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Carcinogenic Properties of certain Rubber Additives

E. BOYLAND, R. L. CARTER, J. W. GORROD* and F. J. C. ROE

Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, London S.W.3

INTRODUCTION

UNEQUIVOCAL evidence is now available to show that there is an increased incidence of tumours of the bladder amongst workers in the rubber industry [1–3]. The original data, presented by Case and Hosker [1], were "consistent with the hypothesis that the risk was introduced with a certain antioxidant in 1928 which was known to cause bladder tumours in those workmen who manufactured it." The antioxidant in question was a formaldehyde condensation product of α- and β-naphthylamine which contained approximately 2·5% of uncombined α-naphthylamine and 0·25% β-naphthylamine. Use of this material in the rubber industry was stopped in 1949 but whether this was the only carcinogenic substance responsible for this disturbing situation is unknown.

In the course of manufacture of rubber products, various substances are added to the raw materials to modify its physicochemical properties; such compounds are numerous and the processes in which they participate are complex but four main groups of additives may be considered: *organic accelerators, carbon-black reinforcing agents, organic anti-oxidants,* and a miscellaneous group which includes *plasticisers, softeners and dyestuffs* [1]. The groups of organic accelerators and antioxidants overlap to some extent and comprise a wide variety of chemicals including aromatic amines, nitrosamines and their derivatives. The relationship between aromatic amines and tumours of the bladder has long been familiar [4–6] but it is now recognised that nitrosamines can also induce bladder tumours [7–9]. It seemed, therefore, desirable to examine the carcinogenic properties of some of the compounds used as organic accelerators or antioxidants and, in the present paper, we describe experiments with four derivatives of nitrosamines.

*Present address: Department of Pharmacy, Chelsea College of Science and Technology, London S.W.3.

MATERIALS AND METHODS

Rats. One hundred and twenty male CB stock rats were used, aged 6–7 weeks and weighing 200–230g. They were kept in metal cages, 6 animals in each, and were maintained on standard cubed diet no. 86 (Withers Ltd., Godstone, Surrey) and water *ad libitum.*

Rubber additives. The following rubber additives were examined:

1. *N-nitrosodiphenylamine* ("Vulcatard")
2. *N,N-dinitrosopentamethylenetetramine* ("Vulcacel") Both kindly supplied by I.C.I. Ltd., England.
3. *Polymerised N-nitroso* 2,2,4-*trimethyl*-1,2-*dihydroquinoline.*
4. *Nimethyl-N,*4-*dinitrosoaniline* Both kindly supplied by Monsanto Chemicals Ltd., England.

Conduct of experiment

The rats were divided into 5 equal groups, composed of 24 animals. Each of the 4 rubber additives was freshly dissolved in polyethylene glycol 400 and 0·25 ml was injected intraperitoneally at weekly intervals for six months. One group of animals acted as solvent controls and were injected with 0·25 ml polyethylene glycol only. The doses of additives which were administered were as follows:

Group 1. *Polymerised N-Nitroso* 2,2,4-*trimethyl*-1,2-*dihydroquinoline*—25 mg/week; total 650 mg.

Group 2. *N-methyl-N*,4-*dinitrosoaniline*—5 mg/week; total: 130 mg.

Group 3. *N,N-dinitrosopentamethylenetetramine*—25 mg/week; total: 650 mg.

Group 4. *N-nitrosodiphenylamine*—2·5 mg/week; total: 325 mg.

The rats were examined daily and all sick animals were killed at once. The experiment was terminated after two years. A standard post-mortem examination [10] was performed and all tumours and tissues showing macroscopic abnormalities were fixed in Bouin's solution. Paraffin sections were cut at 5μ and were stained with haematoxylin and eosin and, in some instances, with haematoxylin and Van Gieson, Masson's trichrome, Gordon and Sweet's Silver technique for reticulin and the McManus Periodic acid–schiff (PAS) technique.

RESULTS

1. *Intraperitoneal tumours*

The number of intraperitoneal tumours which developed in the four experimental groups is shown in Table 1. Eight tumours were seen—six in group 1 and two in group 2—in rats treated with *polymerised N-nitroso* 2,2,4-*trimethyl*-1,2-*dihydroquinoline* and with *N-methyl-N*,4-*dinitrosoaniline*, respectively. No tumours were found in rats in groups 3, 4 or 5. The times of death of tumour-bearing animals are also recorded in Table 1 but, since intraperitoneal neoplasms are rarely detectable during life, the time of induction of these lesions remains uncertain.

Apart from the presence of tumours, certain pathological features were common to rats in all four test groups, irrespective of which rubber additive had been injected. At autopsy, rats from all groups showed fibrous adhesions in the peritoneal cavity, binding the liver, diaphragm, stomach, spleen, small intestine, omentum and the anterior abdominal wall. Retroperitoneal fibrosis was sometimes apparent around the kidneys and pancreas. Such changes varied in extent in different rats and in different experimental groups but they appeared to be most consistent and most intense in animals in the group (Group 4) treated with *N-nitrosodiphenylamine*. Traces of injected material were frequently visible in visceral adhesions and small amounts of ascitic fluid were occasionally present.

On microscopic examination, the adhesions were found to be infiltrated with inflammatory cells consisting mainly of lymphocytes and macrophages (Fig. 1). Plasma cells and also polymorphs were occasionally seen but no discrete abscesses were found. Injected material was present in variable amounts; it was predominantly intracellular, lying within macrophages which sometimes accumulated in large numbers (Fig. 2). There was no evidence of granuloma formation. Dilated lymphatic vessels were sometimes seen, especially in the omentum and in thickened hepatic and splenic capsules. Comparable but less intense changes were also observed in several control rats, injected with polyethylene glycol only, and it appeared that repeated intraperitoneal injections frequently led to a low-grade chronic peritonitis, irrespective of the material administered.

In two of the experimental groups, intraperitoneal sarcomas were also encountered. The tumours were somewhat different in the two groups and they are therefore discussed separately:

(A) Of 24 rats injected with *N-methyl-N*,4-*dinitrosoaniline*, one developed a fibromyxosarcoma and one developed a spindle cell sarcoma (Figs. 3–5). The fibromyxosarcoma showed extensive myxoid degeneration together with zones of haemorrhage, necrosis and ectopic calcification. Viable tumour elements consisted mainly of spindle cells with occasional multinucleate giant cells. Mitoses were inconspicuous. The second tumour was a large spindle cell sarcoma which was adherent to the body wall and had invaded the caecum. Spindle cells predominated but more pleomorphic cells and mitoses were common; nuclear chromatin, arranged in a coarse stippled fashion, was unusually prominent. Both tumours showed a moderate amount of collagen formation and had a well-developed reticulin framework. Neither tumour contained any of the injected material. No metastases were seen.

