08736 from the National Heart Institute, and NB-00024 from the National Institute of Neurological Diseases and Blindness, U.S. Public Health Service.

Dr. M. P. Drake is supported by U.S. Public Health Service fellowship 5F3AM-11,175 from the National Institute of Arthritis and Metabolic Diseases.

* Publication no. 270 from the Graduate Department of Biochemistry, Brandeis University.

† Department of Biochemistry, Brandeis University.

‡ Rogosin Laboratories, Department of Medicine, Cornell University Medical Center.

[1] Orekhovitch, V. N., A. A. Tustanowski, K. D. Orekhovitch, and N. E. Plotnikova, *Biokhimiya*, **13**, 55 (1948).

[2] Veis, A., J. Anesey, and J. Cohen, *J. Am. Leather Chemists' Assoc.*, **55**, 548 (1960).

[3] Gross, J., J. H. Highberger, and F. O. Schmitt, these PROCEEDINGS, **40**, 679 (1954).

[4] Boedtker, H., and P. Doty, *J. Am. Chem. Soc.*, **78**, 4267 (1956).

[5] Hall, C. B., and P. Doty, *J. Am. Chem. Soc.*, **80**, 1269 (1958).

[6] Schmitt, F. O., J. Gross, and J. H. Highberger, *Exptl. Cell Res., Suppl.*, **3**, 329 (1955).

[7] Ramachandran, G. N., in *Aspects of Protein Structure* (New York: Academic Press, 1963), p. 39.

[8] Piez, K. A., E. Weiss, and M. S. Lewis, *J. Biol. Chem.*, **235**, 1987 (1960).

[9] Grassmann, W., K. Hannig, and J. Engel, *Z. Physiol. Chem.*, **324**, 284 (1961).

[10] Altgelt, K., A. J. Hodge, and F. O. Schmitt, these PROCEEDINGS, **47**, 1914 (1961).

[11] Hodge, A. J., and F. O. Schmitt, these PROCEEDINGS, **44**, 418 (1958).

[12] Hodge, A. J., and J. A. Petruska, in *Aspects of Protein Structure* (New York: Academic Press, 1963), p. 289.

[13] Rubin, A. L., D. Pfahl, P. T. Speakman, P. F. Davison, and F. O. Schmitt, *Science*, **139**, 37 (1963).

[14] Hodge, A. J., J. H. Highberger, G. G. J. Deffner, and F. O. Schmitt, these PROCEEDINGS, **46**, 197 (1960).

[15] Rubin, A. L., M. P. Drake, P. F. Davison, D. Pfahl, P. T. Speakman, and F. O. Schmitt, in preparation.

[16] Drake, M. P., P. F. Davison, S. Bump, A. L. Rubin, and F. O. Schmitt, in preparation.

[17] Wasserman, E., and L. Levine, *J. Immunol.*, **87**, 290 (1961).

[18] Gallop, P. M., S. Seifter, and E. Meilman, *Nature*, **183**, 1659 (1959).

[19] Hörmann, H., *Leder*, **11**, 173 (1960).

[20] Orekhovitch, V. N., in *Procollagens, Their Chemical Structures, Properties, and Biological Role* (Moscow, 1952).

## *INHIBITION OF CARBOXYDISMUTASE BY IODOACETAMIDE**

BY BRIAN R. RABIN† AND PATRICK W. TROWN

BIO-ORGANIC CHEMISTRY GROUP, LAWRENCE RADIATION LABORATORY, UNIVERSITY OF CALIFORNIA, BERKELEY

*Communicated by Melvin Calvin, January 9, 1964*

The initial step in the fixation of carbon dioxide by autotrophic organisms is the reaction of carbon dioxide with RuDP to give 3-phosphoglyceric acid.[1, 2] This reaction is catalyzed by carboxydismutase, which is inhibited by parachloromercuribenzoate,[3] suggesting that SH groups are required for its activity. In order to define the function of these groups more precisely, we have investigated the irreversible inhibition of the enzyme by iodoacetamide.

