A closer study of modified membranes must be left for a later communication. It is apparent, however, from the data given in this paper that much greater over-all selectivity for separations can be achieved by use of a membrane of the optimum pore size. When mixtures containing molecules of widely differing size are to be separated a series of membranes with different porosities will be needed.

From our data at present with cellophane membranes and 0.01 *N* acetic acid five standard sizes are easily available as follows. (1) A stretched 20/32 membrane which readily passes native ovalbumin. (2) The 20/32 Visking cellophane which readily passes native proteins up to ovalbumin. (3) The 18/32 Visking cellophane which will not pass proteins as large as insulin. (4) Acetylated 20/32 Visking which will not pass subtilin but will pass bacitracin. (5) Acetylated 18/32 Visking which will scarcely pass bacitracin. A study of the optimum way to integrate the use of these different sizes with the countercurrent approach is in progress.

The porosity of the membrane does not correlate with the thickness as can be seen from the data in Table II, which gives five sizes of cellophane studied with insulin. The thickness given is that found with a micrometer on the dry membrane before use.

TABLE II

COMPARISON OF THICKNESS, SIZE AND POROSITY OF SEVERAL SIZES OF VISKING TUBING

Size	Thickness, mm.	Result with insulin
8/32	0.064	Slowly passes
18/32	.0175	Does not pass
20/32	.020	Rapidly passes
23/32	.0275	Does not pass
27/32	.025	Passes less rapidly than with 20/32

NEW YORK, N. Y.

[CONTRIBUTION FROM THE DEPARTMENT OF PHYSIOLOGICAL CHEMISTRY, UNIVERSITY OF CALIFORNIA SCHOOL OF MEDICINE]

The Methylation of Deoxyuridine[1]

BY ELIZABETH A. PHEAR AND DAVID M. GREENBERG

RECEIVED JANUARY 15, 1957

The methylation of deoxyuridine, with formaldehyde as the methyl group precursor, has been demonstrated with cell-free preparations from various tissues. The most active of the extensively studied tissues was the thymus gland. Employing a Dowex-1-Cl^--treated, dialyzed rat thymus preparation, the primary product of the reaction appeared to be thymidylic acid, which was then partially converted to thymidine. Tetrahydrofolic acid, reduced phosphopyridine nucleotide, adenosine triphosphate and Mg^{++} were required as cofactors for the reaction.

Friedkin and Roberts[2] showed that deoxyuridine (UDR)[3] was incorporated into DNA-thymine and free TDR by suspensions of chick embryo and by rabbit and chick bone marrow. They concluded that the TDR was formed by a folic acid-dependent methylation. Reichard[4] also showed that deoxyuridine was incorporated into DNA-thymine. The observations of Friedkin and Wood[5] suggested that CDR also may be incorporated into DNA-thymine by the same or a similar reaction.

This paper describes studies of the methylation of UDR with $H_2C^{14}O$ in a cell-free preparation of rat thymus gland, and a survey of the distribution of this enzyme system in various other tissues.

Experimental

Materials.—Thymus glands from 1 month old Long-Evans rats were used for most of the work. In the determination of the distribution of the enzyme in various tissues, spleen and bone marrow from 1 month old rabbits, and marrow from pork, lamb and veal bones,[6] and thymus glands from steer, lamb and pig[7] were tested.

The sources of the following chemicals were: DPNH from Sigma Co., ATP from Nutritional Biochemical Co., UDR and TDR from California Foundation for Biochemical Research, folic acid from Mann, CF was a gift of the Lederle Laboratories, and the $H_2C^{14}O$ was purchased from the Bio-Rad Co., Berkeley. THF was prepared by the reduction of folic acid by the method of O'Dell, *et al.*[8]

Enzyme Methods.—Cell-free tissue preparations were obtained by homogenizing the minced tissue with 9 volumes of 0.05 *M* veronal buffer at *p*H 7.6 in a Potter–Elvehjem glass homogenizer, followed by centrifugation for 10 minutes at 20,000 × *g*. The thymus preparations were then batch-treated with Dowex-1-Cl^-,[9] centrifuged at 59,000 × *g*, and dialyzed overnight against veronal buffer.

