would grow to a significant steady state value. This would be evident by an initial lag in the production of allene. The earliest possible measurements, made when less than 1% of the suboxide had been decomposed, showed no significant increase in the ratio CO/allene.

Preliminary results indicate that a carbon atom can also be inserted into carbon–hydrogen bonds. The photolysis of carbon suboxide in the presence of methane yields ethylene, while in the presence of cyclopropane both ethylene and acetylene are formed. Further experiments are in progress.

Carbon atoms produced by nuclear transformations react with hydrocarbons to give products different from those observed here.[7] This discrepancy could be due to the high energies of the nucleogenic carbon atoms, or to the fact that the CCO radical, and not a carbon atom, is the reactive species in the photolysis of carbon suboxide.

I wish to thank Professor F. E. Blacet for the use of his equipment and for several stimulating discussions.

(7) C. MacKay and R. Wolfgang, Abst. of Papers, 138th Meeting, Am. Chem. Soc., 1960, p. 83-P; C. MacKay and R. Wolfgang, *J. Am. Chem. Soc.*, **83**, 2399 (1961).

DEPARTMENT OF CHEMISTRY
UNIVERSITY OF CALIFORNIA AT LOS ANGELES
LOS ANGELES 24, CALIFORNIA KYLE BAYES

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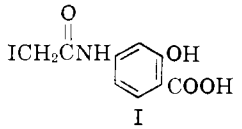
POTENTIAL ANTICANCER AGENTS.[1] LXVI. NON-CLASSICAL ANTIMETABOLITES. III.[2] 4-(IODOACETAMIDO)-SALICYLIC ACID, AN *EXO*-ALKYLATING IRREVERSIBLE INHIBITOR OF GLUTAMIC DEHYDROGENASE

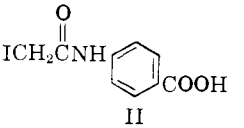
Sir:

Strong evidence for the proposition[2-4] that inhibitors can be constructed which fit the active site of an enzyme reversibly, then become irreversibly bound by alkylation of the enzyme adjacent to the active site (*exo*-alkylation) has now been observed experimentally.

Since salicylate reversibly inhibits GDH[5] and LDH with I_{50} values[6] of 19 and 20, respectively, 4-(iodoacetamido)-salicylic acid (I)[7] was investigated as a possible irreversible inhibitor of these two enzymes.



I



II

When a solution of GDH·DPNH in tris buffer at *p*H 7.4 was incubated at 37° with 2 mM. concentration of I, the amount of inhibition—compared to a control solution without inhibitor run simultaneously—increased with time[8] (Fig. 1).

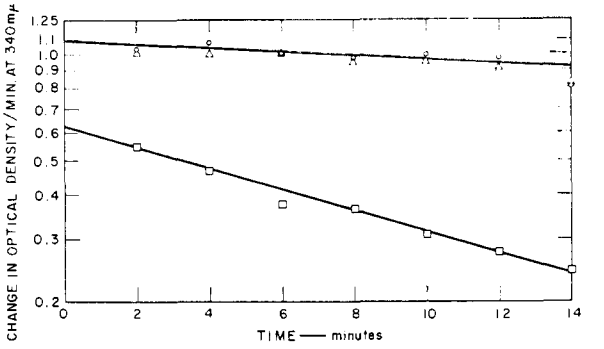
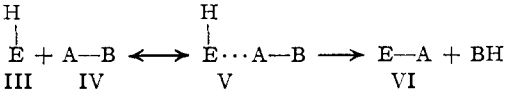


Fig. 1.—Rate of inactivation of GDH at 37° in 0.05 *M* tris buffer (*p*H 7.4) containing 0.23 mM. DPHN: O, no other addition; Δ, 4.48 mM. iodoacetamide; □, 2.00 mM. 4-(iodoacetamido)-salicylic acid.

In order to take advantage of the specificity of the enzyme site,[2,3,9] there is inherent in the design of *exo*-alkylating irreversible inhibitors the requirement that a reversible complex (V) between inhibitor (IV) and enzyme (III) must form prior to the internal formation of a covalent bond as in VI. It can be argued that I irreversibily alkylated



GDH to give VI without prior formation of a reversible complex (V), in the same manner that iodoacetamide alkylates enzymes[10] (tail alkylation); if such were the case, then the N-substituent on the iodoacetamide should not affect the rate of inactivation, providing the activity of the halogen remains the same and there is no added steric hindrance. In contrast, if a reversible complex (V) must be formed first, then the rate of inactivation is dependent upon the amount of reversible complex (V).

