Biochem. J. (1969) **111**, 615
Printed in Great Britain

Study of the Methylation and Lack of Deamination of Deoxyribonucleic Acid by *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine

By VALDA M. CRADDOCK
Toxicology Research Unit, Medical Research Council Laboratories, Carshalton, Surrey

(*Received* 25 *October* 1968)

1. DNA labelled with ^{14}C in the purine residues was prepared by treating newborn rats with [^{14}C]formate and killing them for preparation of nucleic acids at 11–17 months. This DNA was incubated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, and then analysed for products of methylation and deamination reactions. 2. Evidence was found for the formation of 7-methylguanine and a smaller amount of 3-methyladenine, and, after preliminary denaturation of the DNA, 1-methyladenine was detected. The presence of cysteine increased the extent of methylation. No evidence was found for the formation of xanthine or hypoxanthine, even at pH 5·5.

For about 20 years MNNG* was used as a reagent for diazomethane (McKay, 1948) until it was shown to be a potent locally acting carcinogen (Schoental, 1966; Sugimura, Nagao & Okada, 1966; Druckrey *et al.* 1966). When given to rats by stomach tube it causes not only stomach tumours but also the formation of multiple large cysts in the liver (Craddock, 1968c). It also has anticarcinogenic activity (Skinner, Gram, Greene, Greenberg & Baker, 1960), and is a mutagen for bacteria (Mandell & Greenberg, 1960) and for plants (Müller & Gichner, 1964). It produces chromosome aberrations in plant cells (Gichner, Michaelis & Rieger, 1963) at a concentration as low as 0·01 mM (Sax & Sax, 1966). To study the chemical mechanisms involved in carcinogenesis, in particular to determine whether cancer might originate in a mutation-like process, it was of interest to study which reactions of MNNG might be responsible for the mutations produced over the wide pH range used, and which might be relevant in carcinogenesis. An understanding of the mechanism of action of MNNG is of obvious practical importance in determining under what conditions it will produce the different related effects, i.e. when it will be carcinogenic and when carcinostatic.

Under alkaline conditions MNNG breaks down to form diazomethane, which would methylate nucleic acids, and under acid conditions it gives rise to nitrous acid, which would cause deamination. Although both alkylation and deamination of DNA are known to cause mutation, there is no agreement on the part played by these reactions in the less extreme conditions under which MNNG causes mutagenesis. After study of the effect of pH on mutagenicity in yeast, it was considered possible that under acid conditions (pH 2) the effect is due to nitrous acid, whereas above pH 6 it is due to diazomethane (Zimmerman, Schwaier & Laer, 1965). However, MNNG is a mutagen at pH 5, where it has maximum stability, with minimum formation of nitrous acid or of diazomethane, and so it was suggested that mutagenesis is due to MNNG itself rather than to a breakdown product and that, in the case of prophage induction, the formation of diazomethane and of nitrous acid was 'ruled out by the conditions of the experiment' (Malke, 1967).

Experiments were therefore carried out to determine whether in fact MNNG methylated or deaminated DNA at different H^+ concentrations. The fact that a direct reaction between MNNG and DNA can take place is shown by its inactivation of transforming DNA *in vitro* (Terawaki & Greenberg, 1965). Under conditions such as those that occur in the intact animal during carcinogenesis caused by a single administration of MNNG, i.e. by using MNNG in solution, and a short incubation time, the extent of reaction of MNNG with DNA is small. Although MNNG labelled in the methyl group can be used to study the methylation reactions, to measure low levels of deamination it is necessary that the DNA itself should be radioactive. In the experiments described below, the DNA used was prepared from rats treated as neonatal animals with [^{14}C]formate, and was therefore labelled in the purine residues and in the methyl group of thymine. Previous evidence has been given for the methylation of DNA by MNNG (Craddock, 1968*a*; McCalla, 1968; Lawley, 1968). There is also evidence that other reaction products are formed with DNA

* Abbreviation: MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

(Craddock, 1968*a*; McCalla, 1968) and with RNA (Singer & Fraenkel-Conrat, 1967).

A preliminary account of this work has been presented (Craddock, 1968*b*). Evidence was obtained for methylation of DNA, but not for deamination, even at pH 5·5.