(B) The six intraperitoneal sarcomas which were observed in rats treated wtih *polymerised N-nitroso* 2,2,4-*trimethyl*-1,2-*dihydroquinoline* differed in several respects from those just described. The tumours varied in size (some of them measuring up to 4 cm dia.) and many appeared to be multifocal. Their gross pattern was also variable, ranging from solid masses to flat plaques which tended to infiltrate muscle planes and grow along peritoneal membranes. The tumours were composed mainly of pleomorphic polygonal and spindle cells with moderate numbers of mitoses (Fig. 6). Multinucleate giant cells of an unusual type

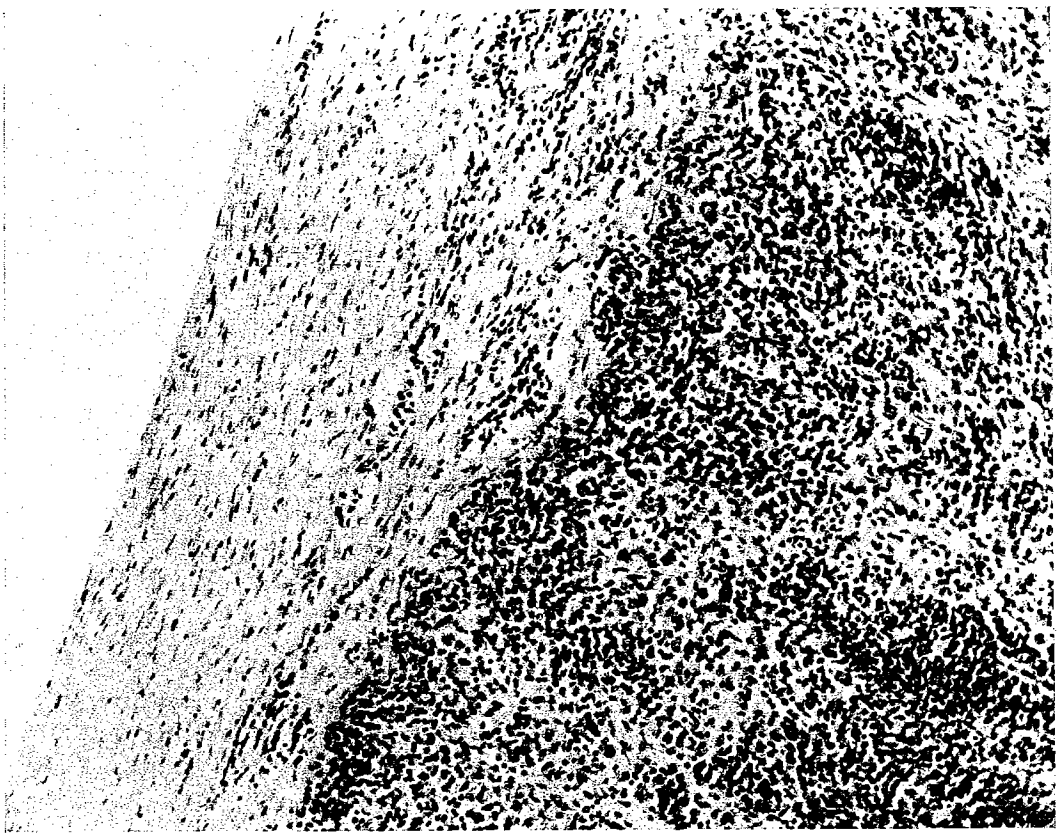

Fig. 1. *Spleen from rat injected with* N-nitrosodiphenylamine. *The capsule is thickened and shows a diffuse inflammatory infiltrate and dilated lymphatic channels. (N.B.—All photomicrographs are shown at a magnification of* ×160 *and stained with haematoxylin and eosin unless otherwise stated).*

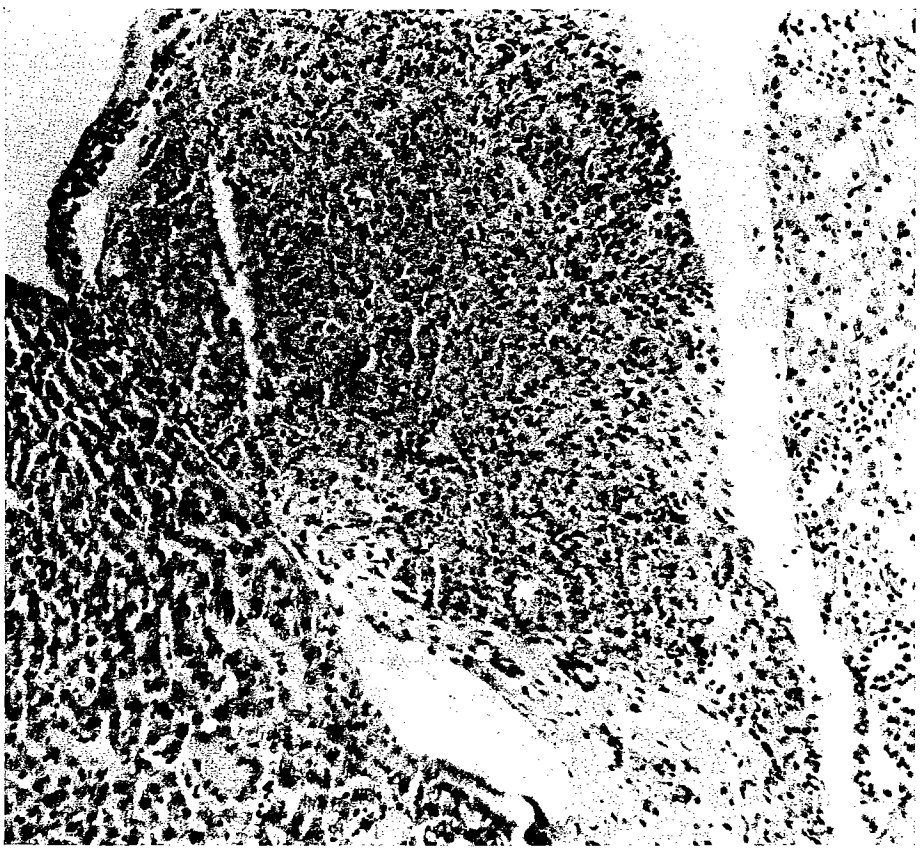

Fig. 2. *Adhesions binding together the liver and kidney from a rat injected with polymerised* N-nitroso 2,2,4-trimethyl-1,2-didydroquinoline. *There is a dense accumulation of macrophages in which ingested material is clearly seen.*