The incubations were run for 2 hr. in 25-ml. beakers at 37° under nitrogen in a Dubnoff shaking metabolic incubator. Each vessel contained 2 ml. of enzyme (5–20 mg. protein) and substrates and cofactors at the following levels: $H_2C^{14}O$, 5 μmoles (98,400 c.p.m. per μmole); UDR, 10 μmoles; carrier TDR, 1.2 μmoles; ATP, 10 μmoles; DPNH, 2 μmoles; $MgSO_4$, 10 μmoles; THF 500 μg. The total volume was 2.7 ml. and when any of the above constituents was omitted the volume was made up with buffer; 0.05 *M* veronal buffer at *p*H 7.6 was used throughout unless otherwise indicated. Carrier thymidylic acid was added after incubation, since it inhibited the reaction, but the addition

(1) Aided by research grants from the National Cancer Institute (CY-2915 and 3175), National Institutes of Health, U. S. Public Health Service and the Cancer Research Funds of the University of California.

(2) M. Friedkin and D. Roberts, *Federation Proc.*, **14**, 215 (1955); *J. Biol. Chem.*, **220**, 653 (1956).

(3) Other abbreviations employed: TDR, free thymidine; TA, "thymidylic acid"—see Analytical Methods section; TTC, total acid-soluble compounds yielding thymine on hydrolysis; CDR, deoxycytidine; CF, citrovorum factor, leucovorin; ATP, adenosine triphosphate; DPN, DPNH, oxidized and reduced diphosphopyridine nucleotide; TPN, TPNH, oxidized and reduced triphosphopyridine nucleotide; TCA, trichloroacetic acid; DNA, deoxyribonucleic acid; RNA, ribonucleic acid; THF, tetrahydrofolic acid.

(4) P. Reichard, *Acta Chem. Scand.*, **9**, 1275 (1955).

(5) M. Friedkin and H. Wood, IV, *J. Biol. Chem.*, **220**, 639 (1956).

(6) Kindly supplied by Swift and Co., San Francisco.

(7) These were a gift from James Allen and Sons, 3rd Street, San Francisco.

(8) B. L. O'Dell, J. M. Vandenbelt, E. S. Bloom and J. J. Pfiffner, THIS JOURNAL, **69**, 250 (1947).

(9) B. E. Wright and T. C. Stadtman, *J. Biol. Chem.*, **219**, 863 (1956).

of carrier TDR before incubation had no effect on the reaction, and counteracted breakdown of TDR.[10] The incubations were run in pairs, with UDR added to one member of the pair only. The TDR, TA and TCC formed from the UDR were deduced from the difference in activity of these compounds isolated from the two incubations.

The reaction was stopped by adding TCA to give a final concentration of 10%. The precipitate was washed with 5% TCA, and the supernatant and washings combined.

Analytical Methods. Total Thymine-containing Compounds (TTC).[11]—These were estimated in the incubation extract after removal of the TCA with ether. The liquid was dried and hydrolyzed for 15 min. with 0.2 ml. of 12% perchloric acid, and the latter removed with KCl. To eliminate amino acids and salts, the preparation was adjusted to *p*H 2.3 and eluted from a Dowex-50-H^+ column with water at *p*H 3. The eluate was neutralized with NH_4OH, dried and chromatographed on Whatman No. 1 paper with butanol–water (86:14) as solvent. After 18 hr. of diffusion, the thymine spots were located by ultraviolet light. These, together with paper blanks, were cut out, weighed and eluted with warm water at *p*H 3. The concentration and purity of the samples was estimated photometrically, by use of their absorption densities at 250, 260 and 280 mμ in acid solution.

