every 7 glucose residues; for glucans B and P, 1 mol. of formic acid was liberated from 10 glucose residues (Bell & Northcote, 1950; Peat *et al.* 1958*a*). The present result of 14% of triol groups is in good agreement with the combined yield of non-reducing end groups (6·4%) and (1→6)-linked glucose residues (7·5%) by methylation analysis.

Additional information on the arrangement of the two types of linkage was obtained by enzymic degradation studies. Incubation of glucan A with a bacterial laminarinase preparation kindly supplied by Glaxo Research Ltd. (Greenford, Middlesex) gave glucose, laminaribiose and laminaritriose, together with about 10% of an enzymically resistant and water-soluble glucan (limit dextrin); gentiobiose was not released. The enzyme preparation did not hydrolyse gentiobiose or the linear β-(1→6)-glucan, pustulan (see Lindberg & McPherson, 1954). The limit dextrin consisted largely of β-(1→6)-linked glucose residues since: (*a*) a partial acid hydrolysate was similar to that from pustulan, and contained a mixture of sugars with the R_{Glc} values of glucose, gentiobiose, gentiotriose and gentiotetraose; (*b*) by methylation analysis, the major tri-*O*-methyl-D-glucose formed was the 2,3,4-isomer; (*c*) the limit dextrin was slowly hydrolysed by a β-(1→6)-glucanase preparation from *Penicillium brefeldianum* QM1872, kindly supplied by Dr E. T. Reese and Dr M. Mandels.

It is therefore concluded that yeast glucan contains main chains of β-(1→6)-linked D-glucose residues to which are attached linear side chains of β-(1→3)-linked D-glucose residues. It seems probable that different samples of glucan differ in the degree of substitution of the main chain, and in the lengths of the side chains. For example, in a sample of glucan examined by Misaki & Smith (1963), almost all the β-(1→6)-linked D-glucose residues carry side chains that contain, on the average, 8 glucose residues. In sample A about half of the glucose residues in the main chains carry side chains, the average length of which is 15 glucose residues, whereas in sample B the side chains contain about 8 glucose residues (cf. Bell & Northcote, 1950).

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Aspinall, G. O. (1963). *J. chem. Soc.* p. 1676.
Bell, D. J. & Northcote, D. H. (1950). *J. chem. Soc.* p. 1944.
Kuhn, R., Trischmann, H. & Low, I. (1955). *Angew. Chem.* **67**, 32.
Lindberg, B. & McPherson, J. (1954). *Acta chem. scand.* **8**, 985.
Misaki, A. & Smith, F. (1963). *Abstr. Amer. chem. Soc.*, 144*th Meet.*, p. 14c.
Northcote, D. H. (1963). *Pure appl. Chem.* **7**, 669.
Peat, S., Turvey, J. R. & Evans, J. M. (1958*b*). *J. chem. Soc.* p. 3868.
Peat, S., Whelan, W. J. & Edwards, T. E. (1958*a*). *J. chem. Soc.* p. 3862.
Warsi, S. A. & Whelan, W. J. (1957). *Chem. & Ind.* p. 1573.

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Studies on Methylazoxymethanol, the Aglycone of Cycasin: Methylation of Nucleic Acids *in vitro*

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Cycasin, the azoxyglucoside found in *Cycas circinalis* and *C. revoluta* (Nishida, Kobayashi & Nagahama, 1955; Riggs, 1956), is carcinogenic when fed to rats (Laqueur, Mickelsen, Whiting & Kurland, 1963). The active component is the aglycone, MAM* [$CH_3 \cdot N(O) : N \cdot CH_2 \cdot OH$], and not cycasin itself (Kobayashi & Matsumoto, 1965).

* Abbreviation: MAM, methylazoxymethanol.

There is a striking similarity in the lesions of rats given cycasin and those given dimethylnitrosamine (Laqueur, 1964). Dimethylnitrosamine affects primarily the endoplasmic reticulum of the liver cell (Emmelot & Benedetti, 1960). Liver cells of rats given cycasin show histological evidence of breakdown in the endoplasmic reticular structure, with accompanying decrease in liver RNA content (Williams, 1964). Liver nucleic acids are methyl-

ated *in vivo* by [^{14}C]dimethylnitrosamine (Magee & Farber, 1962).

Dimethylnitrosamine may be converted metabolically into the alkylating agent diazomethane (Magee & Hultin, 1962; Schoental, 1960), a carcinogen. MAM is isomeric with hydroxydimethylnitrosamine and thus it may also be transformed into diazomethane (Miller, 1964). Kriek & Emmelot (1963) used systems of aqueous solutions of microsomal or soluble RNA treated with diazomethane in ether, with vigorous stirring, to demonstrate methylation and breakdown of ribonucleic acids. The present communication reports on the methylation of nucleic acids and other compounds by MAM in a solution buffered at pH 7 at 37°.