METHODS AND MATERIALS

Preparation of ^{14}C-labelled DNA. Newborn female rats were given a series of injections of [^{14}C]formate in 0·9% NaCl (100μc, 0·63mg./ml.). The neonatal animals were injected at first daily and then twice daily during weekdays for 23 days, the route being at first subcutaneous and later intraperitoneal. The volume injected was increased from 0·05ml. to 0·2ml. during this period, each animal receiving a total of 205μc (1·29mg.). The rats were weaned at 30 days, and were maintained on normal M.R.C. diet 41B (Bruce & Parkes, 1956) until they were killed, two animals at 11 months and one at 17 months. It has been found that, after this treatment, most of the radioactivity remaining in the animal is in the DNA of various tissues and in brain lipid (Craddock & Magee, 1967*a*). DNA was prepared from liver and small intestine by a method of Kirby (1962), modified as described by Craddock, Villa-Treviño & Magee (1968).

Incubation procedure. ^{14}C-labelled DNA (10mg.) was dissolved in 0·02M-sodium phosphate buffer, pH 7·5 (10ml.). MNNG (30mg.) was dissolved in the same buffer (10ml.) and immediately added to the DNA solution. The mixture was incubated in the dark, with shaking, at 38° for 30min. The solution was then cooled, the pH measured and the DNA precipitated by addition of 20ml. of 1% Cetab (cetyltrimethylammonium bromide). It was washed successively in water, 2% (w/v) sodium acetate in 70% (v/v) ethanol overnight in the cold, ethanol, ethanol–ether and ether. The dried product was weighed.

When the incubation was carried out in the presence of cysteine, a solution of cysteine (0·1g./5ml. of appropriate buffer; 1ml.) was added to the DNA solution immediately before addition of MNNG.

When heat-denatured DNA was used, the solution, prepared as described above, was placed in a boiling-water bath for 10min. and then cooled rapidly. Preliminary experiments recording 'melting' and cooling curves in 0·02M-sodium phosphate buffer, pH 7·5, showed that little re-annealing occurred even during slow cooling.

Analysis of DNA. The dried sample of DNA from the incubation procedure was hydrolysed with 1N-HCl at 100° for 1hr. 7-Methylguanine and in some cases xanthine and hypoxanthine were added to act as markers, and the hydrolysate was analysed on a column (1cm. × 10cm.) of Dowex 50 (H^+ form) with 1N-HCl as eluent for the first 300ml. and then a 1–4N-HCl linear gradient, the rate of flow being 12·5ml./hr. The E_{260} was determined on each fraction of the effluent, 8ml. of each fraction was used for determination of radioactivity as described by Craddock & Magee (1967*b*) and the remaining 2ml. was used for paper chromatography. The solvent systems used were propan-2-ol–conc. HCl–water (34:9:7, by vol.), methanol–conc. HCl–water (7:2:1, by vol.) and butan-1-ol–aq. NH_3 (sp.gr. 0·88)–water (85:2:12, by vol.) For separation of adenine from 1-methyladenine the solvent was 3-methylbutan-1-ol–5% (w/v) Na_2HPO_4 (1:1, v/v).

RESULTS AND DISCUSSION

Preliminary experiments with normal DNA from salmon testis or rat liver gave no evidence for any reaction with MNNG under the incubation conditions employed. 'Melting' curves of rat liver DNA treated with MNNG were not significantly different from those of untreated DNA and gave no evidence that cross-linking had occurred. The results of an experiment with ^{14}C-labelled DNA are shown in Fig. 1. Most of the radioactivity occurs, as with untreated DNA, in guanine, adenine and presumably the methyl group of thymine. A peak of radioactivity follows the 7-methylguanine carrier, and there is a shoulder where 3-methyladenine is known to be eluted from the column. Paper chromatography with the propan-2-ol–hydrochloric acid and methanol–hydrochloric acid