Thymidine (TDR) was measured in the presence of 1.2 μmoles of carrier. Samples of the dried, TCA-free acid-soluble residues after incubation were streaked onto Whatman No. 1 paper and chromatographed for 18 hr. in butanol–water (86:14). The thymidine spots were eluted, dried and rechromatographed for 5 hr. in 0.1 *M* borate buffer at *p*H 9.3. The spots (located with ultraviolet light) together with paper blanks were cut out, weighed, and the concentration of thymidine estimated from its absorption density at 260 mμ.

"Thymidylic Acid" (TA).—The majority of counts were present in a substance which behaved similarly to, and moved together with thymidylic acid on chromatography in isopropyl alcohol–HCl (isopropyl alcohol 17 ml., concentrated HCl 41 ml., water 39 ml.), isobutyric acid–NH_3 (10 vol. isobutyric acid, 6 vol. 0.5 *N* NH_4OH), 0.1 *M* borate at *p*H 9.3, and butanol–water. However it was not possible to isolate and characterize this compound as thymidylic acid. It was estimated by chromatography in butanol–water in the presence of 5.6 μmoles of carrier thymidylic acid. The immobile area in this solvent was eluted, and rechromatographed in isopropyl alcohol–HCl. The TA spot was eluted, hydrolyzed, passed through a Dowex-50-H^+ column, eluted with water at *p*H 3, chromatographed on Whatman No. 1 paper in butanol–water, and estimated spectrophotometrically as thymine. The sum of the activity of *Thymidine and "Thymidylic acid"* (Table I) was obtained after chromatography of the TCA-free residue from incubations in isopropyl alcohol–HCl in the presence of carriers. The spot which contained both these compounds was eluted, hydrolyzed and estimated, after chromatography on Dowex-50-H^+, and on paper in butanol–water, as thymine. Radioactivity was measured in duplicate, on infinitely thin samples dried on polythene planchets, to 5% accuracy with a gas flow counter. $H_2C^{14}O$ activity was estimated after its conversion to the dimedon derivative.[12] Protein was estimated with the biuret reagent.[13]

It was shown that TTC formation was proportional to the protein concentration over the range 3–20 mg. protein per incubation, so results have been expressed as μmoles of TTC, TA and TDR formed per mg. protein.

The activity of the TTC formed in the absence of added UDR in Dowex-treated samples varied from 0–8% of that formed in its presence. Results using the paired incubation method for TTC estimation agree to within 10% of those obtained using 2-C^{14}-uridine prepared by the method of Friedkin and Roberts.[2]

Table I shows that of the total activity in TTC, most was present in the TA fraction, with about 10% in the TDR. As the work was concerned primarily with the methylation of UDR, TTC only was usually measured.

Results

A test for the incorporation of UDR and $H_2C^{14}O$ into the purine and pyrimidine bases of DNA and RNA was performed following an incubation by isolating the nucleic acids according to the method of Schmidt and Thannhauser.[14] After hydrolysis free bases were isolated by the method of Cohn.[15] In confirmation of the results of Friedkin and Wood,[5] only DNA-thymine contained significant activity. TTC was more active than the DNA-thymine, so further studies were confined to this fraction.

Substrate Specificity.—UDR was found to be a better substrate than CDR or deoxycytidylic acid for TTC formation, and thus was used throughout the work. In one experiment in which 10 μmoles of these bases were compared as substrates, a thymus preparation, formed 22.2×10^{-4} μmole of TTC per mg. protein from UDR and only 2.2×10^{-4} and 3.2×10^{-4} μmole from CDR and cytidylic acid, respectively.

TABLE I

DISTRIBUTION OF RADIOACTIVITY BETWEEN TTC, TA AND TDR

Thymine activity estimated as	μmoles formed per mg. protein × 10^4
TTC	19.5
TDR	1.7
TA	16.8
TA and TDR	20.1

Occurrence of the Enzyme System.—Table II shows that cell-free preparations of spleen were more active enzymatically than bone marrow preparations from various sources. The comparative activity of other tissues was investigated in the rat, and the results are shown in Table III. An immature rat (100 g. wt.) was used in this experiment, since the tissues of young animals were found to be more active than those of older animals.