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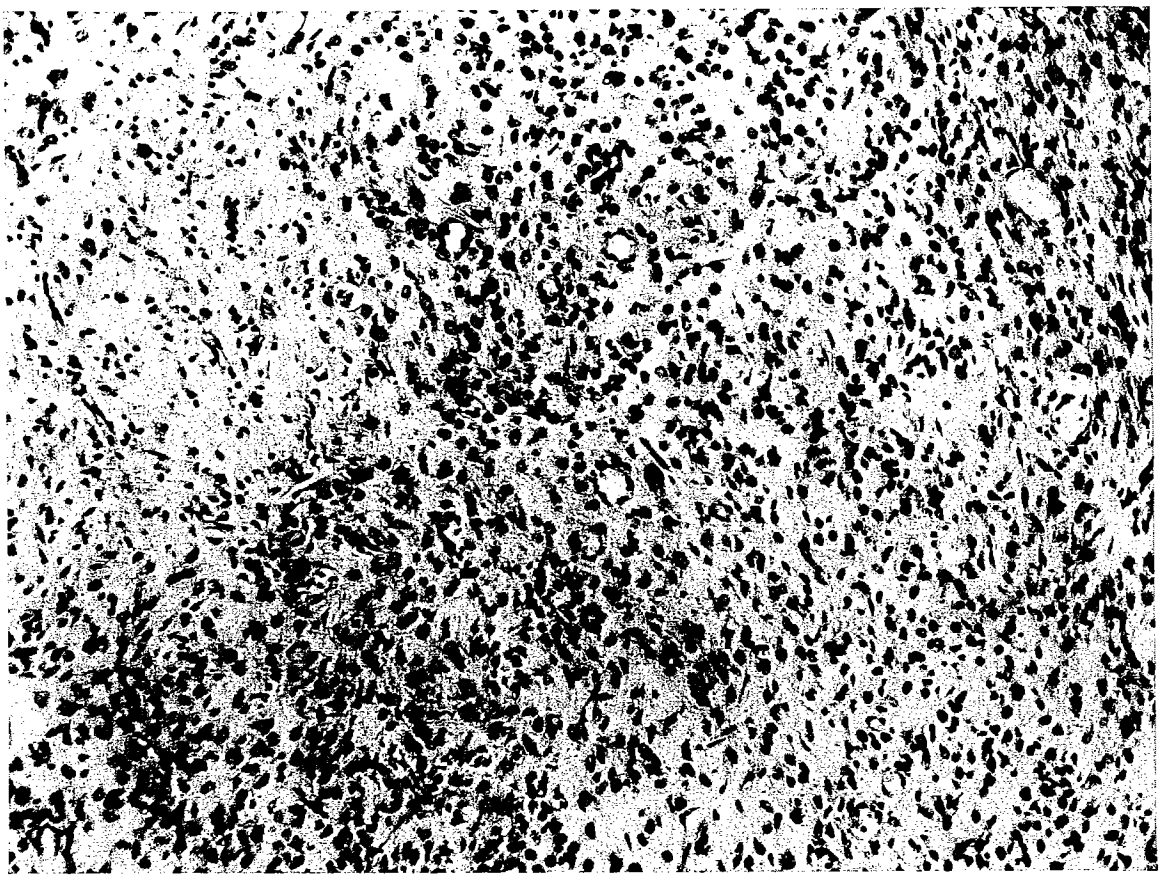
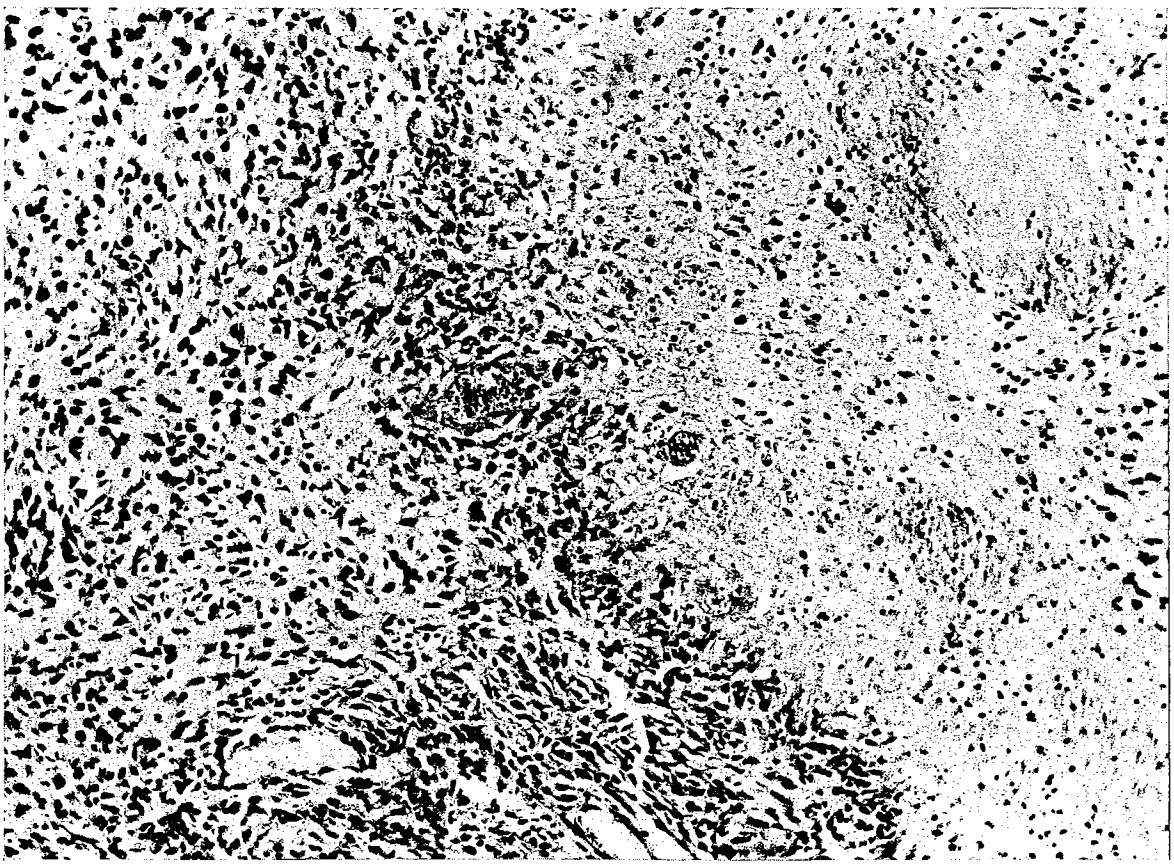


Figs. 3 *and* 4. *Intraperitoneal myxosarcoma from rat injected with* N-methyl-N,4-dinitrosoaniline.