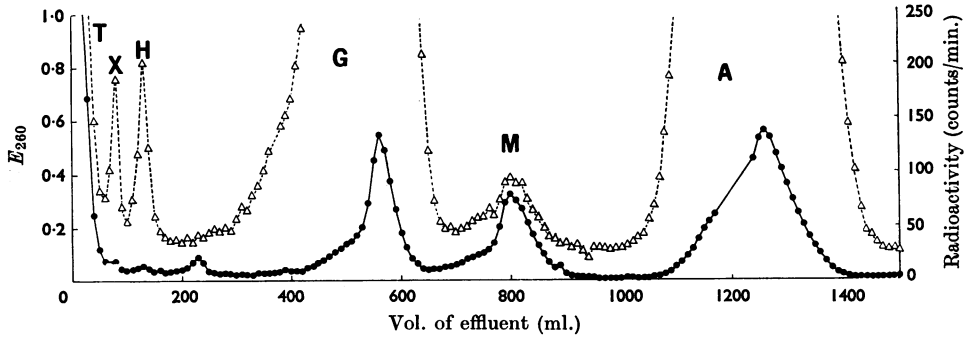

Fig. 1. Chromatographic analysis, on a column (1cm. × 10cm.) of Dowex 50 (H^+ form), with a 1–4N-HCl linear gradient, of an acid hydrolysate of ^{14}C-labelled DNA treated with MNNG for 30min. at 38° at pH 7·5. Carrier 7-methylguanine was added. ●, E_{260}; △, radioactivity. T, Pyrimidine deoxyribonucleotides; X, xanthine; H, hypoxanthine; G, guanine; A, adenine; M, 7-methylguanine carrier.

Table 1. *Extent of methylation and deamination of* ^{14}C*-labelled DNA by treatment with MNNG, calculated from total radioactivity under the relevant peaks after column chromatography*

Conditions are described in the text.

	Percentage conversion			
	Methylation		Deamination	
Incubation conditions	Guanine → 7-methylguanine	Adenine → 3-methyladenine	Guanine → xanthine	Adenine → hypoxanthine
Control, untreated DNA	—	—	0·8	1·0
pH7·5	1·8	0·5	1·0	1·0
pH7·5, cysteine present	5·6	1·1	0·9	1·0
pH7·5, cysteine present, DNA denatured	3·2	0·2	0·8	1·0
pH5·5, cysteine present	3·1	1·0	1·0	1·2
pH7·5, solid MNNG added*	3·0	0·9	0·9	0·7

* Results of experiment described by Craddock (1968*a*).

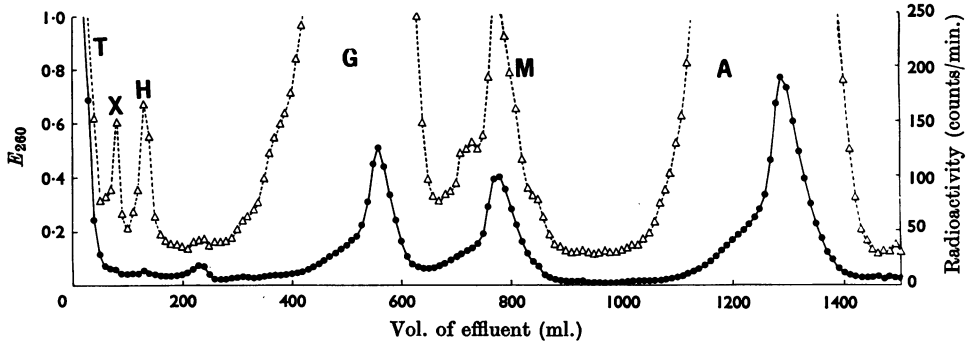

Fig. 2. Chromatographic analysis of an acid hydrolysate of ^{14}C-labelled DNA treated with MNNG for 30 min. at 38° at pH7·5 in the presence of 10 mM-cysteine. Method and key are as given in Fig. 1.

solvents gave further evidence that the radioactivity is in fact in 7-methylguanine. In butan-1-ol–ammonia some of the radioactivity of the 7-methylguanine peak moved more slowly than did 7-methylguanine. This minor product has not been characterized. From the total radioactivity under the relevant peaks, the percentage conversion of guanine into 7-methylguanine and of adenine into 3-methyladenine could be calculated (Table 1). The results give only approximate values, in view of the incomplete separation of 7-methylguanine from 3-methyladenine and the presence of a minor unknown component. As no 7-methylguanine has been detected in normal rat liver DNA (Craddock *et al.* 1968), the results represent methylation by MNNG.

Relatively large peaks of radioactivity occurred in the regions of xanthine and hypoxanthine. As the peaks were well-defined the extent of formation of xanthine from guanine and of hypoxanthine from adenine could be calculated. Unlike the situation with regard to methylation, some deamination occurs as an artifact during hydrolysis with mineral acid. However, by comparing the results (Table 1) with the control values it seems clear that, if incubation with MNNG does increase the amount of deamination, the extent of the reaction is very small. The results of an experiment in which DNA was treated with solid MNNG are also shown; in this case the conversion of adenine into hypoxanthine was slightly less than in the control experiment. In another experiment, in which DNA was treated with MNNG and hydrolysed with perchloric acid to minimize deamination occurring as an artifact (Wyatt, 1952), the radioactivity in the xanthine region of the column effluents was not above background, and the amount of hypoxanthine formed (0·75% deamination of adenine) was similar to that in control experiments with perchloric acid. Generally hydrochloric acid hydrolysis was used, to lessen the chance of destruction of other possible reaction products.

