COMMUNICATIONS TO THE EDITOR

INCORPORATION OF THYMIDINE TRIPHOSPHATE INTO DEOXYRIBONUCLEIC ACID BY A PURIFIED MAMMALIAN ENZYME

Sir:

Previous work from this laboratory demonstrated the incorporation of thymidine into DNA[1] catalyzed by the high-speed supernatant fraction from regenerating rat liver homogenates.[2] The incorporation of thymidine was stimulated by a mixture of DAMP, DGMP, and DCMP. Enzymes catalyzing formation of higher phosphates of deoxynucleotides have been observed by several investigators.[3] To elucidate the deoxynucleotide effect on thymidine incorporation, supernatant fraction was examined for ability to phosphorylate DAMP, DGMP and DCMP. When fortified with 5 mM. adenosine triphosphate, 5mM. Mg^{++}, and 6 mM. 3-phosphoglycerate regenerating rat liver supernatant fraction phosphorylated 5 mM. DAMP, DGMP, or DCMP at rates of approximately 1 μmole/hr./mg. protein, forming DATP, DGTP, or DCTP (respectively) as principal product. H^3-Thymidine also was phosphorylated to H^3-TTP by regenerating liver supernatant fraction, but at a much lower rate (approximately 0.03 μmole/hr./mg. protein) and this rate was lower by a factor of at least 10 in normal supernatant fraction.[4]

Deoxynucleoside triphosphates, synthesized by crude supernatant fraction as outlined above, isolated by ion-exchange,[5] and purified where necessary by chromatography on cellulose,[3c] were then incubated with a polymerizing enzyme purified about ten fold from regenerating rat liver.[6] The experiment presented in Table I demonstrates a requirement for Mg^{++}, DNA,[7] and the presence of all four deoxynucleoside triphosphates for maximal activity of purified enzyme in incorporating H^3-TTP into DNA.

Omission of a single deoxynucleoside triphosphate reduced incorporation of H^3-TTP by 66–70%. An earlier experiment with slightly less active enzyme and a different H^3-TTP preparation gave the same general result with a less definite requirement for Mg^{++}. These findings are offered as explanation for the deoxynucleotide stimulation reported in crude preparations.[2] Requirement for DNA, Mg^{++}, and presence of all four deoxynucleoside triphosphates, together with the fact that this enzyme is inhibited by pyrophosphate,[8] suggest that the reaction mechanism described for the *E. coli* enzyme[9] will also hold for rat liver enzyme.

TABLE I

REQUIREMENTS FOR H^3-THYMIDINE TRIPHOSPHATE INCORPORATION INTO DNA

Complete system contained in 0.25 ml.: DNA, 250 μg; enzyme, 380 μg. protein; H^3-TTP, 3.3 mμmoles (22,300 c.p.m.); DGTP, 8 mμmoles; DATP, 10 mμmoles; DCTP, 9 mμmoles; Mg^{++}, 2 μmoles; and TRIS:HCl, *p*H 8.0, 10 μmoles. After 60 minutes incubation at 37° 1.0 ml. cold 10% TCA was added. TCA insoluble material was washed two times with 0.5 ml. 5% TCA, hydrolyzed 20 minutes at 80° in 0.2 ml. 0.2 *M* NaOH, DNA and protein reprecipitated with dilute HCl and 5% TCA, and precipitate washed with 95% EtOH. Insoluble material dissolved in 0.5 ml. 90% formic acid and 0.1 ml. plated. (This rapid method of DNA isolation gives quantitative recovery.)

Reaction mixture	Total radioactivity[a] in DNA, c.p.m.
Complete	1,100
Omit DGTP	330
Omit DATP	400
Omit DCTP	400
Omit DGTP, DATP, and DCTP	175
Omit Mg^{++}	25
Omit DNA[b]	35
Complete, 60 minutes at 0°	45

[a] Radioactivity assayed in windowless flow counters. Since the same amount of material was plated from each reaction mixture no correction has been made for self-absorption. [b] 250 g. DNA added after incubation.

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REACTION OF CHLORODIFLUOROMETHANE WITH LINDE MOLECULAR SIEVE 5A

Sir:

Chlorodifluoromethane reacts at room temperature with a synthetic zeolite (Linde Molecular Sieve 5A).[1] Chlorofluoromethanes have been reported stable to temperatures in excess of 400°.[2] When chlorodifluoromethane was adsorbed at 25.0° on

(1) These abbreviations are used: DNA, deoxyribonucleic acid; DAMP, DGMP, DCMP, and TMP for the mono- and DATP, DGTP, DCTP, and TTP for the triphosphates of deoxyadenosine, deoxyguanosine, deoxycytosine, and thymidine; TCA, trichloroacetic acid; and TRIS, tris-(hydroxymethyl)-aminomethane.

