Short Communications

Methylation of Adenine in Deoxyadenylic Acid or Deoxyribonucleic Acid at N-7

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Alkylation of nucleic acids in neutral aqueous solution has been shown to occur principally at N-7 of guanine moieties, but positions of less reactivity have been found at N-1 and N-3 of adenine and N-1 of cytosine (Lawley & Brookes, 1963). As yet N-7 of adenine has not been implicated as a reactive site. Although Reiner & Zamenhof (1957) claimed to have obtained 7-methyladenine from methylation of adenine and of DNA, Pal (1962) showed that the product in question was in fact 3-methyladenine, which Lawley & Brookes (1963) have further shown to be the principal minor product from methylation of DNA.

Since 3-methyladenine is hydrolysed from methylated DNA at neutral pH (Lawley & Brookes, 1963), it was expected that the minor alkylation product of deoxyadenylic acid, 3-methyldeoxyadenylic acid, would be hydrolysed at neutral pH to yield 3-methyladenine, whereas the principal product, 1-methyldeoxyadenylic acid, would yield 6-methylaminopurine deoxyriboside phosphate (Lawley & Brookes, 1963), but the rate of the former reaction had not been determined. Experiments designed to investigate this led to the unexpected finding that two methylated bases split off from methylated deoxyadenylic acid at neutral pH; one was the expected 3-methyladenine and the other, as reported below, has been identified as 7-methyladenine. It has also been shown that alkylation of DNA by dimethyl sulphate or diethyl sulphate gives a small yield of the corresponding 7-alkyladenines.

The sodium salt of deoxyadenosine 5'-monophosphate (0.1 m-mole) was treated with dimethyl sulphate (0.5 m-mole) in 0.4N-sodium phosphate buffer, pH 7.0 (5 ml.), at 37°. After 1, 2, 4, 18 and 120 hr., the final pH being 6.2, 0.5 ml. portions were chromatographed two-dimensionally on Whatman no. 3 paper with as first solvent *n*-butanol-aq. ammonia (sp.gr. 0.88)-water (85:2:13, by vol.), then methanol-conc. hydrochloric acid-water (7:2:1, by vol.) [solvents (2) and (1) respectively of Lawley & Brookes (1963)]. Purine bases liberated by hydrolysis at neutral pH are moved away from the origin by the butanol-ammonia solvent (2), the residual non-hydrolysed nucleo-

tides remaining at the origin; in the second dimension, the deoxynucleotides are hydrolysed by the acid in solvent (1) to yield their corresponding bases. Two methyladenines were found to be liberated by hydrolysis in the neutral reaction mixture in amounts increasing with time up to 18 hr., the half-lives of both hydrolyses being about 1.5 hr. These products were eluted from the paper into 0.1N-hydrochloric acid and their ultraviolet-absorption spectra measured. One was identified from its spectra and R_F values as 3-methyladenine (Brookes & Lawley, 1960), and from the known extinction coefficients the molar yield of this product was determined as 1.2% of the original deoxyadenylic acid. The second product liberated at neutral pH was identical in spectra (Fig. 1) and R_{Ad} values, i.e. R_F relative to that of adenine [in solvent (1), 1.2; in solvent (2), 0.8], with authentic 7-methyladenine, kindly supplied by Dr R. Denayer, the yield being 2.1%. The nucleotides that remained at the origin after chromatography in solvent (2) were hydrolysed by the acid in solvent (1), liberating adenine and 1-methyladenine, the principal methylation product (Brookes & Lawley, 1960), in 26% yield.

These experiments also indicate why 7-methyladenine has not previously been detected as a product from methylation of adenine nucleotides, since its R_F values are almost identical with those of 1-methyladenine. Its elution from Dowex 50 (H^+ form) by hydrochloric acid showed also its close similarity to 1-methyladenine. However, if these two methyladenines were both present in methylated DNA they could be distinguished by the release of 7-methyladenine at neutral pH, whereas 1-methyladenine would remain attached to the macromolecule, and could further be converted into 6-methylaminopurine by alkaline treatment (Brookes & Lawley, 1960), which leaves 7-methyladenine unchanged.