A carcinogenic alkyl nitroso compound related to MNNG, methylnitrosourethane, is known to react with thiols (Schoental & Rive, 1965), and the presence of cysteine increases the extent of methylation of DNA by this compound (Schoental, 1967). Thiols are known to react with MNNG at neutral pH with evolution of nitrogen (Schoental, 1966). On the other hand, it was found that the inhibition of growth of *Escherichia coli* caused by MNNG is prevented by a variety of amino acids and thiols, and it was postulated that reaction of MNNG with the thiol group might protect the cell constituents from alkylation (Greenberg & Morris, 1961). The effect of 10mM-cysteine on the reaction of MNNG with DNA is shown in Fig. 2. It is seen (Table 1) that the extent of methylation is considerably increased, whereas the amount of deamination is similar to that occurring in the previous experiment and in untreated control samples of DNA. The mechanism of the effect of cysteine has not been elucidated, but it is possible that cysteine reacts with MNNG to form diazomethane, which then reacts with DNA or with another molecule of cysteine to form *S*-methylcysteine. The effect of cysteine on the stability of MNNG was studied by means of the absorption spectrum of the nitroso compound. MNNG at pH 7·5 has $\lambda_{max.}$ 279mμ, due presumably to the guanidino part of the molecule, and a peak at 400mμ due to the nitroso group. During decomposition at 39° at pH 7·5 the peak at 400mμ decreases, whereas that at 279mμ shifts to 264mμ. When cysteine is added the peak at 400mμ disappears rapidly, within a few minutes. At pH 5·5 cysteine causes a less marked increase in the rate of disappearance of the peak at 400mμ, and at pH 3 cysteine has no detectable effect.

The secondary structure of DNA is known to influence its reactions with alkylating agents (Lawley & Brookes, 1963). When DNA is denatured the 1-position of adenine becomes alkylated, whereas in native DNA reaction at this site is diminished by hydrogen-bonding. Thus, although MNNG reacts with free nucleotides more extensively in the case of adenine to form 1-methyladenine (Rau & Lingens, 1967), this cannot be taken as evidence that a similar reaction would occur in DNA. The idea that the conformation of DNA is important in its reaction with MNNG in biological systems is supported by the evidence that MNNG causes mutation of the replicating point more than of other regions of the chromosome (Cerda-Olmedo, Hanawalt & Guerola, 1968). The effect of denaturation of DNA on the reaction with MNNG in the presence of cysteine is shown in Fig. 3. The extent of formation of 7-methylguanine and 3-methyladenine is decreased, whereas the extent of deamination, presumably occurring as an artifact, is unaltered (Table 1). The presence of 1-methyladenine would be obscured on column chromatography by the large peak of adenine. Fractions from the first part of the adenine peak, i.e. the region where 1-methyladenine is known to occur if it is present, were pooled and examined by paper chromatography in 3-methylbutan-1-ol–sodium phosphate, in which 1-methyladenine moves some way ahead of adenine. A small fraction of the radioactivity, representing 0·6% of the adenine, was found in the position of 1-methyladenine.

When MNNG is used a mutagen, bacteria are often treated at pH 5·5, when deamination of nucleic acids rather than methylation might be expected to occur. DNA was incubated with MNNG at pH 5·5, and xanthine and hypoxanthine were added to the hydrolysate before column chromatography. It was found that 7-methylguanine and 3-methyladenine had been formed (Fig. 4), but paper chromatography of the appropriate fractions from the adenine peak showed that no 1-methyladenine was detectable. The amount of deamination was not greater than that found at pH 7·5.

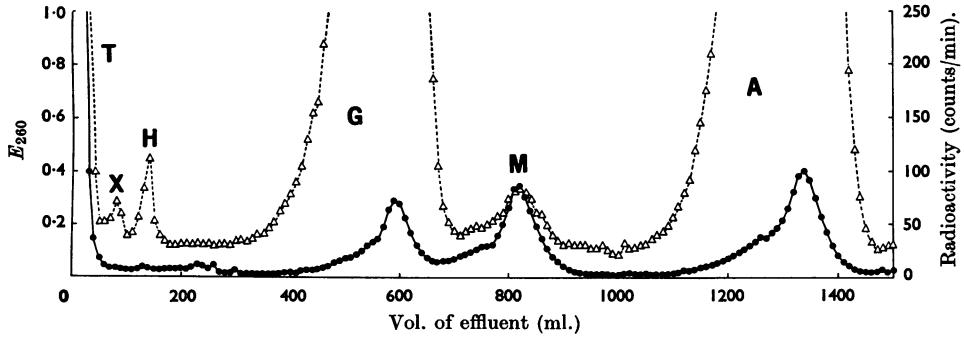

Fig. 3. Chromatographic analysis of an acid hydrolysate of heat-denatured ^{14}C-labelled DNA treated with MNNG for 30min. at 38° at pH 7·5 in the presence of 10mM-cysteine. Method and key are as given in Fig. 1.