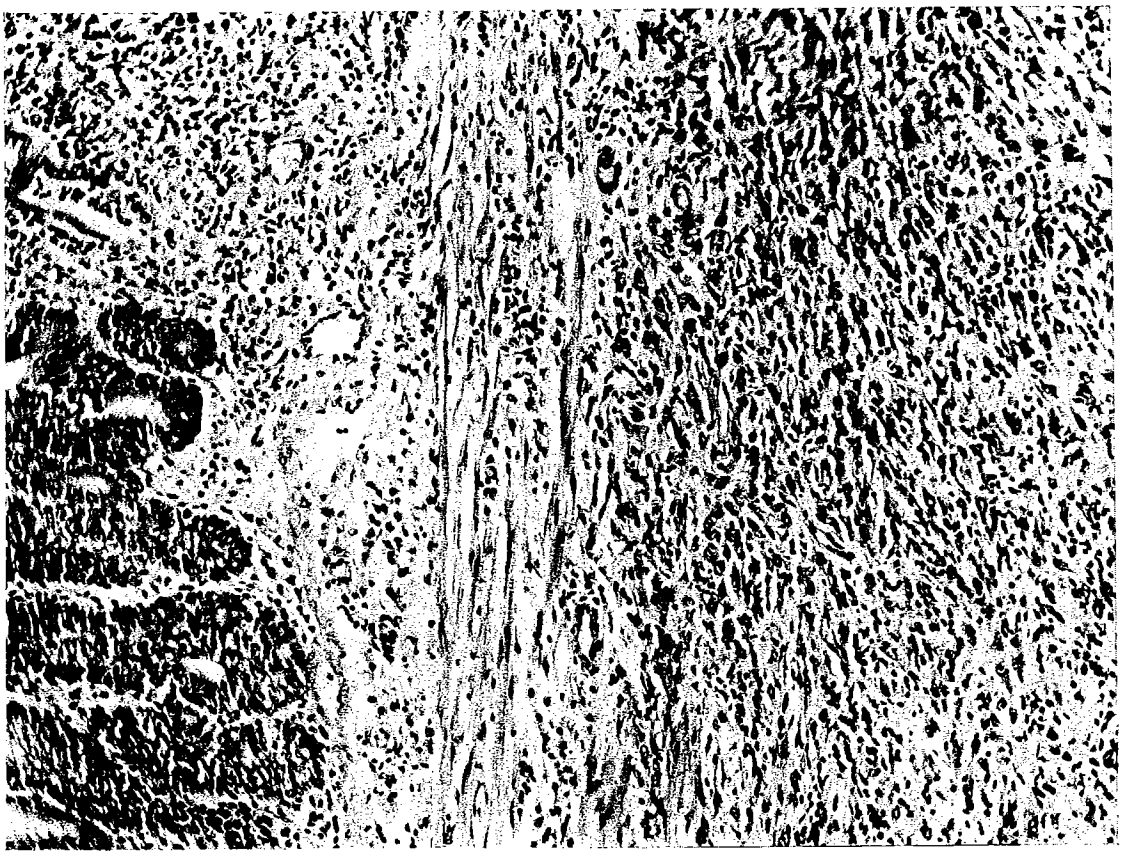


Fig. 5. *Spindle cell sarcoma from rat injected with* N-methyl-N,4-dinitrosoaniline.
The tumour is invading the wall of the caecum.

Figs. 6, 7 *and* 8. *Intraperitoneal sarcomas from rats injected with polymerised* N-nitroso 2,2,4-trimethyl-1,2-dihydroquinoline.