These differences were, therefore, applied to investigate whether adenine moieties of DNA are alkylated at N-7. Salmon-sperm DNA (70 mg.; 0.18 m-mole of DNA P) was dissolved in 5 mN-sodium phosphate buffer, pH 7 (7 ml.), and then

Fig. 1. Absorption spectra in 0.05N-hydrochloric acid (a) and 0.05N-sodium hydroxide (b) of 7-methyladenine (—) and of products obtained chromatographically as described in the text from the methylation in neutral aqueous solution of deoxyadenylic acid (O), and of DNA (Δ).

2.5M-sodium acetate (2.5 ml.) and dimethyl sulphate (0.15 ml.) were added. After 45 min. at 37° the DNA was precipitated by the addition of ethanol (20 ml.), redissolved in 25 mM-sodium acetate, pH 6.3 (10 ml.), and kept at 37° for 4 days. The precipitation was then repeated, the supernatant being retained, and the precipitated DNA was treated with 0.1N-sodium hydroxide (10 ml.) for 16 hr. at 37°. The residue from the evaporated supernatant was redissolved in water (0.8 ml.) and after standing overnight at 4° yielded a crystalline precipitate of 7-methylguanine that was removed by centrifugation, and a portion (0.25 ml.) of the residue was chromatographed as described for methylated deoxyadenylic acid. The supernatant was shown to contain, in addition to 7-methylguanine, 3-methyladenine and 7-methyladenine. The alkali-treated DNA was then precipitated after neutralization and a portion was hydrolysed for 10 min. at 100° with N-hydrochloric acid, liberating adenine, guanine and 6-methylaminopurine, which were separated and estimated by two-dimensional paper chromatography. The 6-methylaminopurine had been derived from alkaline rearrangement of 1-methyladenine. The molar proportions of methylated purines were determined spectroscopically from eluates of the spots as 7-methylguanine:3-methyladenine:1-methyladenine:7-methyladenine = 75:18:5:2; i.e. 7-methyladenine comprised about 2% of the total methylated purines, which amounted to 0.14 mole/mole of DNA P.

In an analogous experiment diethyl sulphate (0.2 ml.) was used in place of dimethyl sulphate and,

in addition to 7-ethylguanine, 3-ethyladenine [R_{Ad} in solvent (1), 1.8; in solvent (2), 1.5] and 7-ethyladenine [R_{Ad} in solvent (1), 1.5; in solvent (2), 1.3] were identified as having been hydrolysed off the ethylated DNA into the supernatant: (3-ethyladenine: at pH 2, λ_{max} 274 mμ, ϵ 18400, $\epsilon_{280}/\epsilon_{260}$ 1.32; at pH 12, λ_{max} 273, ϵ 13400, $\epsilon_{280}/\epsilon_{260}$ 1.33; 7-ethyladenine: at pH 2, λ_{max} 272, ϵ 12400, $\epsilon_{280}/\epsilon_{260}$ 1.025; at pH 12, λ_{max} 270, ϵ 9500, $\epsilon_{280}/\epsilon_{260}$ 0.76). The yields estimated spectroscopically after elution of the spots were: 3-ethyladenine, 0.6 mmole/mole of DNA P; and 7-ethyladenine, 0.16 mmole/mole of DNA P. Thus, though the ethylating agent attacks the same groups as the methylating agent, the extent of reaction is much less, in agreement with previous work (Lawley & Brookes, 1963).

We conclude, therefore, that the reactive groups of adenine in deoxyadenylic acid are, in order of decreasing reactivity, N-1 > N-7 > N-3; and those of adenine in DNA, N-3 > N-1 > N-7.