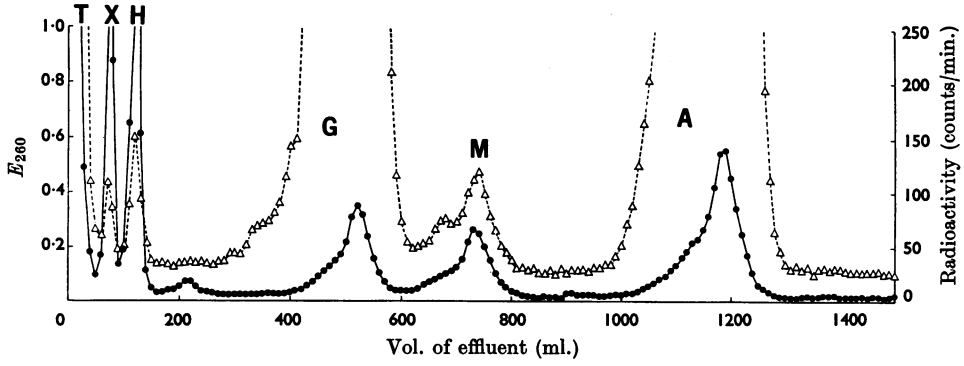

Fig. 4. Chromatographic analysis of an acid hydrolysate of ^{14}C-labelled DNA treated with MNNG for 30min. at 38° at pH 5·5 in the presence of 10mM-cysteine. Carrier xanthine, hypoxanthine and 7-methylguanine were added. Method and key are as given in Fig. 1.

Thus MNNG reacts with DNA to form 7-methylguanine and to a much smaller extent 3-methyladenine, and under certain conditions a small amount of 1-methyladenine is formed, but very little if any deamination was detectable even at pH 5·5. There is some evidence for the formation of other minor reaction products. The peak of 7-methylguanine was found by paper chromatography in butan-1-ol–ammonia to contain a radioactive product moving more slowly than 7-methylguanine. Also, the paper chromatography in 3-methylbutan-1-ol–sodium phosphate that showed the formation of 1-methyladenine revealed the presence of a minor slow-moving radioactive product. This was not N^6-methyladenine, which is known to be formed from 1-methyladenine, even at pH 5·6 (Lawley & Brookes, 1963), nor was it 7-methyladenine. These minor products have not been characterized. The occurrence of methylation from pH 5·5 to pH 7 is consistent with genetic evidence (Cerda-Olmedo & Hanawalt, 1968) that, above pH 5, the lethality and mutation produced by MNNG are both due to the action of diazomethane on chromosomes, the lethal reaction being susceptible to repair, but not the mutagenic reaction.

In mammalian systems it may be that the variety of effects caused by MNNG is due to methylation reactions, the carcinogenic event being related to that causing mutation in bacteria, the carcinostatic event related to the lethal reaction in bacteria. Methylation of RNA may cause the inhibition of protein synthesis. In cell-free systems there is evidence that methylation of polynucleotides is responsible for the inhibition of protein synthesis (Lingens, Sussmuth, Wacker & Chandra, 1967; Chandra, Wacker, Sussmuth & Lingens, 1967). In the intact animal, additional reactions of MNNG with cell constituents must take place. Unlike the situation with bacteria, animal cells contain histones, which might be relevant to the action of MNNG; it has in fact been shown (Skinner *et al.* 1960) that MNNG reacts with lysine to form probably N^{ϵ}-nitroamidinolysine. The situation is further complicated by the possibility of metabolism of MNNG, by the effect of the lipophilic compound on membrane systems and by the possibility of repair of one or more of the alterations in DNA. The reactions of MNNG in intact cells are therefore being studied. Preliminary experiments were carried out with intact rats in which the stomach nucleic acids were labelled by giving control animals [^{3}H]adenine or [^{3}H]formate and test animals [^{14}C]adenine or [^{14}C]formate. The ^{14}C-labelled rats were given MNNG by stomach tube, and 5hr. later the test and control animals were killed, the stomachs pooled and the nucleic acids isolated and analysed in a single sample, with simultaneous counting of ^{14}C and ^{3}H. The ^{3}H-labelled nucleic acids thus acted as an internal control for deamination occurring as an artifact and for the amount of naturally occurring 7-methylguanine in stomach RNA. In each experiment there was evidence for a higher content of 7-methylguanine in RNA from treated animals than in that from untreated animals.