MAM was prepared by the method of Kobayashi & Matsumoto (1965). RNA was obtained by the phenol extraction procedure of Kirby (1956) from the livers of six albino rats. The ultraviolet spectrum of the purified RNA showed a single peak at 258mμ. DNA was a commercial product obtained from calf thymus.

RNA (10mg.) was placed in a small test tube (12mm. × 125mm.) and mixed with 1m-mole (90mg.) of MAM in 1ml. of 0·2M-phosphate buffer, pH 7·0, and the mixture was kept at 37° for 16hr. RNA was then precipitated with 2 vol. of ethanol. The precipitate was sedimented in a centrifuge at 2000 rev./min. (480*g*), washed twice with ethanol–water (3:1, v/v) and dried in a vacuum desiccator over P_2O_5. The dried material was hydrolysed with 0·25ml. of N-HCl at 100° for 4hr. in a sealed tube (10mm. × 105mm.). An equal quantity of RNA was incubated without MAM and hydrolysed in the same manner.

DNA was treated in a similar manner with some modifications. The MAM-treated mixture was evaporated to dryness on a steam bath with the aid of a stream of air. The residue was hydrolysed with 0·5ml. of formic acid by the method of Wyatt (1950) at 175° for 1·5hr.

Samples (0·25ml.) of the hydrolysate were spotted on Whatman no. 1 paper (22cm. × 48cm.) and chromatographed by the descending method either in one or two dimensions. Three solvent mixtures, 2N-HCl in aq. 65% (v/v) propan-2-ol (Wyatt, 1951), butan-1-ol–0·6N-NH_3 (6:1, v/v) (Chargaff, Lipshitz, Green & Hodes, 1951) and butan-1-ol–formic acid–water (77:11:12, by vol.) (Markham & Smith, 1949), were used for the one-dimensional system and the last two solvent mixtures were used for the two-dimensional system (Weissmann, Bromberg & Gutman, 1957). The chromatograms were dried and the location of the bases was detected with ultraviolet radiation and outlined with pencil. The spots were cut out and the compounds were extracted by immersing the pieces of filter paper in 3ml. of 0·1N-HCl or 0·1N-NaOH for 20min. Blanks of equal sizes were cut out from adjacent spots where no compound was present. Ultraviolet spectra of the eluates were obtained with a Beckman DK-2 recording spectrophotometer.

A single methylated derivative, identified as 7-methylguanine, was found in the hydrolysates of both RNA and DNA treated with MAM. The derivative and some of the nucleic acid components were identified by comparing R_F values, and ultraviolet-absorption maxima reported by Reiner & Zamenhof (1957) and Brookes & Lawley (1961). Ultraviolet-absorption maxima and R_F values obtained for the derivative, authentic 7-methylguanine and guanine are shown in Table 1.

The ease with which MAM methylated nucleic acids indicated that it may be able to methylate other classes of compounds. The methylating action of MAM on phenol and *p*-chlorobenzoic acid was tested. Phenol (1m-mole) was dissolved in 2ml. of water, neutralized with 0·1N-NaOH and buffered with 2ml. of 0·2M-phosphate buffer. MAM (1m-mole) was added and the mixture was kept overnight at 37°, then extracted with 10ml. of diethyl ether. The ether was evaporated under reduced pressure and the residue was dissolved in 1ml. of chloroform. A portion of the chloroform solution (1μl.) was injected into an Aerograph Hi-Fi 600-D gas–liquid chromatograph with a hydrogen flame detector. The column was packed with 15% diethylene glycol succinate on Chromosorb W treated with hexamethyldisilazane. The flow of H_2 and the carrier gas, N_2, was maintained at 25ml./min. The column temperature was 103° and the injector temperature was 153°. A chromatogram with a single peak was obtained. Authentic anisole (1m-mole) was dissolved in 1ml. of chloroform and chromatographed in the same manner. The retention times of anisole and the derivative prepared by the treatment of phenol with MAM were identical. The yield of anisole produced with MAM was 5%. Riggs (1965) has demonstrated that, with a more vigorous reaction, anisole is produced in 40–50% yield when a solution of cycasin in molten phenol is treated with a drop of conc. H_2SO_4 and warmed until evolution of gas ceased.

p-Chlorobenzoic acid (1m-mole) was dissolved in 1ml. of 1·0N-NaOH and buffered with 0·2M-phosphate buffer. MAM (1m-mole) was added to the mixture, which was then kept at 37° for 16hr. A strong odour of an ester was detected at the end of the incubation period. The mixture was extracted once with 5ml. of chloroform. The chloroform was dried over Na_2SO_4 and the volume was reduced by evaporating the solvent under reduced pressure. The chloroform extract was chromatographed in the same manner as for anisole and a chromatogram with a single peak was obtained.

Table 1. *Ultraviolet-absorption maxima and R_F values of derivative from MAM-treated nucleic acids, 7-methylguanine and guanine*

Solvent 1, butan-1-ol–NH_3; solvent 2, butan-1-ol–formic acid; solvent 3, propan-2-ol–HCl.