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Vitamin A Acid from Retinene

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The interest in vitamin A acid (retinoic acid) follows from the work of Dowling & Wald (1960), who showed that it was completely ineffective in the visual role as it cannot be reduced to the aldehyde retinene, though it had high potency in promoting growth. Subsequent investigators (Harris, 1960; Wolf & Varandani, 1960) have further shown that vitamin A acid would not support reproduction but would maintain normal growth. Dowling & Wald (1960), Moore (1960) and Morton (1960) put forward a hypothesis that vitamin A acid, or some compound derived from it, is the form of vitamin A active systemically. It has been the experience of many workers that it is extremely difficult to find vitamin A acid in the intestinal tissues even after feeding huge doses of it, unless it be given in the esterified form. Dmitrovsky (1961) has shown that vitamin A acid can be formed by the liver, by means of an enzyme that oxidizes retinene. This has been further confirmed by Elder & Topper (1961) and Mahadevan, Murthy & Ganguli (1962).

Although the enzymic route of oxidation of retinene to vitamin A acid has thus been clearly demonstrated, a rapid method of oxidation of retinene to vitamin A acid is still to be found. The only reported method of oxidation of retinene to vitamin A acid is by Miki & Hiraga (1960), who obtained the acid by stirring retinene with silver oxide in sodium hydroxide solution. The method, however, is time-consuming and needs 60 hr. of continuous stirring.

A new and rapid method of synthesizing vitamin A acid from retinene is now reported. Retinene can be prepared from easily available vitamin A alcohol (synthetic, or from naturally occurring material in fish-liver-oil concentrates), according to the method of Ball, Goodwin & Morton (1948). Retinene thus obtained can be oxidized by Tollens reagent, when vitamin A acid is obtained. Oxidation is complete within 15–30 min. A description of the method is given below.

Retinene (100 mg.) was dissolved in about 150 ml. of ethanol and to this was added Tollens reagent (10 g. of silver nitrate in 100 ml. of water plus 10 g. of sodium hydroxide in 100 ml. of water, dilute ammonia solution being added to dissolve the precipitate). The reaction mixture was kept at 30–35°

in the dark for $\frac{1}{2}$ hr. A mirror, or blackening of the solution, resulted. The solution was then decanted and was acidified with aq. 50 % hydrochloric acid with cooling. The acidified solution was decanted and extracted with ether. The ethereal extract was washed with water to remove excess of hydrochloric acid, dried over anhydrous sodium sulphate and evaporated to dryness. The dry residue was dissolved in light petroleum (b.p. 40–60°) ($\lambda_{\max}$. 350 m μ , in light petroleum or ethanol). The light-petroleum solution of the product was chromatographed on a column packed with weakened alumina [8 % (v/w) of water (Barua & Morton, 1949)], when two zones were seen: a slowly moving yellow zone and another yellow zone (containing vitamin A acid) that was adsorbed very strongly near the top of the column. This zone could not be made to flow down even with 20 % (v/v) ether in light petroleum (b.p. 40–60°), and so it was extruded and eluted with ethanol containing a little ammonia. The eluate was acidified with aq. 50 % hydrochloric acid and extracted with peroxide-free ether, and the ether extract was washed with water to remove excess of hydrochloric acid, dried over anhydrous sodium sulphate and evaporated to dryness *in vacuo*. The residue was dissolved in a small quantity of light petroleum (b.p. 40–60°) and kept at 0°, when crystals separated. These were filtered off and dried (yield 50 mg.). The recrystallized material had the following characteristics: $\lambda_{\max}$. 350 m μ (in light petroleum or ethanol); $E_{350\text{ m}\mu}^{1\%, 1\text{ cm.}}$ 1504, (in ethanol); m.p. 179° (uncorr.). When a drop of 0.1 N-sodium hydroxide solution was added to the ethanolic solution, the characteristic shift to 335 m μ of vitamin A acid was observed. The $\lambda_{\max}$. could be brought back to 350 m μ on adding 2 drops of 0.1 N-hydrochloric acid (personal communication from Professor R. A. Morton). The substance was soluble in aqueous sodium carbonate solution. This crystalline material gave with antimony trichloride reagent a purple colour with $\lambda_{\max}$. 572 m μ .