TABLE II

DISTRIBUTION OF METHYLATING ENZYME SYSTEM IN VARIOUS SPLEEN AND MARROW PREPARATIONS

Differences from standard incubation medium: 0.77 μmole $H_2C^{14}O$ per incubation, and 0.15 *M* KCl–$KHCO_3$ buffer, *p*H 7.4.

	Source	μmoles TTC formed per mg. protein × 10^5
Spleen	Rat	34.0
	Rabbit	17.0
Bone marrow	Pork	8.2
	Lamb	7.2
	Rabbit	3.8
	Veal	0.1

The high activity found in the thymus gland led to a survey of the activities of thymus glands of various species. The results are shown in Table IV. Preparations of sheep, steer and hog thymus were made from adult animals. Probably prepara-

(10) L. A. Manson and J. O. Lampen, *Federation Proc.*, **8**, 224 (1949).

(11) Total acid-soluble compounds yielding thymine on hydrolysis with perchloric acid: which could include thymidine, thymidylic acid and other acid-soluble nucleotides.

(12) L. P. Vernon and S. Aronoff, *Arch. Biochem.*, **29**, 179 (1950).

(13) T. E. Weichselbaum, *Am. J. Clin. Path. Tech. Sect.*, **10**, 40 (1946).

(14) G. Schmidt and S. J. Thannhauser, *J. Biol. Chem.*, **161**, 83 (1945).

(15) W. E. Cohn, *Science*, **109**, 377 (1949).

tions from young animals would have had greater activity.

TABLE III

DISTRIBUTION OF METHYLATING ENZYME SYSTEM IN TISSUES OF THE RAT

Tissue	μmoles TTC formed per mg. protein × 10^4
Submaxillary gland	185.0
Thymus	95.1
Liver	6.9
Spleen	2.6
Lung	2.6

TABLE IV

DISTRIBUTION OF METHYLATING ENZYME SYSTEM IN VARIOUS THYMUS PREPARATIONS

Source	μmoles TTC formed per mg. protein × 10^4
Hog	34.2
Rat	24.4
Steer	6.5
Sheep	2.8

Preparations homogenized and centrifuged in 0.25 *M* sucrose exhibited less enzymic activity than those prepared in 0.05 *M* veronal buffer at *p*H 7.4. Acetone powders, and preparations frozen either before or after dialysis were completely inactive.

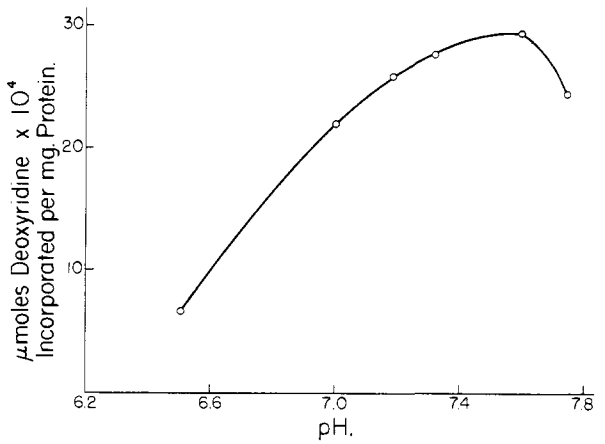

Fig. 1.—*p*H-activity curve for formation of acid-soluble thymine derivatives from deoxyuridine.

Attempts at fractionation of the enzyme system with $(NH_4)_2SO_4$ in 0.05 *M* veronal buffer at *p*H 7.4 were unsuccessful. If nucleic acids were first removed with protamine sulfate or $MnCl_2$,[16] and the $(NH_4)_2SO_4$ fractionation performed in 0.15 *M* KCl–$KHCO_3$ at *p*H 7.4, most of the enzyme activity was precipitated between 33 and 50% saturation. However, both the protamine sulfate and the $MnCl_2$ caused loss of activity. Moreover, recombination of the fraction containing most of the activity with that not precipitated at 60% $(NH_4)_2SO_4$ saturation, which alone had no activity, led to enhanced activity.

