

## ADDITIVE EFFECT IN THE INDUCTION OF KIDNEY TUMOURS IN RATS TREATED WITH DIMETHYLNITROSAMINE AND ETHYLMETHANESULPHONATE\*

R. MONTESANO, U. MOHR, P. N. MAGEE, J. HILFRICH AND H. HAAS

*From the International Agency for Research on Cancer, Lyon, France; Section of Experimental Pathology, Medizinische Hochschule, Hannover, Federal Republic of Germany and Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London, England*

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**Summary.**—Wistar rats were treated with a single dose of 30 mg/kg of DMN or with single doses of 100, 200 or 300 mg/kg of EMS. Tumours of the kidney developed in a few animals receiving EMS and in 33% of the male and 63% of the female rats treated with DMN alone. In the animals receiving DMN and, 8 hours later, a single dose of 100, 200 or 300 mg/kg of EMS, an additive effect was observed in the induction of kidney tumours. This additive effect was more pronounced in female than in male rats. Morphologically, the tumours were of epithelial and mesenchymal type with a preponderance of the former type. The significance of alkylation of the nucleic acids of the kidney observed with these two compounds is discussed in relation to the present findings.

A SINGLE dose of dimethylnitrosamine (DMN), which is completely metabolized within 5–6 hours, is sufficient to produce a significant incidence of kidney tumours in the rat (Magee and Barnes, 1959). Ethylmethanesulphonate (EMS) administered in 3 large doses resulted in an increase of lung adenomata and induced epithelial cell tumours of the kidney in mice (Alexander and Connell, 1963) and tumours of the kidney in rats (Swann and Magee, 1969). Biochemical studies showed that administration of a single dose of DMN or EMS to rats results in the alkylation of some cellular constituents of the kidney, following metabolic activation in the case of DMN and spontaneous breaking down in the case of EMS (Swann and Magee, 1968, 1971).

Although the significance of alkylation of cellular macromolecules in initiating a carcinogenic process is not clear, it appears that it is important for the carcinogenic action of DMN and EMS. The induction

of kidney tumours after treatment with a single dose of these two alkylating agents is a promising model which allows a study of the correlation between biological response and biochemical interactions with cellular components. The following long-term studies were carried out in order to examine a possible additive effect in the induction of kidney tumours, which might be the result of the total level of alkylation of cellular components after combined treatment with DMN and EMS.

### MATERIALS AND METHODS

The materials used were dimethylnitrosamine (DMN), (obtained from Dr F. Krüger), ethylmethanesulphonate (EMS), (T. Schuchardt GmbH Co., Munich, Federal Republic of Germany), and 0.9% sodium chloride. Groups of Wistar rats of both sexes, 9–10 weeks old, from the colony of the Zentral Institut für Versuchstiersucht der Deutschen Forschungsgemeinschaft, Hannover, were

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employed. All animals were observed and weighed once weekly; they were killed 110 weeks after the treatment, by which time all the animals of Group 7 had died spontaneously. Except for a few animals lost through cannibalism, all were autopsied. All the organs, including the central nervous system, were examined macroscopically. Eight graded histological sections were made for each kidney and other pertinent tissues were processed for histological examination.

Groups 1, 2 and 3 received a single i.p. injection of 100, 200 and 300 mg/kg respec-

tively of EMS in 1.0 ml of 0.9% sodium chloride. The solution of EMS was freshly prepared before treatment. Group 4 received a single i.p. injection of 30 mg/kg of DMN in 1.0 ml of 0.9% sodium chloride. The other groups (Groups 5, 6 and 7) received a single i.p. injection of DMN as in Group 4 and 8 hours later an i.p. injection of 100, 200 or 300 mg/kg of EMS respectively.

A control group received a single i.p. injection of 1.0 ml of 0.9% sodium chloride. The number of rats in each group is given in Table I.