Iodoacetamide showed a negligible reversible inhibition of GDH, having an I_{50} of 230 with respect to α-ketoglutarate. When 4.48 mM.[11] iodoacetamide was incubated with GDH·DPNH at 37°, *there was no detectable irreversible inhibition;* two controls of GDH·DPNH, one with and one without 2 mM. 4-(iodoacetamido)-salicylic acid (I), were run simultaneously, all three solutions being made from a master solution of the enzyme (Fig.

(1) This work was carried out under the auspices of the Cancer Chemotherapy National Service Center, Contract No. SA-43-ph-1892.

(2) For paper II see B. R. Baker, W. W. Lee, W. S. Skinner, A. P. Martinez and E. Tong, *J. Med. Pharm. Chem.*, **2**, 633 (1960).

(3) B. R. Baker, Cancer Chemotherapy Reports, No. 4, 1 (1959), paper I on Non-classical Antimetabolites.

(4) H. F. Gram, C. W. Mosher and B. R. Baker, *J. Am. Chem. Soc.*, **81**, 3103 (1959).

(5) Crystalline lactic dehydrogenase (LDH) from rabbit muscle, crystalline L-glutamic dehydrogenase (GDH) from mammalian liver, and reduced diphosphopyridine nucleotide (DPNH) were purchased.

(6) See reference 2 for definition.

(7) C. van der Stelt, A. J. Z. Vourspuij and W. Th. Nauta, *Arzneimittel-Forsch.*, **4**, 544 (1954).

(8) The amount of remaining enzyme was determined by adding 1 mM. α-ketoglutarate and 75 mM. $(NH_4)_2SO_4$ to an aliquot and observing of the rate of disappearance of DPNH at 340 mμ.

(9) H. Fraenkel-Conrat in P. D. Boyer, H. Lardy and K. Myrbäck, "The Enzymes," Academic Press, Inc., New York, N. Y., 1959, Vol. I, pp. 611–613.

(10) M. Dixon and E. C. Webb, "Enzymes," Academic Press, Inc., New York, N. Y., 1958, pp. 376–378.

(11) This concentration of iodoacetamide was employed since the rate of reaction[12] of I with thiosulfate was 2.24 times as rapid as that of iodoacetamide.

(12) Measured by suitable modification of the thiosulfate method of V. K. LaMer and M. E. Kammer, *J. Am. Chem. Soc.*, **53**, 2832 (1931).

1).[13] Under similar incubation conditions, 4-acetamidosalicylic acid showed only an unchanging amount of reversible inhibition with time,[13] thus indicating that the alkylating action of the iodoacetyl group of I is necessary for the irreversible inhibition by I and that the effect of I is not due to chelation or some other action by the salicylate structure.

Further evidence that binding of I to the active site of GDH is a necessary prerequisite for irreversible inhibition was obtained.

(1) The reversible GDH inhibitor, isophthalate,[14] at 16 mM. concentration, reduced the rate of inactivation by I to about one-half.[15] Protection against irreversible inhibition by a competitive inhibitor is considered to be strong evidence that the active site is involved in the irreversible inhibition.[15,17,18]

(2) The rate of inactivation should be dependent upon the dissociation constant for III + IV $\leftrightarrow$ V, that is, the K_i. For this purpose I was compared with 4-(iodoacetamido)-benzoic acid (II).[19] First, the reactivities of the halogen in I and II were shown to be identical.[12] Second, I and II showed reversible inhibition of GDH with average $K_i = 6.9 \times 10^{-4}$ and 5.4×10^{-3}, respectively,[20] an eight-fold difference. When I or II was incubated with GDH·DPNH at 37°, I inactivated the enzyme at eight times the rate of II, in agreement with the ratio of the two K_i values.[21]

(3) It can be calculated from the K_i of I that doubling the concentration of I from 1 mM. to 2 mM. should give only a 1.23-fold increase in the inactivation rate, rather than a two-fold increase if tail-alkylation occurred. Simultaneous incubation experiments did indeed show a rate saturation effect, the observed ratio of the rates being 1.23.[13]

That I was not a general enzyme poison was shown by the observation that hexokinase·ATP was inhibited neither reversibly nor irreversibly under the conditions used for GDH; however, LDH and glucose-6-phosphate dehydrogenase were irreversibly inhibited and studies of the type described for GDH and I are progressing.

This new type of non-classical antimetabolite that most probably operates by *exo*-alkylation should have broad utility for drug design. In addition, properly designed *exo*-alkylating irreversible inhibitors could fill the demand for relatively specific agents needed to label the active sites of enzymes for enzyme mechanism studies.[22,23]

(13) All incubation experiments were duplicated at least twice.

(14) W. S. Caughey, J. D. Smiley and L. Hellerman, *J. Biol. Chem.*, **224**, 591 (1957).