The author thanks Mr R. Hunt for skilled technical assistance, Dr P. D. Lawley for samples of 3-methyladenine and 1-methyladenine and for discussions, and Dr G. Hitchings for a sample of N^6-methyladenine.

REFERENCES

Bruce, H. M. & Parkes, A. S. (1956). *J. Anim. Tech. Ass.* **7**, 54.

Cerda-Olmedo, E. & Hanawalt, P. C. (1968). *Molec. gen. Genet.* **101**, 191.

Cerda-Olmedo, E., Hanawalt, P. C. & Guerola, N. (1968). *J. molec. Biol.* **33**, 705.
Chandra, P., Wacker, A., Sussmuth, R. & Lingens, F. (1967). *Z. Naturf.* **22b**, 512.
Craddock, V. M. (1968*a*). *Biochem. J.* **106**, 921.
Craddock, V. M. (1968*b*). *Abstr. 5th Meet. Fed. Europ. biochem. Soc., Prague*, no. 305.
Craddock, V. M. (1968*c*). *Experientia*, **24**, 1148.
Craddock, V. M. & Magee, P. N. (1967*a*). *Biochim. biophys. Acta*, **134**, 182.
Craddock, V. M. & Magee, P. N. (1967*b*). *Biochem. J.* **104**, 435.
Craddock, V. M., Villa-Treviño, S. & Magee, P. N. (1968). *Biochem. J.* **107**, 179.
Druckrey, H., Preussmann, R., Ivankovic, S., So, B. T., Schmidt, C. H. & Bücheler, J. (1966). *Z. Krebsforsch.* **68**, 87.
Gichner, T., Michaelis, A. & Rieger, R. (1963). *Biochem. biophys. Res. Commun.* **11**, 120.
Greenberg, J. & Morris, J. (1961). *Antibiot. Chemother.* **11**, 52.
Kirby, K. S. (1962). *Biochim. biophys. Acta*, **55**, 545.
Lawley, P. D. (1968). *Nature, Lond.*, **218**, 580.
Lawley, P. D. & Brookes, P. (1963). *Biochem. J.* **89**, 127.
Lingens, F., Sussmuth, R., Wacker, A. & Chandra, P. (1967). *Naturwissenschaften*, **54**, 492.
McCalla, D. R. (1968). *Biochim. biophys. Acta*, **155**, 114.
McKay, A. F. (1948). *J. Amer. chem. Soc.* **70**, 1974.
Malke, H. (1967). *Nature, Lond.*, **214**, 811.
Mandell, J. D. & Greenberg, J. (1960). *Biochem. biophys. Res. Commun.* **3**, 575.
Müller, A. J. & Gichner, T. (1964). *Nature, Lond.*, **201**, 1149.
Rau, J. & Lingens, F. (1967). *Naturwissenschaften*, **54**, 517.
Sax, K. & Sax, H. J. (1966). *Proc. nat. Acad. Sci., Wash.*, **55**, 1431.
Schoental, R. (1966). *Nature, Lond.*, **209**, 726.
Schoental, R. (1967). *Biochem. J.* **102**, 5c.
Schoental, R. & Rive, D. J. (1965). *Biochem. J.* **97**, 466.
Singer, B. & Fraenkel-Conrat, H. (1967). *Proc. nat. Acad. Sci., Wash.*, **58**, 234.
Skinner, W. A., Gram, H. F., Greene, M. O., Greenberg, J. & Baker, B. R. (1960). *J. med. pharm. Chem.* **2**, 299.
Sugimura, T., Nagao, M. & Okada, Y. (1966). *Nature, Lond.*, **210**, 962.
Terawaki, A. & Greenberg, J. (1965). *Biochim. biophys. Acta*, **95**, 170.
Wyatt, G. R. (1952). *J. gen. Physiol.* **36**, 201.
Zimmerman, F. K., Schwaier, R. & Laer, U. V. (1965). *Z. VererbLehr.* **97**, 68.