TABLE I.—*Tumour Induction in Rats following Treatment with DMN and/or EMS*

Groups	Initial no. of animals	Effective no. of animals <sup>a</sup>	Total tumour bearing animals	No. of animals with tumours of				
				Kidney	Nasal cavities	Mammary glands	Lung	Other organs
Control	20 ♂	20	4	—	—	—	—	4 <sup>b</sup>
	20 ♀	20	16	—	—	9	—	10 <sup>c</sup>
1	20 ♂	20	5	2	—	—	—	3 <sup>d</sup>
EMS 100 mg	20 ♀	18	12	1	—	9	—	7 <sup>e</sup>
2	20 ♂	20	8	—	—	1	—	7 <sup>f</sup>
EMS 200 mg	20 ♀	20	14	—	—	13	—	5 <sup>g</sup>
3	20 ♂	20	9	2	—	1	1	7 <sup>h</sup>
EMS 300 mg	20 ♀	20	13	—	—	8	—	9 <sup>i</sup>
4	19 ♂	15	10	5	3	1	2	5 <sup>j</sup>
DMN 30 mg	21 ♀	19	18	12	5	6	—	3 <sup>k</sup>
5	20 ♂	18	13	9	4	1	2	7 <sup>l</sup>
DMN + EMS-100	20 ♀	18	12	11	2	4	1	6 <sup>m</sup>
6	25 ♂	16	12	8	2	—	4	4 <sup>n</sup>
DMN + EMS-200	20 ♀	17	15	10	—	6	1	8 <sup>o</sup>
7	35 ♂	14	11	9	2	2	2	7 <sup>p</sup>
DMN + EMS-300	40 ♀	21	20	15	6	2	2	9 <sup>q</sup>

<sup>a</sup> Survivors at time of first tumour (11 weeks), less animals lost through cannibalism.

<sup>b</sup> 1 malignant lymphoma; 1 adrenal gland adenoma; 1 hepatoma; 1 tumour of the testis.

<sup>c</sup> 6 malignant lymphomata; 1 adrenal gland adenoma; 1 insuloma; 2 uterus carcinomata; 1 lipoma; 1 forestomach papilloma; 1 Zymbal gland squamous cell carcinoma.

<sup>d</sup> 1 malignant lymphoma; 1 adrenal gland adenoma; 1 fibrosarcoma of the heart; 1 intestinal adenocarcinoma.

<sup>e</sup> 2 malignant lymphomata; 2 uterus polyps; 1 squamous cell carcinoma of the vagina; 1 s.c. fibrosarcoma; 1 forestomach papilloma.

<sup>f</sup> 3 malignant lymphomata; 1 phaeochromocytoma; 1 lipoma; 1 hepatocellular carcinoma; 1 thyroid carcinoma; 1 squamous cell carcinoma of the palate.

<sup>g</sup> 1 malignant lymphoma; 1 adrenal gland adenoma; 1 fibrosarcoma of the heart; 1 hepatoma; 1 forestomach papilloma.

<sup>h</sup> 4 adrenal gland adenomata; 1 fibrosarcoma of the heart; 1 squamous cell carcinoma of the palate; 1 carcinoma of the sebaceous glands; 2 papillomata of the tongue; 1 thyroid carcinoma.

<sup>i</sup> 2 malignant lymphomata; 5 fibrosarcomata of the heart; 1 granulosa cell carcinoma; 1 polyp of the cervix; 1 luteoma; 1 bladder papilloma; 1 uterus haemangioma.

<sup>j</sup> 3 malignant lymphomata; 1 fibrosarcoma of the heart; 2 adrenal gland adenomata; 1 s.c. fibrosarcoma.

<sup>k</sup> 1 malignant lymphoma; 1 hepatoma; 1 intestinal haemangioma.

<sup>l</sup> 5 adrenal gland adenomata; 2 fibrosarcomata of the heart.

<sup>m</sup> 1 malignant lymphoma; 1 adrenal gland adenoma; 1 thyroid carcinoma; 1 adenocarcinoma of the ovary; 1 oesophageal papilloma; 1 insuloma.

<sup>n</sup> 2 adrenal gland adenomata; 1 fibrosarcoma of the heart; 1 thyroid carcinoma; 1 papilloma of the tongue.

<sup>o</sup> 4 malignant lymphomata; 3 adrenal gland adenomata; 1 squamous cell carcinoma of the palate; 1 intestinal sarcoma; 1 hepatoma; 1 osteosarcoma.

<sup>p</sup> 3 malignant lymphomata; 2 adrenal gland adenomata; 1 fibrosarcoma of the heart; 1 hepatocellular carcinoma; 1 hepatoma; 1 papilloma of the tongue.