(15) Isophthalate has been shown[17] to be a competitive inhibitor of L-glutamate and a non-competitive inhibitor of α-ketoglutarate. Isophthalate most probably combines with the active site of GDH in both directions, but gives non competitive kinetics in the one direction due to a two-step rate phenomenon related to that recently described for acetylcholinesterase.[16]

(16) R. M. Krupka and K. J. Laidler, *J. Am. Chem. Soc.*, **83**, 1445, 1448, 1454 (1961).

(17) J. A. Thoma and D. E. Koshland, Jr., *J. Mol. Biol.*, **2**, 169 (1960).

(18) That this protection was not due to interaction of isophthalate and I was shown in an independent thiosulfate-type experiment.[12]

(19) S. Sakai, G. Saito, and A. Sato, *Chem. Abstr.*, **51**, 16940 (1957).

(20) The K_i values were calculated from K_p and K_m, obtained from a Lineweaver–Burk plot according to ref. 10, pp. 22–24.

(21) That these results were not due to traces of heavy metal ions was shown by a highly sensitive spectrographic analysis of the I, II and 4-acetamidosalicylic acid used in these experiments.

(22) Reference 10, pp. 486–502.

(23) Ref. 9, p. 618.

(24) School of Pharmacy, University of Buffalo, Buffalo, N. Y.

B. R. BAKER[24]
LIFE SCIENCES DIVISION — WILLIAM W. LEE
STANFORD RESEARCH INSTITUTE — ETHEL TONG
MENLO PARK, CALIFORNIA — LEONARD O. ROSS

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ELECTRON SPIN DENSITY DISTRIBUTIONS IN CONJUGATED SYSTEMS BY N.M.R.

Sir:

It was found[1] that π-electron spin densities on the conjugated ligands of some paramagnetic nickel(II) chelates of aminotroponeimines could be determined from n.m.r. studies. It now appears that substituted nickel aminotroponeimineates can be employed for a general study of conjugative effects and spin density distributions in π-systems. Some preliminary results of this study are reported below. In particular, it is shown that spin densities are transmitted through N, O and S atoms connecting conjugated systems.

A positive spin density is placed on the p π orbital of nitrogen of nickel(II) aminotroponeimineates as a result of the nickel–nitrogen bonding. This spin density is distributed throughout the π-system of the ligand. Spin densities on sp^2 carbon atoms to which hydrogen atoms are bonded are manifested in the proton magnetic resonance spectrum by large high field (positive carbon spin densities) and low field (negative carbon spin densities) shifts.[2] These shifts, $\Delta H/H$,[3] are produced by isotropic hyperfine contact interactions[4] and are related to the nuclear hyperfine coupling constants, a_i, by

$$\frac{\Delta H_i}{H} = -a_i \frac{\gamma_e}{\gamma_H} \frac{g\beta S(S+1)}{6kT} \qquad (1)$$

Each a_i is related to its carbon atom spin density ρ_{ci}, by[5]

$$a_i \cong Q\rho_{ci} \qquad (2)$$

For aromatic C–H fragments, $Q \cong -22.5$ gauss.[6]

Analyses of the n.m.r. spectra of the nickel aminotroponeimineates[7] (obtained on 0.1–0.2 molar solutions of chelate in $CDCl_3$) with respect to assignment of resonances to unique ligand hydrogen atoms were facilitated by relative resonance intensities, nuclear spin-spin structure, and expected signs of spin densities based on simple valence bond considerations. A paramagnetic $\rightleftarrows$ diamagnetic equilibrium exists for these chelates in solution.[1] The spin density distributions shown in Fig. 1 refer to the paramagnetic state ($\mu_{eff} = 3.2$ BM.)

(1) W. D. Phillips and R. E. Benson, *J. Chem. Phys.*, **33**, 607 (1960).

(2) H. M. McConnell and D. B. Chesnut, *ibid.*, **28**, 107 (1958).

(3) Where $\Delta H = H_{chelate} - H_{ligand}$. Both ligand and chelate were internally referred to $(CH_3)_4Si$ to eliminate bulk susceptibility effects.

(4) E. Fermi, *Z. Physik*, **60**, 320 (1930).

(5) H. M. McConnell, *J. Chem. Phys.*, **24**, 632 (1956).

(6) S. I. Weissman, T. R. Tuttle, Jr., and E. de Boer, *J. Phys. Chem.*, **61**, 28 (1957).

(7) Prepared from the appropriate ligand and nickel chloride; see W. R. Brasen, H. E. Holmquist and R. E. Benson, *J. Am. Chem. Soc.*, **83**, 3125 (1961).