*Experimental.—Enzyme:* Crude carboxydismutase was prepared from isolated spinach chloroplasts as previously described.[4] It was purified by fractional ammonium sulfate precipitation, followed by repeated gel filtration on Sephadex G-200.[5] The enzyme was stored as a precipitate

in 50% saturated ammonium sulfate solution at 0°. Prior to each experiment, a small quantity of the suspension was dialyzed overnight against 0.002 *M* tris, pH 8.0. The water, dialysis tubing, and buffer were freed from heavy metal ions by standard procedures.[6, 7]

*Buffers:* Tris(hydroxymethyl)aminomethane buffers of the desired pH and ionic strength were prepared with Trizma (Sigma Chemical Co., St. Louis), redistilled hydrochloric acid, and "Specpure" sodium chloride (Johnson, Matthey and Co., Ltd., Hatton Garden, London). They were prepared as stock solutions (I, 1.0; total tris, 1 *M*), shaken with excess 8-hydroxy quinoline, and extracted three times with metal-free carbon tetrachloride. They gave a negative reaction in the dithizone test.

Di(hydroxyethyl)amine buffers were prepared from the redistilled base, redistilled hydrochloric acid, and "Specpure" sodium chloride. Because of their high pH, they were not extracted with 8-hydroxy quinoline.

*RuDP:* RuDP was purchased from Calbiochem, Los Angeles, as the Ba salt. The preparation was stated to contain 26% RuDP and was used without further purification, except as follows: 10.5 mg were suspended in water (200 $\mu$l) at 0° and HCl (0.1 *M*, 200 $\mu$l) and $Na_2SO_4$ (0.1 *M*, 400 $\mu$l) added. The mixture was stirred, then allowed to stand at 0°. After 10 min, NaOH (0.1 *M*, 200 $\mu$l) was added, and the mixture left at 0° for 15 min. It was then centrifuged, and the supernatant solution containing the Na salt of RuDP (approximately 0.006 *M*) was decanted. The final pH of this solution was 7.0.

*Iodoacetamide:* The recrystallized material was a gift from Dr. R. Hiller.

*Enzyme assay:* This was based on the measurement of acid-stable radioactivity produced in the reaction between RuDP and $NaH^{14}CO_3$.[8] A typical assay contained in 200 $\mu$l: magnesium chloride, 2 $\mu$mole; tris buffer pH 8.0, 15 $\mu$mole; $NaH^{14}CO_3$, 1.5 $\mu$mole (38 $\mu$curie); RuDP, 0.06 $\mu$mole. The reaction was allowed to run for 10 min at 25° and stopped by the addition of acetic acid (50 $\mu$l, 6 *N*). Aliquots (25 $\mu$l) were plated on aluminum disks which were counted in an automatic apparatus. Results are reported as cpm, which are proportional to the concentration of active enzyme.[8]

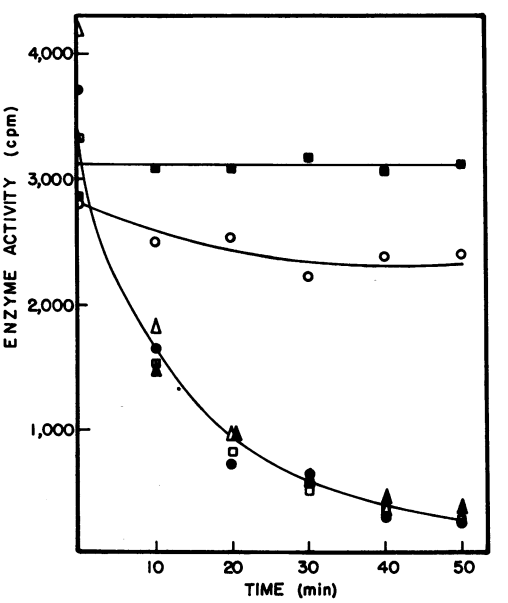



FIG. 1.—Progress curves for the inhibition of carboxydismutase by iodoacetamide at 25° in tris buffer pH 8.0 (0.07 *M*). All reaction mixtures contained: iodoacetamide, 0.5 mM; enzyme, 1 mg/ml. ▲—▲, control, no additions; □—□, plus $MgCl_2$ (10 mM); ●—●, plus $NaHCO_3$ (10 mM); △—△, plus $MgCl_2$ (10 mM) and $NaHCO_3$ (10 mM); ■—■, plus RuDP (1.5 mM) and $MgCl_2$ (10 mM); ○—○, plus RuDP (1.5 mM). Incubation mixtures diluted tenfold with a solution of cysteine (10 mM) before assay.