Dowex-1-Cl^- treatment and overnight dialysis caused no loss of activity, decreased the radioactivity of the TTC in the control vessel and facilitated the study of cofactor requirements. This is the only purification so far achieved with the enzyme system.

Cell-free preparations of rabbit bone marrow were more active in 0.05 *M* veronal than in 0.15 or 0.05 *M* tris-(hydroxymethyl)-aminomethane, glycylglycine or KCl–$KHCO_3$ buffers at *p*H 7.4. No enzyme activity was obtained in phosphate buffer. In veronal buffer the *p*H optimum was 7.4–7.7 (Fig. 1).

Conversion of Thymidylic Acid to Thymidine.—1.2 μmoles of TDR was added to a series of incubations of rat thymus preparation, containing 20 mg. protein per incubation. In these, the initial thymidylic acid carrier content varied from 0–5.6 μmole. The remainder, to give a total of 5.6 μmoles in each vessel, was added at the end of the experiment. Recovery of carrier thymidylic acid decreased serially from 2.9 μmoles in the incubation to which thymidylic acid had all been added after incubation, to 2.3 μmoles in the sample to which it had all been added initially. The TDR recovery varied inversely from 0.7–1.5 μmoles. This experiment shows qualitatively, since recoveries were low, that the TDR increases, and the thymidylic acid decreases in concentration during incubation. Some of the thymidylic acid may have been converted into acid-insoluble compounds, but some must have given rise to TDR. From the results obtained from studies of the relative activities of TA and TDR (*cf.* Table I and V), it seems that about 10% of the TA is converted to TDR during incubation under the present conditions in rat thymus preparations.

Cofactor Requirements.—The results obtained in a study of TA and TDR formation by a Dowex-1-treated, dialyzed rat thymus preparation are shown in Table V. Essentially similar results were obtained for TTC formation by rat thymus, rat spleen and rabbit bone marrow preparations. Table V shows that ATP, DPNH, THF and Mg^{++} are required for the formation of the methyl group of thymine compounds from UDR and formaldehyde.

THF was more effective than CF as the cofactor in this reaction, and other experiments (not given in the table) showed that folic acid at levels of 500 or 250 μg. was without effect.

In this table the need for DPNH in the presence of 500 μg. of THF was not clearly demonstrated. Paper chromatography of samples to which DPNH had not been added revealed the presence of DPN, which had presumably remained attached to the protein in the preparation. THF reduces DPN,[17] and the residual DPN is probably present in its oxidized form at the end of the manipulations prior to incubation. If the amount of THF is reduced to 166 μg., Table V shows that added DPNH definitely stimulated the reaction. Presumably at the higher level, the THF acts both as a reducing agent for the residual DPN in the preparation, and as cofactor, while at the lower level it functions mainly as a co-factor.

The requirement for DPNH also was shown in an experiment in which 6 ml. of rat thymus prepa-

(16) S. Korkes, A. del Campillo, I. C. Gunsalus and S. Ochoa, *J. Biol. Chem.*, **193**, 721 (1951).

(17) L. Jaenicke, *Biochim. Biophys. Acta*, **17**, 588 (1955).