<sup>q</sup> 5 malignant lymphomata; 2 adrenal gland adenomata; 1 fibrosarcoma of the heart; 1 hepatocellular carcinoma; 1 forestomach papilloma; 1 luteoma; 1 papilloma of the tongue.

## RESULTS

During the first 10 weeks, no difference in survival rates was observed between control and treated groups, except for Groups 6 and 7 receiving the highest dose of EMS in combination with DMN, where a high mortality occurred within the first week due to toxicity of the combined treatment. Subsequently, a similar mortality occurred among the rats of all groups up to 60 weeks. From this time, a greater mortality was noticed in Groups 6 and 7, due to the appearance of tumours. Since all the animals of Group 7 were dead by 110 weeks, we decided to sacrifice the animals surviving among the other groups as well at this time.

In the control groups, none of the animals developed kidney tumours. Tumours were found in 4 of the males; they were a malignant lymphoma at 11 weeks, one adrenal cortical adenoma at 102 weeks, one hepatoma and a testicular tumour at 110 weeks. Out of 20 females, 9 developed mammary gland tumours and 6 had malignant lymphomata between 85 and 110 weeks. A few additional tumours scattered in various organs, developed after the 110 weeks (Table I).

*Treatment with DMN or EMS alone*

The tumour incidences for all groups are given in Table I. In Groups 1 and 3, receiving a single dose of 100 or 300 mg/kg of EMS, 5 out of 78 animals of both sexes developed kidney tumours at an average latent period ranging from 90 to 95 weeks. No tumours of the kidney were observed in Group 2. One rat of Group 3 showed a lung tumour at 97 weeks. The incidence of mammary gland tumours, malignant lymphomata as well as other tumours was similar to that observed in the control group.

In Group 4, receiving a single dose of 30 mg/kg body weight of DMN, tumours of the kidney developed in 33% of the males and in 63% of the females, the average time of appearance being 85–90 weeks. In addition, 8 rats developed

tumours of the nasal cavities and 2 out of 34 tumours of the lung. Tumours of other sites showed similar incidences to those in the control group.

*Combined Treatment with DMN and EMS*

In Groups 5, 6 and 7 the final incidences of kidney tumours were 50, 50 and 64% in the males and 61, 59 and 71% in the females respectively (Table I).

These data showed an increased incidence of kidney tumours in the males compared with the 33% incidence observed in the males of Group 4 receiving DMN alone. The females of Group 5 and 6 showed a similar final incidence of kidney tumours to that of Group 4 (63%), whereas the highest tumour yield (71%) was observed in Group 7, receiving the highest dose of EMS in combination with DMN.

Comparison of the percentage of tumour incidences based on the initial number of animals can be misleading if deaths due to causes other than the observed tumours occur at different rates in the various experimental groups. For this reason, the progression with time of the cumulative probability for observing a kidney tumour at death has been calculated by the actuarial method described by Kaplan and Meier (1958). The actuarial probabilities for observation of kidney tumours at death in animals of Groups 1, 3, 4, 5, 6 and 7 have been plotted on Fig. 1, 2 and 3. When males and females (Fig. 1) are examined together, a considerable shortening of the latent period for these tumours was observed in the rats receiving the combined treatments when compared with rats receiving DMN or EMS alone. However, some differences appear when males and females are considered separately. In the females (Fig. 2), all the rats receiving the combined treatment show a shortening of the latent period with no variation among these 3 groups, whereas in the males (Fig. 3) an earlier appearance of kidney tumours was confined to Group 7. Statistically,