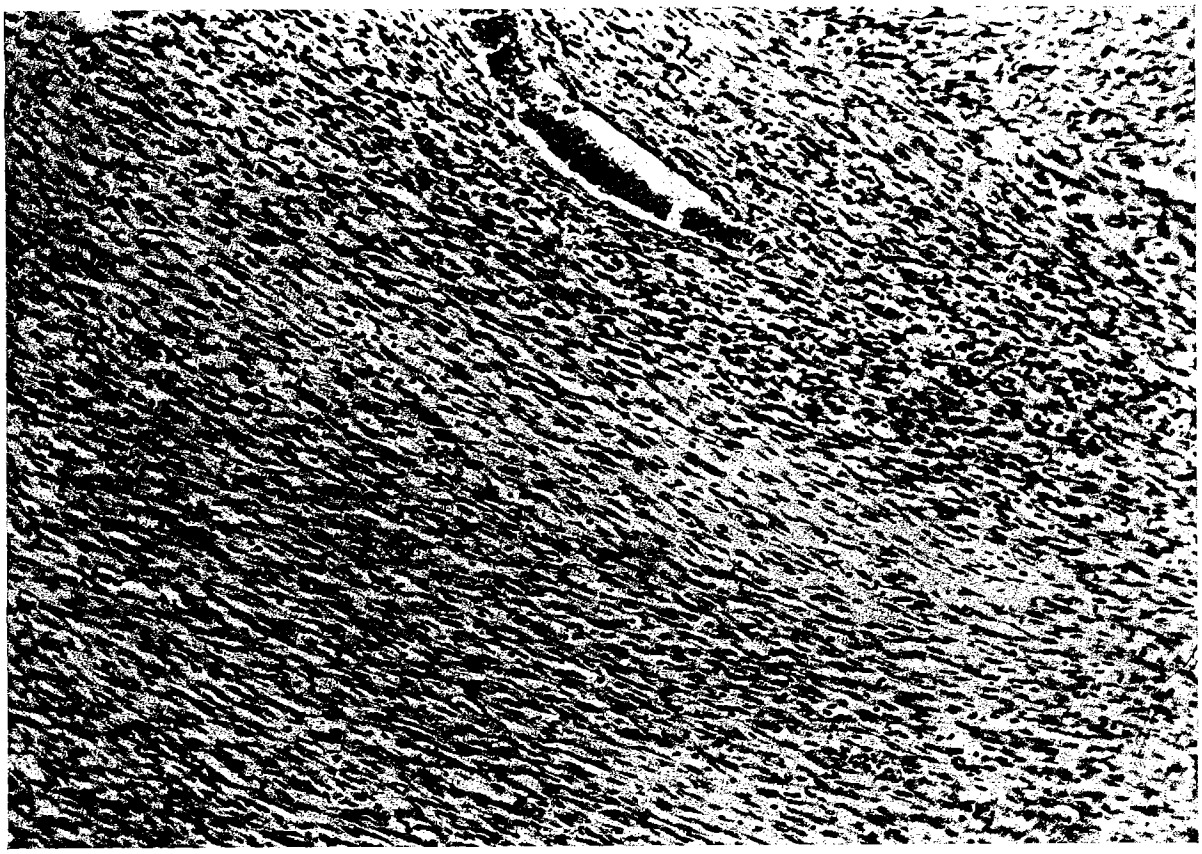


Fig. 6. *A sarcoma composed predominantly of spindle cell elements.*

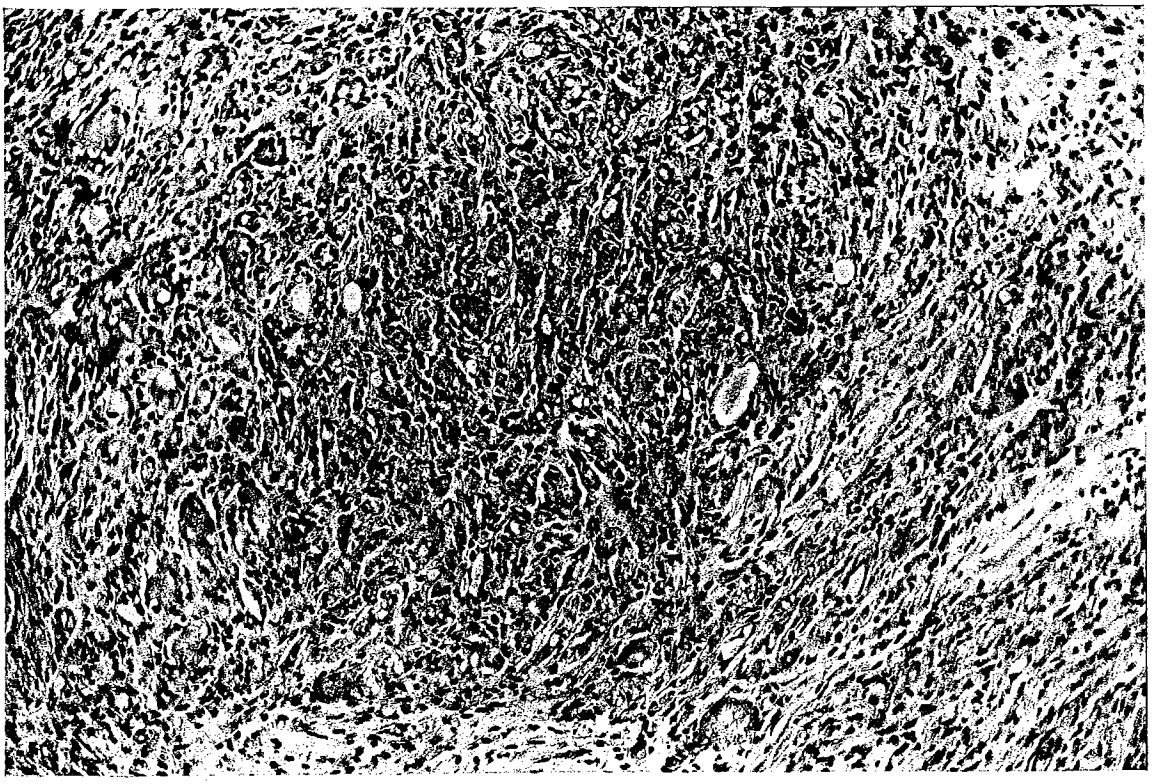


Fig. 7. *A more pleomorphic tumour containing numerous multinucleate giant cells.*

