-enzymatic decarboxylation,[12] proceeds by way of a Schiff base (or Schiff base salt) intermediate. The identification of the group on the enzyme which is reponsible for the formation of the intermediate is actively under investigation.

(11) R. R. Redfield, *Biochem. et Biophys. Acta*, **10**, 344 (1953).
(12) K. J. Pederson, *J. Phys. Chem.*, **38**, 559 (1934).

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THE HIGH TEMPERATURE CONTAINMENT OF SUBSTANCES ABOVE THE MELTING POINT OF THE CONTAINER[1]

Sir:

Present high temperature research is close to the practical limit of solid refractory containers set by their melting points. The highest melting metal is tungsten, melting point, 3643°K., the highest oxide is ThO_2, melting point, 3300°K., and the highest melting carbide is TaC, with a melting point 4200°K. A special case is graphite, with a sublimation point of 3800°K.

In practice at our laboratories, these maxima usually cannot be reached due to (1) chemical reaction between container and substance (2) eutectic mixtures, which lower the melting point and (3) thermal shock.

While the limit of thermal stability of chemical compounds is about 5000°K., it was demonstrated[2,3,4] recently that the *liquid range* (*i.e.*, the range from their melting point to their critical point) of some metals extends to 20,000°K. As elementary substances, they cannot decompose, in contrast to chemical compounds. Thus in the case of many liquid metals, experiments could be carried out far

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(2) A. V. Grosse, R. I. T. U. Report of Sept. 5, 1960; Paper No. 2159, A.R.S.'s Space Flight Report to the Nation, N. Y. City, Oct. 9–15, 1961.
(3) A. V. Grosse, *J. Inorg. Nucl. Chem.*, **22**, 23 (1961).
(4) A. V. Grosse, *Inorg. Chem.*, **1**, 436 (1962).