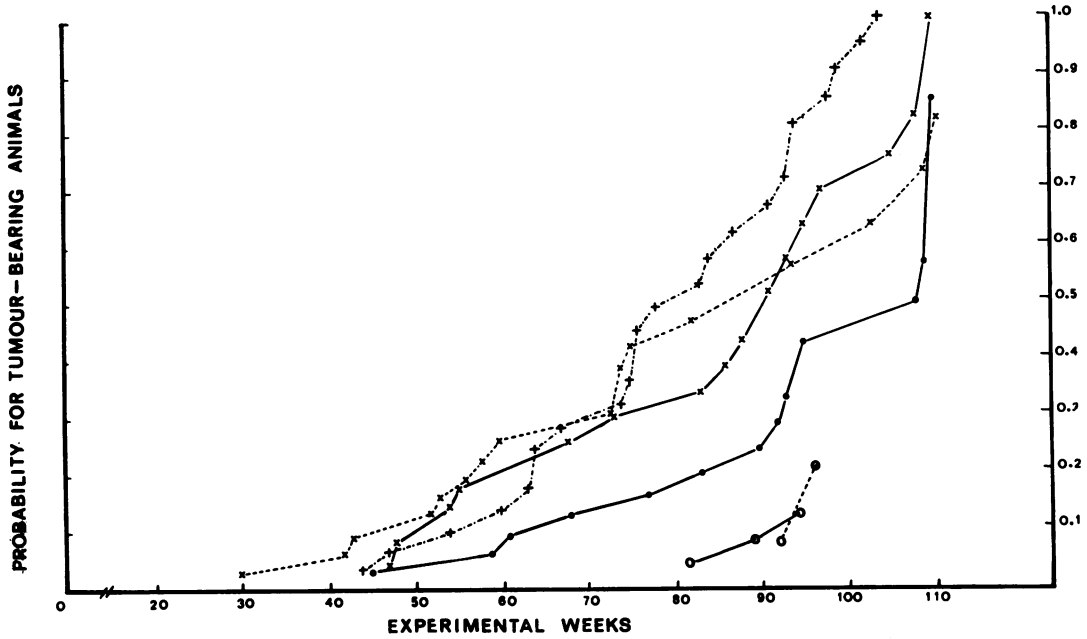


FIG. 1.—Probability for the observation of a kidney tumour in male and female rats at death, calculated according to Kaplan and Meier (1958). ○—○, Group 1; ○- - - ○, Group 3; ●—●, Group 4; ×—×, Group 5; ×- - - ×, Group 6; +---+, Group 7. No kidney tumours were observed in Group 2.

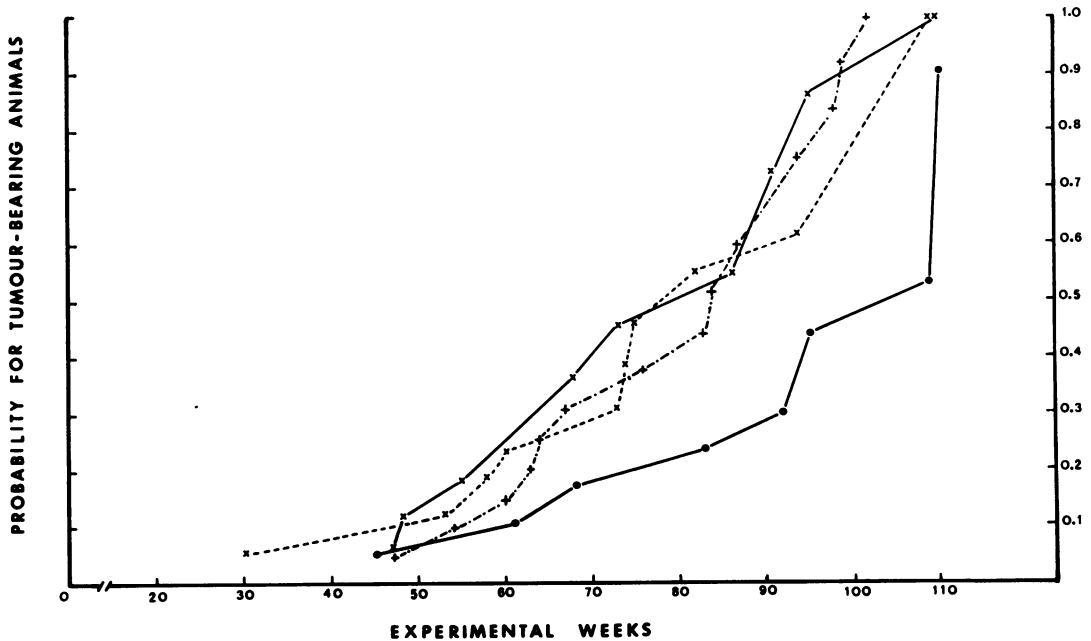


FIG. 2.—Probability for the observation of a kidney tumour in female rats at death, calculated according to Kaplan and Meier (1958). ●—●, Group 4; ×—×, Group 5; ×- - - ×, Group 6; +---+, Group 7. One kidney tumour was observed in a rat of Group 1 at 89 weeks.