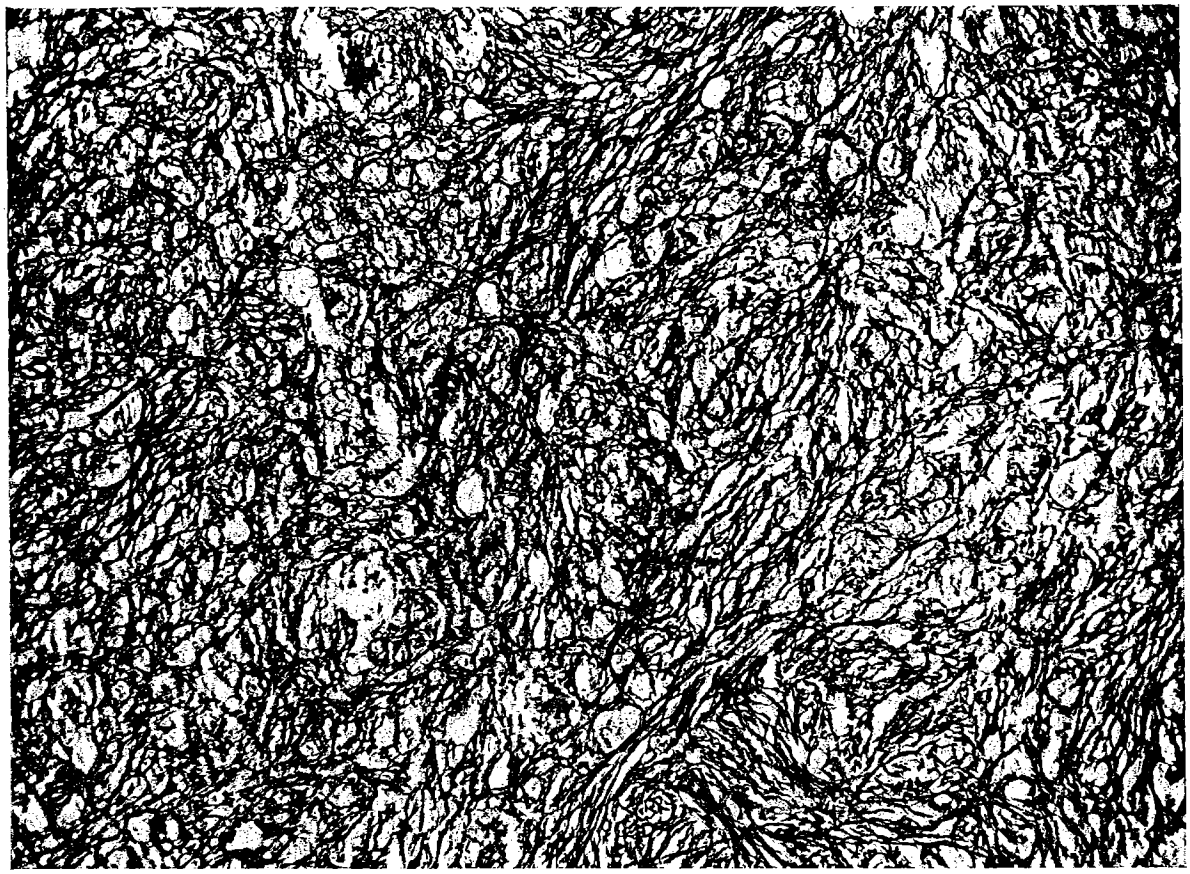


Fig. 8. *Reticulin preparation showing the dense reticulin framework typical of these tumours.*

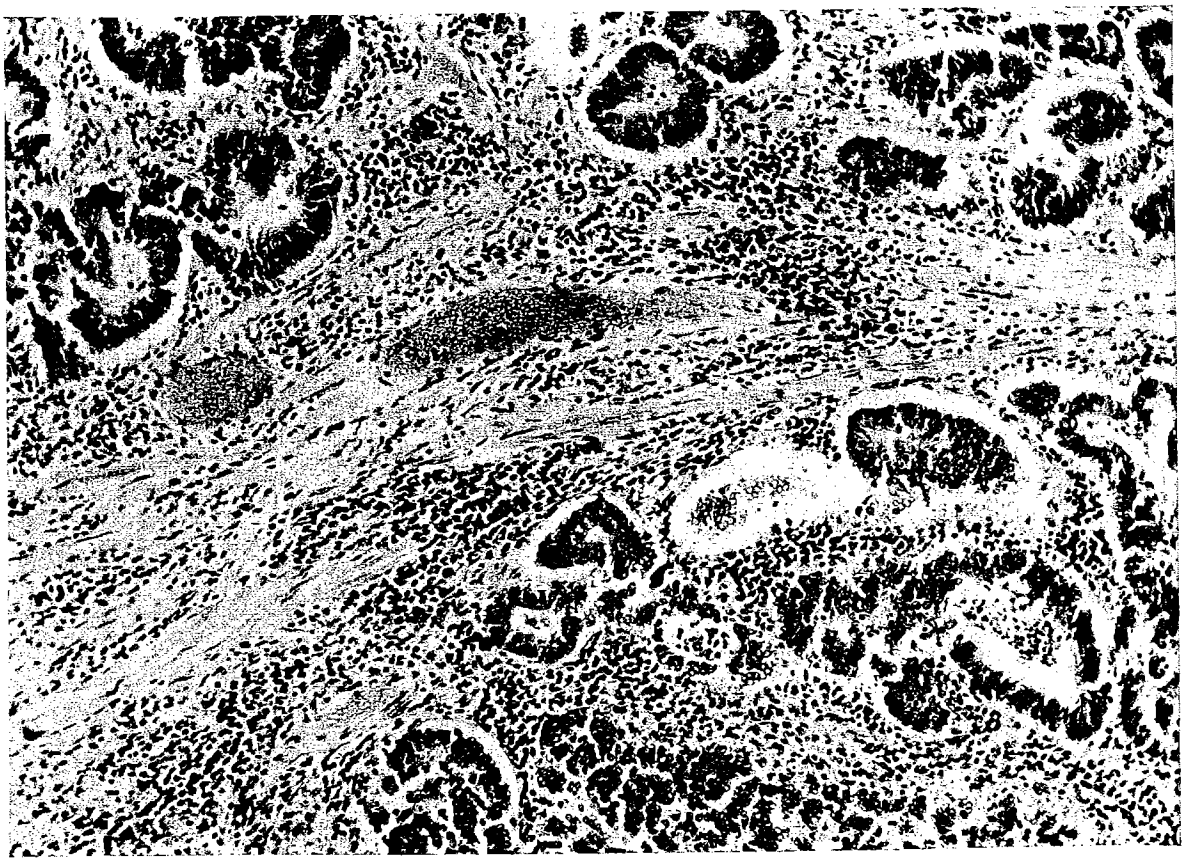


Fig. 9. *Adenocarcinoma of rectum from rat injected with polymerised* N-nitroso 2,2,4-trimethyl-1-2-dihydroquinoline.