ration was passed through a 6 × 1 cm. Dowex-1-Cl^- column at *p*H 7.2 before centrifugation, dialysis and incubation. 15.0 and 21.1 × 10^{-4} μmoles of TTC were then formed in the absence and presence of added DPNH, respectively, when 500 μg. of THF (which gave a maximum yield of TTC) was added. Presumably the column had been more effective in removing DPN than the usual batch treatment. Another possible explanation for the small effect of added DPNH could be that formaldehyde itself might reduce the THF-formaldehyde complex, and in so doing yield an equimolar quantity of HCOOH. To test this possibility, HCOOH was estimated by the method of Sakami.[18] It was found that it was formed in amounts comparable to the TTC, but that its formation was not dependent on the presence of UDR, so that it must have been produced by an independent reaction. Table V does not show clearly a requirement for DPN or TPNH, and shows that TPN is inhibitory. Table VI gives results of an experiment in which only TTC was measured. ATP, Mg and THF were present in all incubation flasks. This experiment shows that DPN is less effective than DPNH as a cofactor. TPN alone or in the presence of DPN was inhibitory. Addition of TPNH increased the yield of TTC only slightly. The requirement for TPNH has not been demonstrated clearly in any experiment, but experiments such as those described above do demonstrate clearly the need for DPNH has not been met with TPNH. Possibly, therefore, this is not a TPNH linked reaction, or possibly residual DPN in the preparation masks the effect of this added cofactor. Thus from the data a requirement for TPNH has not been demonstrated, but reduction can occur by a DPNH linked reaction.

TABLE V

COFACTOR REQUIREMENTS FOR METHYLATING ENZYME SYSTEM

Cofactors were added at the following levels: ATP, 10 μmoles; $MgSO_4$, 10 μmoles; DPNH, 2 μmoles; DPNH,[a] 0.67 μmole; TPNH, TPN and DPN, 0.67 μmole; CF, 500 μg.; THF 500 μg.; THF,[b] 166 μg., 1 μmole carrier thymidylic acid added initially and 4.6 μmoles after incubation.

Cofactors added	μmoles TA formed per mg. protein × 10^4	μmoles TDR formed per mg. protein × 10^4
Mg	0.0	0.0
Mg and DPNH	.1	.3
Mg and ATP	.0	.8
Mg and THF	.1	.0
Mg, THF and DPNH	.4	.8
Mg, THF and ATP	27.0	24.2
Mg, THF, ATP and DPNH	31.7	29.9
THF, ATP and DPNH	12.8	10.7
Mg, ATP and THF[b]	11.4	8.2
Mg, ATP, THF[b] and DPNH	21.5	21.6
Mg, ATP, THF and DPNH[a]	24.0	22.6
Mg, ATP, THF and DPN	21.5	20.7
Mg, ATP, THF, and TPNH	18.4	21.8
Mg, ATP, THF and TPN	0.8	0.7
Mg, ATP, DPNH and CF	3.9	17.6

(18) W. Sakami, *J. Biol. Chem.*, **187**, 369 (1950).

Discussion

From the experimental work reported here it is concluded that the methylation of UDR with formaldehyde is dependent on the presence of THF, ATP, Mg^{++} and DPNH. These same cofactors serve in the biosynthesis of the methyl group of methionine in a system containing formaldehyde and homocysteine.[19]

TABLE VI

EFFECT OF PYRIDINE NUCLEOTIDES ON THE METHYLATING ENZYME SYSTEM

Pyridine nucleotide added 0.28 μmole	μmoles TTC formed per mg. protein 10^4
...	48.3
DPNH	75.2
TPNH	54.5
DPN	62.6
TPN	11.6
DPN and TPN	15.8

THF has been shown to be the most effective folic acid derivative in the transfer of one carbon units to form the β-carbon of serine.[20,21] It is evidently more closely related to the active cofactor than either CF[21,22] or folic acid.[19]

It has been proposed that "active formaldehyde" is N_{10}-CH_2OH·THF or a closely related compound.[22,23] According to Kisliuk[24] formaldehyde reacts non-enzymatically with THF. The DPNH presumably reduces the CH_2OH-intermediate to give the methyl group in the present reaction. ATP, Mg^{++}, THF and DPNH are required for the formation of the β-carbon of serine from formate,[22] but when formaldehyde is the substrate, the reaction is catalyzed by THF alone.[21,22] Thus, it seems likely that in our reaction with formaldehyde as substrate, the ATP is not involved in the one carbon transfer, but rather in the activation of the UDR.