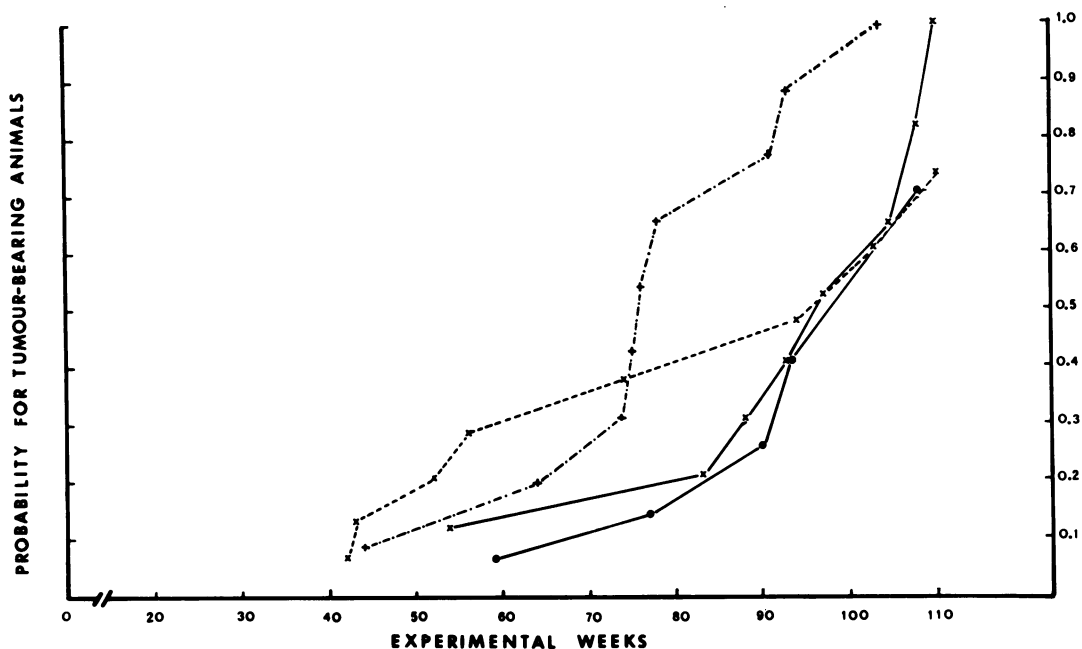


FIG. 3.—Probability for the observation of a kidney tumour in male rats at death, calculated according to Kaplan and Meier (1958). ●—●, Group 4; ×—×, Group 5; ×- - -×, Group 6; +---+, Group 7. Two kidney tumours were observed in rats of Group 1 and Group 3 respectively, dying between 88 and 96 weeks.

the greater incidence of kidney tumours observed in female and male rats of Group 7 was highly significant when compared with the rats of Group 4, as shown in Table III, where the instantaneous tumour mortality rates and the associated  $\chi^2$  values relative to the DMN alone treated rats for the 3 groups receiv-

ing the combined DMN plus EMS treatment were calculated according to Mantel (1966). The rats of Groups 5 and 6 showed a tendency to a greater tumour mortality but these results failed to obtain a statistical significance with the exception of borderline values for the females of Group 5.

TABLE II.—*Multiplicity of Tumours Induced in Rats by DMN and/or EMS*

Group	Total tumour bearing animals	Total no. of tumours	Ratio
Control	4	4	1.0
1	16	23	1.43
2	5	6	1.20
3	12	17	1.41
4	8	9	1.12
5	14	19	1.35
6	9	15	1.66
7	13	22	1.69
	10	19	1.90
	18	47	2.61
	13	33	2.53
	12	34	2.83
	12	32	2.66
	15	41	2.73
	11	40	3.63
	20	64	3.20