	$\lambda_{max.}$ (mμ)			R_F	
Substance	In 0·1 N-HCl	In 0·1 N-NaOH	In solvent 1	In solvent 2	In solvent 3
Derivative from RNA	249	280	0·19	0·21	0·37
7-Methylguanine	249	280	0·18	0·20	0·38
Guanine	248	274	0·13	0·14	0·26
Derivative from DNA	249	280	0·19	0·21	
7-Methylguanine	249	280	0·19	0·21	
Guanine	248	273	0·15	0·16	

The chromatogram was compared with methyl *p*-chlorobenzoate prepared by the methanol–HCl esterification procedure of Stoffel, Chu & Ahrens (1959). The retention times of the products obtained by the esterification of *p*-chlorobenzoic acid with MAM and with methanol were identical. The yield of the ester prepared with MAM was 30%.

MAM is a good alkylating agent in spite of its instability in aqueous solution (Kobayashi & Matsumoto, 1965). The formation of 7-methylguanine is in accord with the action of diazomethane (Kriek & Emmelot, 1963) and dimethyl sulphate (Reiner & Zamenhof, 1957) on nucleic acids. The 7-position of guanine is the most readily alkylated in nucleic acids (Brookes & Lawley, 1961). The mild conditions of pH and temperature under which MAM is able to methylate suggests that diazomethane could be an intermediate in the reaction. The ease with which MAM methylates phenate and carboxylate groups indicates that methylated derivatives of cellular constituents other than purine bases can be expected in tissues treated with MAM *in vivo*.

Teas, Sax & Sax (1965) have demonstrated the radiomimetic effect of cycasin as measured by the frequency of induction of chromosome aberrations in root-tip cells of *Allium cepa*. Presumably, cycasin was hydrolysed by β-glucosidase in the plant and the active constituent was MAM. D. W. E. Smith (personal communication) has found that several histidine-requiring *Salmonella typhimurium* strains reverted to the wild type when treated with MAM–acetate. Thus it is probable that chemical action of MAM *in vivo* results in methylation of nucleic acid bases and induces chromosome aberrations, mutagenesis and carcinogenesis.

Alkylating agents in general are not considered powerful carcinogens (Brookes & Lawley, 1964), with the probable exception of dialkylnitrosamines and MAM. The use of dialkylnitrosamines in the study of carcinogenesis is limited to experiments *in vivo* as it requires enzymic conversion into the active principle. MAM can be used in isolated systems under mild conditions and shows promise as a compound useful in the study of carcinogenesis.

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Brookes, P. & Lawley, P. D. (1961). *J. chem. Soc.* p. 3923.
Brookes, P. & Lawley, P. D. (1964). *J. cell. comp. Physiol.* **64** (Suppl. 1), 111.
Chargaff, E., Lipshitz, R., Green, C. & Hodes, M. E. (1951). *J. biol. Chem.* **192**, 223.
Emmelot, P. & Benedetti, E. L. (1960). *J. biophys. biochem. Cytol.* **7**, 393.
Kirby, K. S. (1956). *Biochem. J.* **64**, 405.
Kobayashi, A. & Matsumoto, H. (1965). *Arch. Biochem. Biophys.* **110**, 373.
Kriek, E. & Emmelot, P. (1963). *Biochemistry*, **2**, 733.
Laqueur, G. L. (1964). *Fed. Proc.* **23**, 1386.
Laqueur, G. L., Mickelsen, O., Whiting, M. G. & Kurland, L. T. (1963). *J. nat. Cancer Inst.* **31**, 919.
Magee, P. N. & Farber, E. (1962). *Biochem. J.* **83**, 114.
Magee, P. N. & Hultin, T. (1962). *Biochem. J.* **83**, 106.
Markham, R. & Smith, J. D. (1949). *Biochem. J.* **45**, 294.
Miller, J. A. (1964). *Fed. Proc.* **23**, 1361.
Nishida, K., Kobayashi, A. & Nagahama, T. (1955). *Bull. agric. chem. Soc. Japan*, **19**, 77.
Reiner, B. & Zamenhof, S. (1957). *J. biol. Chem.* **228**, 475.
Riggs, N. V. (1956). *Chem. & Ind.* **35**, 926.
Riggs, N. V. (1965). *Nature, Lond.*, **207**, 632.
Schoental, R. (1960). *Nature, Lond.*, **188**, 420.
Stoffel, W., Chu, F. & Ahrens, E. A. (1959). *Analyt. Chem.* **31**, 307.
Teas, H. J., Sax, H. J. & Sax, K. (1965). *Science*, **149**, 541.
Weissmann, B., Bromberg, P. A. & Gutman, A. B. (1957). *J. biol. Chem.* **224**, 407.
Williams, J. N. (1964). *Fed. Proc.* **23**, 1374.
Wyatt, G. R. (1950). *Nature, Lond.*, **166**, 237.
Wyatt, G. R. (1951). *Biochem. J.* **48**, 584.