The experimental work reported here shows that, during methylation of UDR, TA and not TDR is the primary product of the reaction. TA is a substance which is so far chromatographically identical with thymidine-5′-phosphate, the naturally occurring thymidylic acid. Thymidylic acid inhibits methylation of UDR, suggesting that it may be involved in the reaction, and in our system both TA (chromatographic material) and thymidylic acid are partially converted to TDR. Thus it seems probable that, since ATP is necessary for the reaction, a phosphorylated derivative and not UDR itself is the natural substrate for the reaction. In support of this, Friedkin and Kornberg[25]

(19) A. Nakao and D. M. Greenberg, THIS JOURNAL, **77**, 6715 (1955).

(20) R. L. Blakely, *Biochem. J.*, **58**, 448 (1954).

(21) N. Alexander and D. M. Greenberg, *J. Biol. Chem.*, **214**, 821 (1955); **220**, 775 (1956).

(22) R. L. Kisliuk and W. Sakami, THIS JOURNAL, **76**, 1456 (1954); *J. Biol. Chem.*, **214**, 47 (1955).

(23) G. R. Greenberg, L. Jaenicke and M. Silverman, *Biochim. Biophys. Acta*, **17**, 589 (1955).

(24) R. L. Kisliuk, *Federation Proc.*, **15**, 289 (1956).

(25) M. Friedkin and A. Kornberg, "Symposium on the Chemical Basis of Heredity," Johns Hopkins Press, Baltimore, Md., 1957, p. 609, quoted by Cohen, *et al.*[26]

(26) S. S. Cohen, M. Green and H. D. Barner, *Biochim. Biophys. Acta*, **22**, 210 (1956).

have found that deoxyuridylic acid is converted to thymine nucleotides by bacteria. It has been suggested recently[27] and partially supported by experimental evidence[26] that the methylation of pyrimidine derivatives may involve first an aldol condensation of the CH_2OH-THF compound with the pyrimidine derivative and then formation of the 5-CH_2OH-dihydro-pyrimidine compound. This is followed by removal of water, yielding the methylene derivative, and finally there is a shift of the double bond. These steps also could possibly involve ATP.

ADDENDUM:—Friedkin and Kornberg[25] and Friedkin[28] have shown that deoxyuridine-5'-phosphate is the substrate for methylation. They postulate a direct condensation of $HOCH_2$-THF with the deoxyuridylic acid, with the splitting out of water, and subsequent cleavage to yield the methyl group. Presumably this would lead to an oxidation of THF to dihydrofolic acid. The function of the reduced phosphopyridine nucleotide might then be to reconvert the latter to THF.

(27) R. L. Hamill, R. L. Herrmann, R. U. Byerrum and J. L. Fairley, *Biochim. Biophys. Acta*, **21**, 394 (1956).

(28) M. Friedkin, *Federation Proc.*, **16**, 183 (1957).

BERKELEY, CALIFORNIA

[CONTRIBUTION FROM THE CHEMISTRY DIVISION OF THE BRITISH COLUMBIA RESEARCH COUNCIL]

Carbodiimides. VII.[1] Tetra-*p*-nitrophenyl Pyrophosphate, a New Phosphorylating Agent

BY J. G. MOFFATT AND H. G. KHORANA

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Tetra-*p*-nitrophenyl pyrophosphate, prepared *in situ* by the reaction of di-*p*-nitrophenyl hydrogen phosphate with di-*p*-tolyl carbodiimide in anhydrous dioxane, has been found to phosphorylate alcohols at room temperature without basic catalysis and, therefore, constitutes a powerful phosphorylating agent. A number of tertiary di-*p*-nitrophenyl phosphate esters were thus prepared in excellent yields. The nitrophenyl groups may be removed by hydrogenolysis in the presence of platinum or by alkaline treatment. Very mild alkaline hydrolysis results in the quantitative formation of alkyl mono-*p*-nitrophenyl hydrogen phosphates. Some other interesting properties of the neutral di-*p*-nitrophenyl phosphate esters have been recorded. A method for the large scale preparation of di-*p*-nitrophenyl hydrogen phosphate is detailed.