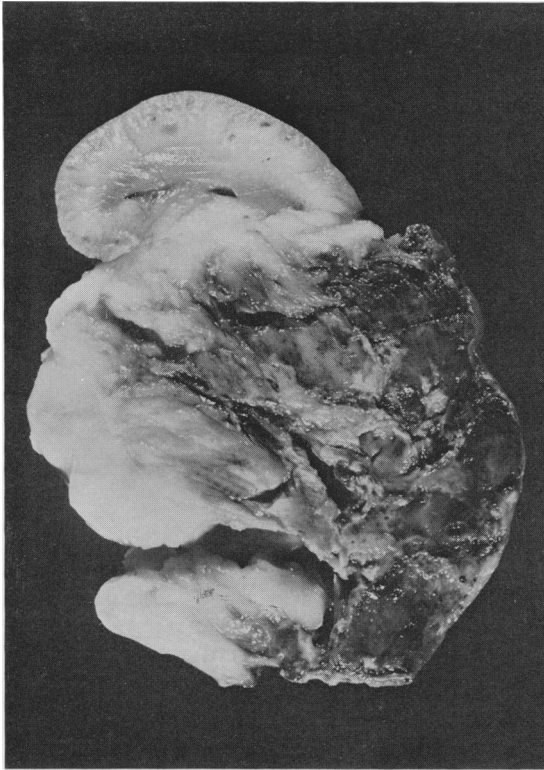


FIG. 4.—Large mesenchymal tumour of the kidney. Group 5.  $\times 1.5$ .

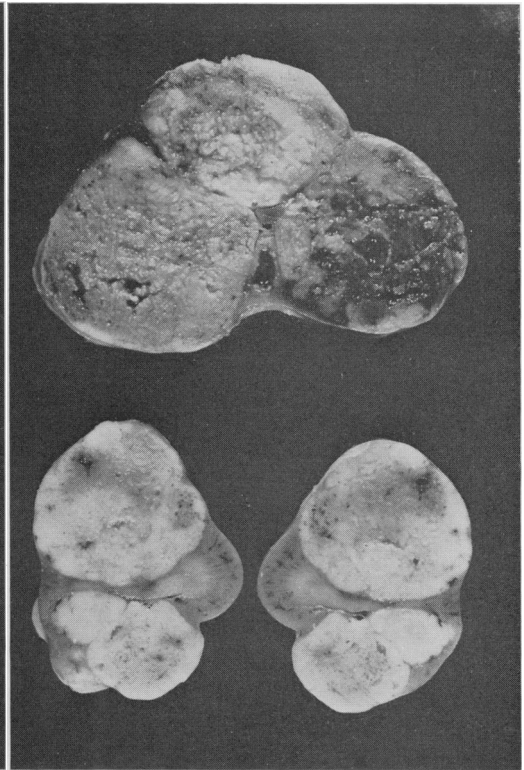


FIG. 5.—Bilateral and multiple adenocarcinoma of the kidney. Group 7.  $\times 1.5$ .

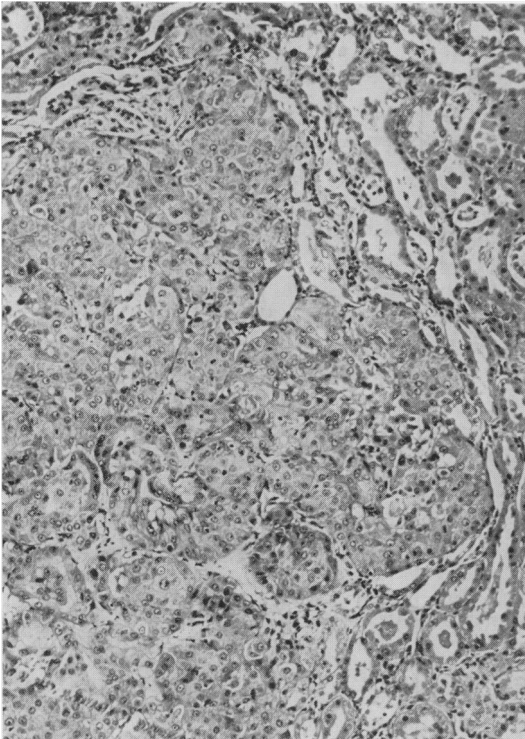


FIG. 6.—Part of a kidney adenoma demonstrating expansive growth. Group 5. H. & E.  $\times 120$ .