The methyl ester of the acid was prepared by refluxing the acid with methyl iodide in ethyl acetate and potassium carbonate for 1 hr. at 50–60°. When the esterified product was chromatographed on 8 % water-weakened alumina, the methyl ester was obtained as a fast-moving compound ($\lambda_{\max}$. 352 m μ , in ethanol; antimony tri-

chloride produced a purple colour with $\lambda_{\max}$. 572 m μ).

When this methyl ester was reduced by lithium aluminium hydride vitamin A alcohol was obtained ($\lambda_{\max}$. 326 m μ , in ethanol; antimony trichloride gave a blue colour with $\lambda_{\max}$. 618 m μ). The vitamin A alcohol, when 'cyclized' with dry ethanolic 0.03N-hydrochloric acid, gave typical resolved bands of anhydrovitamin A, $\lambda_{\max}$. 350, 370 and 390 m μ , with inflexion at 335 m μ (in light petroleum). All these experiments characterized the product as vitamin A acid.

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Improved Rates of Carbon Dioxide Fixation by Illuminated Chloroplasts

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In recent years light-induced carbon dioxide fixation has been repeatedly demonstrated, but the rates reported (e.g. Allen, Arnon, Capindale, Whatley & Durham, 1955; Gibbs & Calo, 1959) have been generally low (0.1–2 μ moles of CO₂ fixed/mg. of chlorophyll/hr.) and have only rarely (Gibbs & Calo, 1959; Losada, Trebst & Arnon, 1960; Havir & Gibbs, 1963) approached 3 % of the maximum achieved by the intact leaf (about 180 μ moles/mg. of chlorophyll/hr.). The failure of isolated chloroplasts to fix carbon dioxide at higher rates has been attributed to losses during the isolation of enzymes concerned in carboxylation (Smillie & Fuller, 1959) and in reduction (Gibbs, Black & Kok, 1961), and has also contributed to the expression of legitimate doubts about the extent to which isolated chloroplasts constitute a complete photosynthetic system *in vitro* (e.g. Stiller, 1962). Somewhat similar doubts were voiced when photosynthetic phosphorylation was first demonstrated, but subsequent slight changes in experimental technique led to substantial improvements in rate. The results reported below suggest that similar improvements in carbon dioxide fixation may also be expected.

Chloroplasts were isolated at pH 6.8 in 0.33M-sorbitol solution containing Na₂HPO₄-KH₂PO₄ buffer (0.1M), NaCl (0.1 %), MgSO₄ (0.1 %) and sodium isoascorbate (0.1 %). Leaves (100 g.) were added to 200 ml. of the chilled solution in a Waring Blendor running at half speed and then homogenized for 5 sec. at full speed. The brei was

squeezed through two layers of muslin and then filtered through eight layers of muslin. The filtrate (approx. 300 ml.) was centrifuged at 0° by allowing the centrifuge to accelerate to 6000 rev./min. (approx. 4000g) and then to return rapidly to rest (total time 100 sec.). The supernatant was discarded. The pellet was washed in cold 0.33M-sorbitol containing NaCl (0.1 %) and MgSO₄ (0.1 %) and finally resuspended, by using cotton wool and a glass rod, in 5 ml. of the same solution. Examination (Zeiss light microscope, magnification $\times$ 1200) showed that the preparations consisted almost entirely of whole chloroplasts and were free of intact cells.

This method differs from those used by most workers in the following respects: (1) the chloroplasts were isolated at pH 6.8 to enhance their stability and used (see below) at pH 7.5 (the reported optimum for CO₂ fixation; Gibbs & Calo, 1959); (2) filtration was substituted for a preliminary centrifugation (to remove cell debris) to minimize loss of whole chloroplasts; (3) leaves were macerated only sufficiently to permit adequate disruption of the cells, and centrifugation was designed to recover a small number of whole chloroplasts in a short time; (4) sorbitol was used to maintain a suitable osmotic pressure because in preliminary experiments it was found to give better results than sodium chloride or sugars such as glucose.