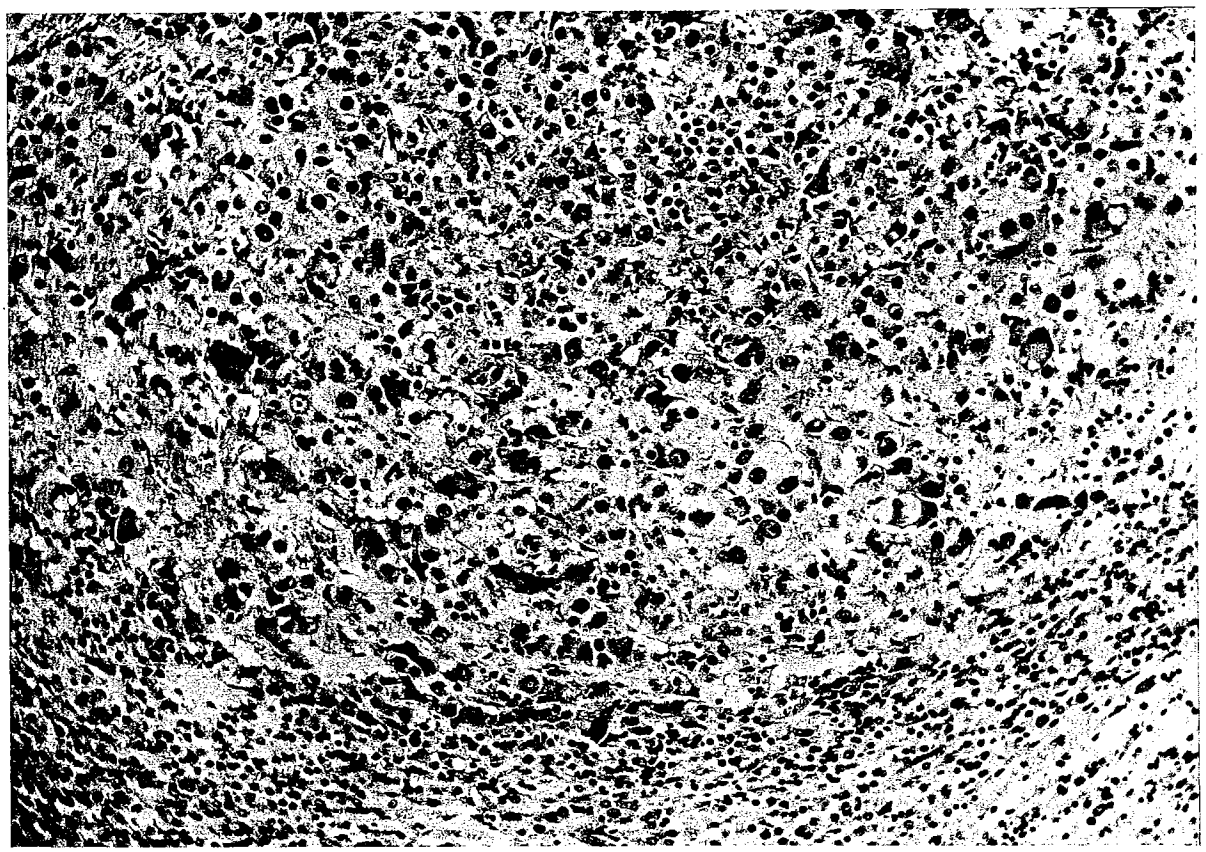


Fig. 10. *Chromophobe adenoma of pituitary gland from rat injected with* N-nitroso-diphenylamine.

Table 1. *Tumours in male CB stock rats following intraperitoneal injections of four rubber additives*

EXPERIMENT GROUP	No. OF RATS	TREATMENT (INTRAPERITONEAL INJECTIONS IN 0·25 ml. POLYETHYLENE GLYCOL, ONCE WEEKLY FOR 6 MONTHS)	TIME OF DEATH (months)						No. OF INTRAPERITONEAL TUMOURS	No. OF TUMOURS AT OTHER SITES
			0–9	10–12	13–15	16–18	19–21	22–24		
I	24	POLYMERISED N-NITROSO 2,2,4,-TRIMETHYL-1,2-DIHYDROQUINOLINE 25 mg.	○○○	■■?	■■○○ [R]	○○○○ ○	■■○○ ○○	○○	6	2
II	24	N-METHYL N, 4-DINITROSOANILINE 5 mg.		○	○○○○ ○○○○ ○	■○○○ [P][T]	■○○○ ○○[E][H]		2	4
III	24	N, N-DINITROSOPENTAMETHYLENETETRAMINE 25 mg.	○	○○○○ [H]	○○○○ ○	○○○○ ○○	○[P]	○○○○ ○	0	2
IV	24	N-NITROSODIPHENYLAMINE 25 mg.	○○○[H] ??	○○○	○○○○	○○○○ ○?	○○[P]	○○	0	2
V	24	POLYETHYLENE GLYCOL 400. (CONTROLS)	○○	○○○○	○○○○ ○	○○○	○○○○ ○○○	○○[H]	0	1

○ = RATS WITHOUT TUMOURS ■ = RATS WITH INTRAPERITONEAL SARCOMATA

E = Exocrine adenoma of pancreas H = Hepatoma P = Pituitary tumour

A = Adenocarcinoma of rectum S = Squamous carcinoma of skin T = Thymoma

? = Full post-mortem examination precluded by decomposition

were frequently observed with a peripheral ring of darkly-staining pyknotic nuclei encircling a large volume of foamy cytoplasm (Fig. 7); no cell inclusions were seen. Numerous thin-walled blood vessels were observed in many tumours. There was quite marked formation of collagen fibres and silver preparations showed a well-developed reticulin framework (Fig. 8). Recognition of metastases from multifocal tumours of this kind is obviously difficult but no tumour masses were seen outside the abdominal cavity.

2. *Tumours other than intraperitoneal sarcomas*

Other tumours encountered in the present study are listed in Table 1. A low incidence was found in all four test groups and there is no evidence that treatment with any of the test materials influenced either their frequency or variety. Certain of these lesions are, however, of interest. Adenocarcinoma of the rectum (Fig. 9) is rare in rats and so is exocrine adenoma of the pancreas—the latter has already been described elsewhere [11]. Chromophobe adenomas of the pituitary gland are more frequently encountered but the considerable cellular atypia in one of them (Fig. 10) is worth recording. It is noteworthy that no tumours of the urinary bladder were encountered.

(3) *Effects of certain rubber additives in mice*

It was originally planned to extend these observations and to study the effects of the same and other rubber additives in young C57 × DBA2 mice. Three substances were tested: *N-methyl-N,4-dinitrosoaniline*; 6-*ethoxy*-2, 2,4 - *trimethyl* - 1,2 - *dihydroquinoline* ("Santoflex AW"); and 6-*dodecyl*-2,2,4-*trimethyl*-1,2-*dihydroquinoline* ("Santoflex DD"). These additives were injected intraperitoneally in arachis oil at weekly intervals for six months and the mice were subsequently observed at regular intervals thereafter. A low incidence of intraperitoneal sarcomas was observed in animals from all three test groups but this results in uninterpretable since similar tumours also appeared in 3 mice injected with solvent alone. This unexpected finding in mice treated with arachis oil is being investigated and the effects of rubber additives (in other solvents) on mice is to be re-examined in the near future.

DISCUSSION

Two of the four rubber additives tested by repeated intraperitoneal injections in rats have been shown to be carcinogenic: treatment with *N-methyl-N,4-dinitrosoaniline* induced two intraperitoneal sarcomas and treatment with *polymerised N-nitroso* 2,2,4-*trimethyl*-1,2-*dihydroquinoline* produced six intraperitoneal tumours. The latter appears to be the stronger carcinogen but it was administered in a larger dose and it is, in any case, a good deal less potent than other nitrosoamines [7–9]. The weak carcinogenic effect of *N-methyl-N,4-dinitrosoaniline* which was found in the present survey confirms similar observations by Weisburger and his associates [12]. No tumours were produced in rats treated with *N,N-dinitrosopentamethylenetetramine* or *N-nitrosodiphenylamine*, or in animals injected with solvent alone.