Methods for the synthesis of esters of phosphoric acid have been the subject of numerous investigations and several phosphorylating agents have been introduced. The reagents commonly employed fall broadly into two groups. To the first group belong phosphorus oxychloride[2] and certain of its monofunctional derivatives such as diphenyl,[3] dibenzyl[4] and di-*p*-nitrobenzyl[5] phosphorochloridates. Common features of the standard procedures employing these reagents are the mildness of conditions and the use of a basic catalyst, *e.g.*, pyridine, which often serves as the medium of reaction. The second group of phosphorylating agents embraces a number of anhydrides[6,7] such as polyphosphoric acid. Reagents of this type are usually employed in large excess and the phosphorylation reactions, which are carried out in the absence of a base, proceed under relatively drastic conditions.[8] Although lacking in elegance, reagents of the second type offer advantages of ready accessibility and simplicity in operation and have proved useful in certain cases.[9]

While the successful application of the existing methods of phosphorylation to a variety of synthetic problems has been recorded, inert hydroxyl functions are sometimes encountered whose phosphorylation in a satisfactory yield presents difficulties. One specific example is that of 2',3'-*O*-isopropylidene guanosine, the phosphorylation of which formed a part of our studies in the nucleotide field. In connection with this problem and our broader interest in developing new and improved methods for the synthesis of phosphate esters of biological interest it was considered desirable to devise a method of phosphorylation which would utilize a mono-functional, powerful and yet mild reagent and which would not require basic catalysis. The present communication describes a method which meets the above requirements and, therefore, combines the desirable features of the two aforementioned types of procedures. The application of this method to a satisfactory synthesis of guanosine 5'-phosphate is recorded in the following communication.[10]

A convenient and efficient method for the synthesis of the symmetrical esters of pyrophosphoric acid

(1) Paper VI. C. A. Dekker and H. G. Khorana, THIS JOURNAL, **76**, 3522 (1954).

(2) See *e.g.*, E. Fischer, *Ber.*, **47**, 3193 (1914); P. A. Levene and R. S. Tipson, *J. Biol. Chem.*, **106**, 113 (1934).

(3) (a) K. Zeile and H. Meyer, *Z. physiol. Chem.*, **256**, 131 (1938); (b) P. Brigl and H. Muller, *Ber.*, **72**, 2121 (1939); (c) H. Bredereck, E. Berger and J. Ehrenberg, *ibid.*, **73**, 269 (1940).

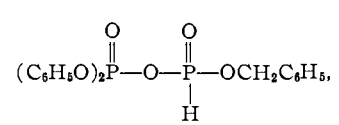
(4) F. R. Atherton, H. T. Openshaw and A. R. Todd, *J. Chem. Soc.*, 382 (1945) and subsequent papers.

(5) L. Zervas and I. Dilaris, THIS JOURNAL, **77**, 5354 (1955).

(6) Broadly speaking all phosphorylating agents are anhydride in character. The above classification serves to emphasize the different types of procedures used in phosphorylation reactions.

(7) For references to earlier literature see G. M. Kosolapoff, "Organophosphorus Compounds," John Wiley and Sons, New York, N. Y., 1950.

(8) The reagent *O*-benzyl phosphorus *O,O*-dibenzylphosphoric anhydride



developed by N. S. Corby, G. W. Kenner and A. R. Todd, *J. Chem. Soc.*, 3669 (1952), for the preparation of mixed secondary phosphites is more akin to the phosphoric acid chlorides in that it is used under mild conditions in the presence of a tertiary base.

(9) See *e.g.*, A. N. Wilson and S. A. Harris, THIS JOURNAL, **73**, 4693 (1951); J. E. Seegmiller and B. L. Horecker, *J. Biol. Chem.*, **192**, 175 (1951); R. H. Hall and H. G. Khorana, THIS JOURNAL, **77**, 1871 (1955).

(10) R. W. Chambers, J. G. Moffatt and H. G. Khorana, *ibid.*, **79**, 3747 (1957).