Reactions were carried out in glass centrifuge tubes (1.5 cm. $\times$ 10 cm.), with rounded ends and 'suba-seal' rubber stoppers, and were started and

stopped at 40 sec. intervals. Reaction mixtures (total vol. 0.3 ml.) were completed by the addition of 10 μ l. of $\text{Na}_2^{14}\text{CO}_3$ solution (20 μC , 0.56 μmole) immediately followed by 0.1 ml. of chloroplast suspension containing approx. 0.1 mg. of chlorophyll [the actual quantity of chlorophyll added was determined spectrophotometrically in samples extracted in 80 % (v/v) acetone].

Each reaction mixture also contained 0.1 ml. of 0.33 M-sorbitol and 0.05 ml. of a solution (at pH 7.5) containing MgSO_4 (5 mM), MnCl_2 (5 mM), KH_2PO_4 (5 mM), NaCl (0.5 M), tris (0.15 M), EDTA (5 mM), sodium isoascorbate (10 mM) and cysteine hydrochloride (10 mM). Where indicated in Table 1 a total of 4 μmoles of 2-phosphoglycerate or sugar phosphate was also added.

The tubes were illuminated for 10 min. by tungsten lamps (approx. 20 000 lux) at 20° in a bath similar to the one described by Hill & Walker (1959). Reactions were stopped, in the bath, by the injection of 0.5 ml. of 0.3 N-HCl. Samples (20 μ l.) were dried on lens filter disks on aluminium planchets and their radioactivities determined with a thin-end-window Geiger-Müller counter. The relationship between radioactivity measured and carbon dioxide fixed was calculated from values obtained when known quantities of labelled sucrose were added to reaction mixtures (containing no

carbonate) that were then acidified and sampled as before. The values for carbon dioxide are minimal and do not take into account losses during drying of samples under acid conditions.

Table 1 gives the values for carbon dioxide fixed in an experiment designed to show the effects of added sugar phosphates and is typical of the rates obtained in the presence of these additives. Dark-fixation was less than 1 % of the maximum rate. The chloroplasts were prepared from 13-day-old pea leaves (*Pisum sativum* var. Laxton Superb) that had been kept in the dark for 12 hr. before use.

Two-dimensional chromatography has shown that the major labelled products in this and similar experiments were triose phosphates, together with some pentose phosphates and hexose phosphates. Other products included malate and aspartate and also glycollate, which, under some conditions, contained 20 % or more of the radioactivity fixed.

I am grateful to Professor C. P. Whittingham, at whose suggestion the work was undertaken and in whose department it was carried out, and for the skilled technical assistance of Mr Carl Baldry. The work was partly supported by grants from the University of London Central Research Fund and the Department for Scientific and Industrial Research.

Table 1. Carbon dioxide fixation by illuminated chloroplasts

Added substrate	CO_2 fixed ($\mu\text{moles/mg. of}$ chlorophyll/hr.)
None	1.1
Glucose 1-phosphate	4.7
2-Phosphoglycerate	6.2
Fructose 1,6-diphosphate	15.2
Ribose 5-phosphate	24.3
Ribose 5-phosphate + fructose 1,6-diphosphate	17.9

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The Rate of Formation and Decomposition of Phosphoryl-phosphatase (*Escherichia coli*)

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It has been shown by Engstrom (1961, 1962) and Schwartz & Lipmann (1961) that alkaline phosphatases from various sources are phosphorylated

on incubation with [^{32}P]orthophosphate in the pH range 5–6 when the enzyme is not active. At pH 10, when the enzyme is fully active, little or no

labelling occurs and phosphoryl-enzyme is rapidly dephosphorylated. Analyses of hydrolysis products of the acid-denatured labelled protein yielded $O[^{32}P]$ -phosphorylserine. Schwartz & Lipmann (1961) presented circumstantial evidence that phosphorylation with orthophosphate at pH 5 occurred at the active site.