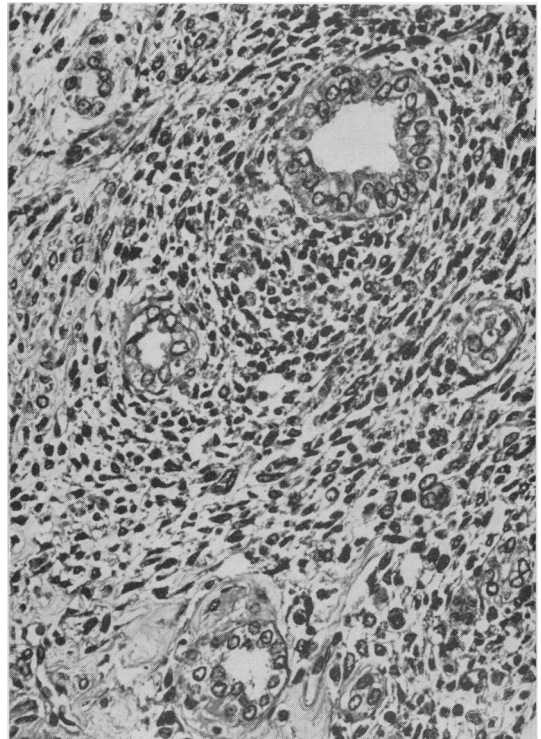


FIG. 7.—Mesenchymal tumour of the kidney. Group 4. H. & E.  $\times 300$ .



The additive effect between EMS and DMN in the induction of kidney tumours is more pronounced in the female rats (Fig. 2), where a higher tumour yield is present in all DMN plus EMS treated groups than in males (Fig. 3) where this effect is confined to the group receiving the highest dose of EMS combined with DMN. The higher multiplicity of tumours as well as the number of lung tumours in rats receiving the combined treatment (Table I and II) suggest that the carcinogenic additive effect is not confined to the kidney but is present to a lesser extent in other organs as well.

The induction of kidney tumours in rats treated with a single dose of various alkylating agents has already been exploited to correlate quantitatively the levels of alkylation in this organ and the tumour yield. Swann and Magee (1968, 1971) examined the amount of alkylation in kidney nucleic acids by dimethylnitrosamine, N-nitroso-N-methylurea, methylmethanesulphonate and the corresponding ethyl derivatives and they found a lack of correlation between the amount of alkylation of N-7 in guanine residues by each compound and their carcinogenic activity. In particular, the extent of conversion of guanine to 7-methylguanine was closely similar in the kidneys with dimethylnitrosamine and nitrosomethylurea, both of which induce tumours in this organ. With methylmethanesulphonate, however, the yield of 7-methylguanine in the kidney nucleic acids was of the same order as that found with the 2 nitroso compounds, but no kidney tumours were observed in the rats surviving a single dose of this compound. Similar results were obtained with the corresponding ethylating agents, among which a single dose of 270 mg/kg of EMS produced 5-10 times more 7-ethylguanine in rat kidney DNA than a carcinogenic dose of diethylnitrosamine or ethylnitrosourea. In these studies, a dose as large as 350 mg/kg body weight of EMS failed to induce kidney tumours; in the present studies, however, a single dose of 100

mg/kg body weight was sufficient to induce a low incidence of kidney tumours. However, these experiments (Swann and Magee, 1968, 1971) compared only the extent of alkylation of N-7 of guanine residues, which is the major alkylation site in nucleic acid bases, by these compounds. The question whether other alkylated sites in nucleic acids may be more important in the initiation of the carcinogenic process has been reviewed by Lawley (1972). As originally suggested by Loveless (1969) and substantiated by Gerchman and Ludlum (1973), alkylation of the 0-6 atom of guanine can cause mispairing of bases and thus induce transition mutations. O'Connor, Capps and Craig (1973) have shown that 0-6-methylguanine is present in hepatic DNA from rats treated with DMN, but none was detected after treatment of the animals with methylmethanesulphonate. EMS is known to react *in vitro* to the 0-6 position of guanine residues of DNA (Lawley, 1972).

The additive effect in the induction of kidney tumours in rats by a single dose of DMN and EMS, demonstrated in these studies, suggests that it might be the result of the total nucleic acid alkylation by these two compounds. However, which specific site(s) of alkylated nucleic acid bases is (are) involved in initiating the carcinogenic process remains to be established. Another factor, that could be related to the present findings is the impairment by the EMS of the cellular immune response which is known to occur with various alkylating agents (Krüger, 1972).

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