(2) F. J. Bollum and V. R. Potter, Abstracts, 132nd meeting, American Chemical Society, 19-C (1957).

(3) (a) H. Z. Sable, P. B. Wilber, A. E. Cohen and M. R. Kane, *Biochim. Biophys. Acta*, **13**, 156 (1954); (b) L. I. Hecht, V. R. Potter and E. Herbert, *ibid.*, **15**, 134 (1954); (c) H. Klenow and E. Lichtler, *ibid.*, **23**, 6 (1957); (d) E. S. Canellakis and R. Mantsavinos, *ibid.*, in press; (e) I. Leberman, A. Kornberg and E. S. Simms, *J. Biol. Chem.*, **215**, 429 (1955); (f) A. Kornberg, "The Chemical Basis of Heredity," ed. W. D. McElroy and B. Glass, Johns Hopkins Press, Baltimore, Md., 1957, p. 579; (g) S. Ochoa and L. Heppel, *ibid.*, p. 615.

(4) F. J. Bollum, P. A. Morse and V. R. Potter, unpublished.

(5) R. B. Hurlbert, H. Schmitz, A. F. Brumm and V. R. Potter, *J. Biol. Chem.*, **209**, 23 (1954).

(6) F. J. Bollum, *Federation Proc.*, **17**, in press (1958).

(7) The requirement for DNA was also demonstrated with crude supernatant fraction, see ref. 2.

(8) Inhibition was 50% at 10 m*M* and 100% at 100 m*M* pyrophosphate, tested with the crude enzyme from regenerating liver. Details to be published soon.

(9) M. Bessman, I. R. Lehman, E. S. Simms and A. Kornberg, *Federation Proc.*, **16**, 153 (1957).

(10) Postdoctoral Fellow of the National Cancer Institute, USPHS. This investigation was supported by a grant (C-646) from the National Cancer Institute, USPHS, to Prof. Van R. Potter. I wish to express sincere appreciation to Prof. Potter for interest and helpful suggestions during the course of this work. I am indebted to Miss Geraldine DeGrazia for doing the many spectrophotometric analyses required in the isolation, characterization, and purification of the deoxynucleoside triphosphates, and in checking out the validity of the rapid method for isolating DNA.

(1) Empirical formula $CaO \cdot Al_2O_3 \cdot 2SiO_2 \cdot nH_2O$; see also D. W. Breck *et al.*, THIS JOURNAL, **78**, 5963 (1956).

(2) A. B. Trenwith and R. H. Watson, *J. Chem. Soc.*, 2368 (1957).

Linde Molecular Sieve 5A which had been evacuated at 10^{-5} mm. and 350° for 15 hours, a type I isotherm was obtained, in which the flat was attained at a low relative pressure (P/P was 0.00-506 at 25°). The corresponding adsorption was 0.20 g. sorbate per g. of sorbent. Each point required about 24 hours for equilibration. When desorption was commenced, a large loss of sorbate with decreasing pressure was observed, placing the desorption isotherm below the adsorption isotherm, rather than above it, the latter normally being expected. Considerable divergence of the two curves was seen down to a pressure of approximately 20 mm. Equilibration took longer on desorption and the residual solid was very gassy. The desorption isotherm was superposable on but not coincident with a carbon dioxide-Sieve 5A isotherm.[3] Carbon dioxide was the only gaseous reaction product from this system. The sample was placed in an absorption train, and yielded only carbon dioxide gas, at temperatures below 150°, where pyrolysis of any residual $CHClF_2$ would not be anticipated.

Chlorodifluoromethane also was stored over outgassed Sieve 5A for three weeks at 25°, and analyzed chromatographically; it was found to contain 0.4% by volume of carbon dioxide which previously had not been present. The residual solid material showed predominantly carbon dioxide evolution when heated in a mass spectrometer to 1000°. No halogen acid or carbon monoxide was seen.

Similar experiments conducted with Linde Molecular Sieve 4A did not show the same change in the isotherms, but infrared spectra showed a strong carbonyl absorption at 4.20μ and unassigned new bands at 14μ in the final gas.

Both series of experiments resulted in a loss of surface area of the solid (*e.g.* from an initial value 566.6 sq.m./g. to a new value of 290.0 sq. m./g. for carbon dioxide on 4A at 25.0°) but no gross structural changes in the sieves caused by the action of $CHClF_2$ were seen by X-ray diffraction.