The present investigation was designed to determine the rate of dephosphorylation of phosphoryl-phosphatase under conditions when the enzyme was active. The rapid-flow quenching technique (Barman & Gutfreund, 1964) used allows the study of reactions with half-times of the order of 5 msec.

Escherichia coli phosphatase (Worthington Biochemical Corp., Freehold, N.J., U.S.A.) was obtained in the form of a suspension of 2.5 mg. of protein/ml. of 0.65-saturated ammonium sulphate solution. For phosphorylation 0.2 ml. of this enzyme suspension was added to 1.3 ml. of medium: 0.05M-sodium acetate-acetic acid buffer, pH 5.4, containing magnesium acetate (33 mM) and $[^{32}P]$ -orthophosphate (1.0 mM; 0.025 mc). The rate of combination of $[^{32}P]$ orthophosphate with the enzyme is first-order (0.046 sec^{-1} at 0°). The $[^{32}P]$ -phosphoryl-enzyme was used within 3 hr. of preparation. Prolonged incubation under these conditions leads to a gradual increase of $[^{32}P]$ orthophosphate bound. For the study of the rate of dephosphorylation the above solution of phosphoryl-enzyme at pH 5.5 was mixed in the rapid-flow

system with an equal volume of either the same acetate buffer or 0.1M-sodium carbonate-bicarbonate containing bovine serum albumin (6 mg./ml.) to give final pH 5.5, 7.3 or 8.4. After specified flow times (see Fig. 1) the reactions were quenched by rapid mixing of the reaction mixture with 7N-perchloric acid. The kinetic experiments were carried out at 20° . The phosphorylated protein was isolated by centrifuging and separated from contaminating $[^{32}P]$ orthophosphate essentially by the method of Schwartz & Lipmann (1961). The rate of dephosphorylation was calculated from the decrease in the percentage of ^{32}P -labelled enzyme during the time between mixing the phosphoryl-enzyme solution with the appropriate buffer and quenching in perchloric acid. The blank values without enzyme were about 10 % of the maximum bound during several hours at room temperature.

In Fig. 1 the results are shown as first-order plots of the data from experiments carried out at pH 8.4 and 7.3. The half-time of the dephosphorylation at pH 8.4 is 6 msec., which corresponds to a first-order constant 115 sec^{-1} , and at pH 7.3 the half-time is 43 msec., corresponding to a rate constant of 16 sec^{-1} . If the model (Schwartz, 1963):

is used to illustrate the proposed mechanism of hydrolysis of the ester (ROP) to ROH and orthophosphate (P) catalysed by the enzyme (E), forming the phosphorylated intermediate EP, one can draw the following conclusions: at pH 8.4 and 20° k_3 is 115 sec^{-1} , and the overall turnover of *p*-nitrophenyl phosphate by the enzyme (k_0) is approx. 30 sec^{-1} . Using the kinetic scheme suggested for such a mechanism by Gutfreund & Sturtevant (1956) one can calculate k_2 as 41 sec^{-1} from $k_2 = k_0 k_3 / (k_3 - k_0)$. The precise values for the rate constants will need revision when more accurate information is obtained about the turnover per mole of pure enzyme under the conditions of pH, temperature and buffer composition of the present experiment. It should be noted that the first-order constant k_3 may be even larger in the absence of orthophosphate, particularly so at pH 7.3. The qualitative picture shows therefore that the rate constant for the dephosphorylation of phosphoryl-phosphatase is consistent with the proposed mechanism for this enzyme, which involves its transient phosphorylation.

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Fig. 1. First-order plot of the dephosphorylation of phosphoryl-phosphatase at pH 7.3 (●) and 8.4 (○). Conditions were as described in the text.

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