*Reaction with iodoacetamide:* A solution of iodoacetamide in water was added at zero time to a mixture of enzyme, buffer, and other components of the reaction mixture in a stoppered tube. Aliquots (50 $\mu$l) were removed and added to a solution of cysteine (10 mM) in tris buffer (50 $\mu$l, pH, 8.0; 0.1 *M* with respect to total tris) at 0°. The other components of the assay were then added, and the tubes transferred to a bath at 25°. Control experiments showed that this procedure stopped the alkylation of the enzyme instantaneously without interfering with the assay.

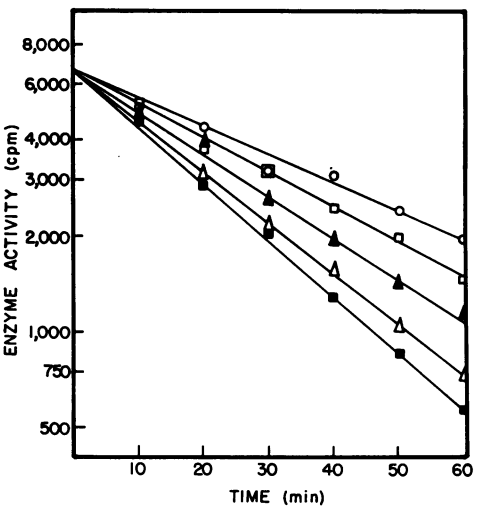



FIG. 2.—Pseudo-first-order decay of carboxydismutase activity at ionic strength 0.1 and 25° in di(hydroxyethyl)amine buffer in the presence of iodoacetamide (0.1 mM). O—O, pH 8.74; □—□, pH 9.04; ▲—▲, pH 9.34; △—△, pH 9.73; ■—■ pH 10.00. Enzyme concentration, 0.25 mg/ml.

*Results.*—The progress curves of the inhibition of the enzyme by iodoacetamide in the presence and absence of various combinations of substrates and cofactor are shown in Figure 1. RuDP at the concentration indicated protected the enzyme completely against alkylation. The decay of activity is pseudo-first-order in enzyme as illustrated by the log plots in Figure 2. Pseudo-first-order rate constants were obtained from thes lopes of similar plots. At fixed pH, temperature, and ionic strength these constants are a linear function of the concentration of iodoacetamide as shown in Figure 3. The second-order rate constant was obtained by dividing the pseudo-first-order constant by the molar concentration of iodoacetamide. The variation of this constant with pH is shown in Figure 4. The data can be fitted to the expression $k_2 = \bar{k}_2/(1 + [H^+]/Ka)$ with $\bar{k}_2 = 833$ l mole$^{-1}$ min$^{-1}$ and $pK_a = 8.82$.

*Discussion.*—The reaction of the enzyme with iodoacetamide is first-order in enzyme even when more than 90 per cent of the catalytic activity has been lost. This suggests that the groups on the protein which become alkylated with loss of activity constitute a homogeneous population and that a single chemical event is being investigated. The group alkylated has a pK value in the region expected for an SH and close to that determined for the SH group at the active site of the plant proteolytic enzyme ficin.[9] Only the conjugate base is alkylated, and the nucleophilic reactivity is approximately twice that of the sulfur at the catalytic site of creatine phosphokinase.[10, 11] It is reasonable to assume that an SH group is involved in the alkylation reaction, but a final proof must, of course, await the isolation of carboxymethyl cysteine.

The sulfhydryl group at the active site of carboxydismutase behaves as an acid with a pK of 8.82, in sharp contrast to similar groups at the active sites of creatine phosphokinase[12] and yeast alcohol dehydrogenase[13] which show no acid dissociation properties. The reasons for these differences are not understood.