Halogen acid products subsequently were found in the solid residuum: they presumably are present in a fully ionized form, since they were not seen in the mass spectrometer.

The reaction appears similar to that observed by Park, *et al.*,[4] who obtained carbon dioxide on heating wet $CHClF_2$, the water in the present case being the constitutional water of the zeolite. However, the mechanism appears open since Barrer and Brook[5] suggested dehydrofluorination to explain the attack of $CHClF_2$ on natural zeolites, and Ayscough and Emeléus[6] remarked on the formation of carbon dioxide and SiF_4 when CF_3 radicals were studied in quartz. The evidence of Neilson and White[7] on the association of liquid $CHClF_2$, and the peculiar geometry of the Molecular Sieve lattice[1] make it impossible to rule out here an exchange mechanism based on the polarization of $CHClF_2$.

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(3) "Molecular Sieve Data Sheets," Linde Air Products Co., Tonawanda, N. Y.

(4) J. D. Park, *et al.*, *Ind. Eng. Chem.*, **39**, 354 (1947).

(5) R. M. Barrer and D. W. Brook, *Trans. Faraday Soc.*, **49**, 940 (1953).

(6) P. B. Ayscough and H. J. Emeléus, *J. Chem. Soc.*, 3381 (1954).

(7) E. F. Neilson and D. White, THIS JOURNAL, **79**, 5620 (1957).

THE SITES OF REDUCTION AND BASE-CATALYZED HYDROGEN-EXCHANGE IN N[1]-METHYLNICOTINAMIDE IODIDE[1]

Sir:

Previous work on the dithionite-reduction of N[1]-methylnicotinamide indicated that hydrogen is added to the 4-position.[2,3,4,5] Our nuclear magnetic resonance (n.m.r.) data substantiate this, and further show that base-catalyzed hydrogen-exchange occurs at the 2- and 6-positions. Such exchange was noticed only at the 2-position of diphosphopyridine nucleotide (DPN).[6]

The assignment of sites was accomplished by comparing the n.m.r. spectra of N[1]-methylnicotinamide iodide (I), 2,6-dideuterio-N[1]-methylnicotinamide iodide (II), N[1]-methylnicotinamide chloride partially 4-deuteriated (III),[3] and N[1]-benzylnicotinamide chloride (IV). II was formed by dissolving 0.025 g. of I in 0.5 cc. of D_2O, containing 0.075 g. of Na_2CO_3. The exchange was completed by heating to 100°.

The spectrum of the ring protons of I consists of a single peak with relative area 1 unit at −114 cycles (benzene = zero cycles. Field strength 40 Mc.), three peaks at −104, −100 and −95 cycles with total relative area 2 units, four peaks at −77, −71, −69 and −62 cycles with total relative area 1 unit. In the spectrum of II, the peaks at −114 and −100 cycles had disappeared, the peak at −104 cycles was reduced in size, and the peaks centered about −70 cycles had collapsed into a 1:1 doublet. In III, the peak at −95 cycles was greatly reduced in size, and the peaks centered about −70 cycles partially collapsed into a doublet. The spectrum of IV is similar to that of I.

Because of the electronegativity and the formal charge of N[1], the 2- and 6-protons are shifted down field.[7] The carboxamide group should similarly affect the 2- and 4-protons. Thus the peak at −114 cycles may be assigned to the 2-proton and the group centered about −70 cycles to the 5-proton. The 2-proton could give a single line since there are no hydrogens on the adjacent atoms. The 5-proton line should be split by the 4- and 6-protons into two overlapping doublets or approximately a 1:2:1 triplet. The remaining spectrum, centered about −100 cycles, must therefore be assigned to the 4- and 6-protons.

Since N[1]-methylpyridinium iodide exchanges its 2- and 6-protons in aqueous Na_2CO_3 solution[8] and

(1) This work was supported in part by a grant from Research Corporation.

(2) M. E. Pullman, A. San Pietro and S. P. Colowick, *J. Biol. Chem.*, **206**, 129 (1954).

(3) G. W. Rafter and S. P. Colowick, *ibid.*, **209**, 773 (1954).

(4) M. B. Yarmolinsky and S. P. Colowick, *Biochim. et Biophys. Acta*, **20**, 177 (1956).

(5) D. Mauzerall and F. H. Westheimer, THIS JOURNAL, **77**, 2261 (1955).

(6) A. San Pietro, *J. Biol. Chem.*, **217**, 589 (1955).

(7) This is consistent with the results of W. G. Schneider, H. J. Bernstein and J. A. Pople, *Can. J. Chem.*, **35**, 1487 (1957).

(8) H. E. Dubb, M. Saunders and J. H. Wang, to be published.