The sulfhydryl is clearly not involved in binding bicarbonate or magnesium ions since these have no effects on its reactivity. The results strongly suggest that it is

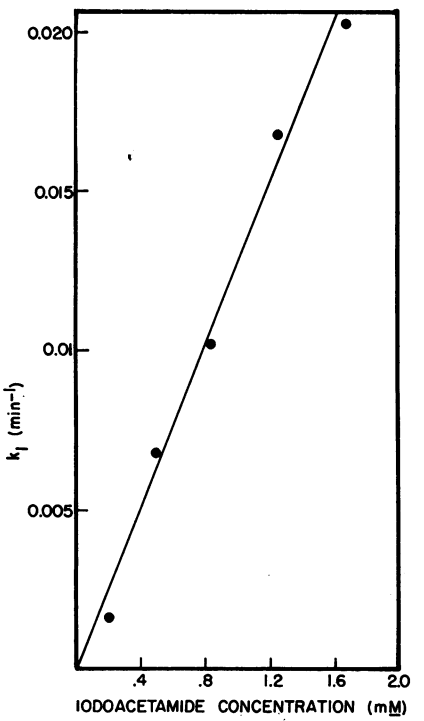



FIG. 3.—Variation of pseudo-first-order rate constant with iodoacetamide concentration at pH 7.18, ionic strength 0.1 and 20°.

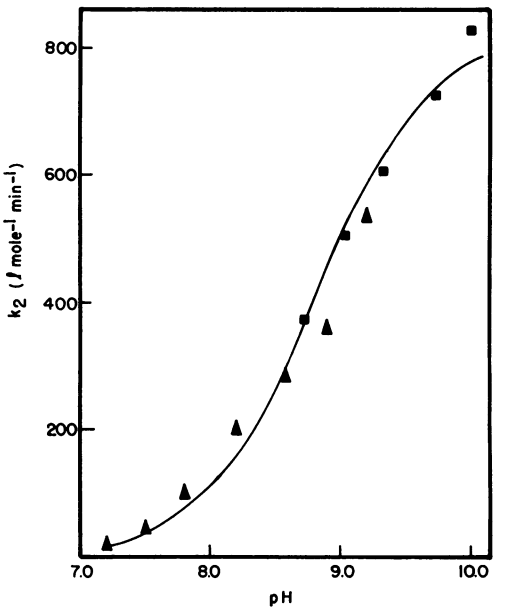
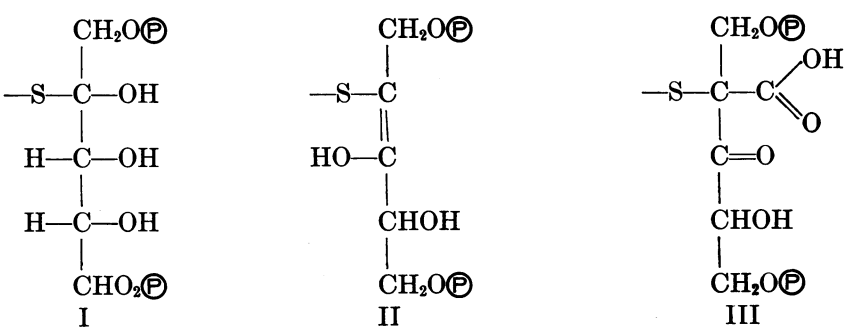



FIG. 4.—Variation of second-order rate constant for the inhibition of carboxydismutase with iodoacetamide with pH at 25° and ionic strength 0.1. ▲, tris buffers; ■, di(hydroxyethyl)amine buffers. The points are experimental and the line theoretical assuming a pK of 8.82 and second-order rate constant for the base species ($\bar{k}_2$) of 833 l mole$^{-1}$.

concerned in interaction with RuDP, perhaps to form a thio-hemiacetal structure (I). This should readily eliminate water:




to give an enol (II), which could be carboxylated by enzyme-bound bicarbonate to give a β-keto acid (III) in an analogous manner to that previously suggested.[14, 15] The β-keto acid is attached to the enzyme as a thio-ether and thus cannot be detected as an intermediate by the usual procedures.

It is of interest to consider these findings in relation to the use of inhibitors in investigations of photosynthesis *in vivo*. It is clear that the pool sizes of the substrates can have important, and perhaps dominating, effects on the sensitivity

of enzymes toward inhibitors. In the present instance, it would be possible to envisage conditions *in vivo* in which carboxydismutase would be insensitive toward iodoacetamide and other alkylating agents.

The authors are indebted to Professor M. Calvin for stimulating advice and for the interest he has shown throughout this investigation. One of the authors (BRR) is indebted to the Wellcome Trust for a travel grant.

The following abbreviations are used: Tris, Tris(hydroxymethyl)aminomethane; RuDP, ribulose-1,5-diphosphate.

* The work described in this paper was sponsored by the U.S. Atomic Energy Commission.

† On leave of absence from the Department of Biochemistry, University College, London.

[1] Bassham, J. A., A. A. Benson, L. D. Kay, A. Z. Harris, A. T. Wilson, and M. Calvin, *J. Am. Chem. Soc.*, **76**, 1760 (1954).

[2] Quayle, J. R., R. C. Fuller, A. A. Benson, and M. Calvin, *J. Am. Chem. Soc.*, **76**, 3610 (1954).

[3] Mayaudon, J., A. A. Benson, and M. Calvin, *Biochem. Biophys. Acta*, **23**, 342 (1957).

[4] Pon, N. G., B. R. Rabin, and M. Calvin, *Biochem. Z.*, **358**, 7 (1963).

[5] Trown, P. W., manuscript in preparation.

[6] Hughes, T. A., and J. M. Klotx, in *Methods of Biochemical Analysis*, ed. D. Glick (1956), vol. 3, p. 265.

[7] Thiers, R. C., in *Methods of Biochemical Analysis*, ed. D. Glick (1957), vol. 5, p. 273.

[8] Pon, N. G., Ph.D. thesis, University of California, UCRL 9373 (1960).

[9] Hollaway, M., private communication.

[10] Watts, D. C., and B. R. Rabin, *Biochem. J.*, **85**, 507 (1962).

[11] Watts, D. C., B. R. Rabin, and E. M. Crook, *Biochem. J.*, **82**, 412 (1962).

[12] Rabin, B. R., and E. P. Whitehead, *Nature (London)*, **196**, 658 (1962).

[13] Rabin, B. R., and D. C. Watts, *Nature (London)*, **188**, 1163 (1960).

[14] Calvin, M., *Fed. Proc.*, **13**, 697 (1954).

[15] Calvin, M., and N. G. Pon, *J. Cell. Comp. Physiol.*, Suppl. 1, **54**, 51 (1959).

---

## *DETERMINATION OF DIFFUSION COEFFICIENTS AND MOLECULAR WEIGHTS OF RIBONUCLEIC ACIDS AND VIRUSES**

BY WIM J. MÖLLER

DEPARTMENT OF BIOPHYSICS, SCHOOL OF MEDICINE, JOHNS HOPKINS UNIVERSITY

*Communicated by J. L. Oncley, January 2, 1964*

It is widely believed that only a small fraction of the total number of large ribonucleic acid (RNA) molecules present in the cell can serve as direct information carriers in protein synthesis.[1] The formation of specific coat protein under the direction of RNA isolated from f2-bacteriophage[2] in a cell-free system from *E. coli* places viral RNA in this category. No clear function has been found for the bulk of the high molecular weight RNA which is present in the ribosomes, the site of protein synthesis. Better physical characterization of the different types of RNA molecules in the cell might help to clarify this situation and to establish what, if any, direct relationships exist between these different large RNA molecules. For this reason we were interested in finding whether existing techniques might be modified so as to give for small amounts of material, reliable molecular weights of RNA, both free and attached to protein. Light scattering, sedimentation-viscosity, sedimentation-diffusion, density-gradient centrifugation, electron-micros-