Certain pathological changes may be emphasised. First, the additives tended to persist in the peritoneal cavity for long periods and could be readily recognised macroscopically at post-mortem examination, many months after the last injection. Secondly, intraperitoneal injections induced a low-grade peritonitis in a high proportion of cases including rats which were injected with solvent only. No association was seen between the intensity of the inflammatory changes and the subsequent appearance of sarcomas; the most extensive inflammation was seen in rats treated with *N-nitrosodiphenylamine* and none of these animals developed intraperitoneal tumours. Thirdly, it is clear that the neoplasms induced by polymerised *Ninitroso* 2,2,4- *trimethyl*-1,2-*dihydroquinoline* presented certain unusual features. They were frequently multifocal and showed considerable pleomorphism. Their cell of origin is obscure: in some cases a diagnosis of mesothelioma was considered but, in the absence of any positive evidence, non-committal terms such as "spindle cell" or "pleomorphic" sarcomas have been preferred. Lastly, it should be noted that although the number of spontaneous neoplasms was not increased by treatment with rubber additives, certain unusual lesions were encountered. In this context, it is interesting that, in the one other report on the carcinogenic effects of *N-methyl-N,4-dinitrosoaniline* [12], one most uncommon tumour—a carcinoma of the thyroid—was found. Some of these pathological findings indicate the shortcomings of the intraperitoneal route for testing carcinogenic substances, particularly the high incidence of low-grade chronic infection.

It is unfortunate that the data from tests using C57 × DBA 2 mice cannot be interpreted at the present time (see results). A low level of carcinogenic activity was found in all three substances which were examined but these results are vitiated by the development of similar intraperitoneal sarcomas in mice treated with solvent alone.

Despite their rather low activity, there is no doubt that two of the four rubber additives which have been tested are carcinogenic in rats. This results, together with the epidemiological findings which were noted earlier [1–3], emphasise the need for a systematic examination of the carcinogenic activity of the materials used as rubber additives. The scope of such an investigation is very large but some of the possible carcinogens which should (in our opinion) be examined in the future are listed in Table 2.

Acknowledgements—We are deeply indebted to Dr C. E. Dukes for his opinions on the histology; to Mr K. G. Moreman and the staff of the Photographic Department for preparing the illustrations; and to Miss Jill Merryweather, Miss Anne Walsh and Mr. G. Munroe for technical assistance.

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Table 2. *Ingredients for rubber which have not been tested for carcinogenic activity or are suspect*

(*a*)	*Aromatic amine derivatives*
	Aldol-1-naphthylamine
	N-Phenyl-2-napththylamine
(*b*)	*Benzidine dyes*
	3,3-Dichlorobenzidine coupled with 2, 4-acetoxylidide
	3,3-Dichlorobenzidine coupled with acetoacetalinide
(*c*)	*Nitrosamines*
	N,N-Dinitroso N,N-dimethyltereph-thalamide
	Dinitrosopentamethylenetetramine
	N-Methyl-N,4-dinitrosoaniline
	Polymerized N-nitroso 2,3,4-trimethyl-1,2-dihydroquinoline
	1,-2-dihydroquinoline
	Nitrosofurfuramide
(*d*)	*Thiourea derivatives*
	Trimethylthiourea
	Diethylthiourea
(*e*)	*Cross linking agents*
	bis (2-Chloroethyl) formaldisulphide
(*f*)	*Acid anhydrides*
	Phthalic anhydride
(*g*)	*Hydrazides*
	pp′, Hydroxy bis (benzenedisulphonyl-hydrazide)
	Benzenesulphonylhydrazide
(*h*)	*Nitrosophenols*
	Ferric complex of 1-nitroso-2-naphthol
(*i*)	*Inorganic materials*
	Asbestos
	Cadmium sulphide
	Selenium

RESUME

Cent-vingt rats mâles du stock CB reçurent des injections intrapéritonéales de divers additifs du caoutchouc, dissous dans le polyéthylène glycol: la N-nitroso 2,3,4, trim-thyl-1,2–dihydroquinoline polymérisée, *la* N-méthyl-N,4-dinitrosoaniline, *la N,N-dinitosopenta methylène-tétramine et la N-nitrisodiphénylaminé. Ces substances furent administrées une fois par semaine, pendant* 6 *mois, et les animaux furent ensuite maintenus en observation pendant* 2 *ans. Aucune tumeur ne fut observée chez les rats traités par la* N,N-dinitrosopentaméthylène tétramine, *par la* N-nitrosodi-phénylamine, *ou par le* polyéthylène glycol *seul. Des sarcomes intrapéritonéaux*

apparurent chez 2 *rats traités par la* N-méthyl-N,4-dinitrosoaniline *et chez* 6 *rats traités par la* N-nitroso 2,2,4-triméthyl-1,2-dihydroquinoline *polymérisée. Ces derniers avaient des tumeurs multifocales qui présentaient divers caractères histologiques inhabituels. Des signes discrets de péritonite chronique furent observés chez certains rats de tous les groupes.*

La N-méthyl-N,4-dinitrosoaniline, *ainsi que le* 6-éthoxy-2,2,4-triméthyl-1,2-dihydroquinoline *et la* 6-dodécyl-2,2,4-triméthyl-1,2-dihydroquinoline, *furent également testés, selon les mêmes modalités, chez des souris* C57 × DBA2. *Un petit nombre de tumeurs intrapéritonéales furent observées dans les* 3 *groupes traités, mais ces résultats ne sont pas interprétables, parce que* 3 *tumeurs de même type apparurent dans le groupe témoin traité par le solvant.*

Ces observations montrent la nécessité d'une étude détaillée des additifs du caoutchouc quant à leur éventuelle cancérogénicité. Elles indiquent aussi que, pour des études ultérieures, la voie d'administration intrapéritionéale n'est peut-être pas la plus favorable.

SUMMARY

One hundred and twenty male CB stock rats were injected intraperitoneally with the following rubber additives dissolved in polyethylene glycol-polymerised N-nitroso 2,3,4-trimethyl-1,2-dihydroquinoline; N-methyl-N,4-dinitrosoaniline; N,N dinitrosopentamethylenetetramine; N-nitrosodiphenylamine. *These substances were given once weekly for six months and the animals were then observed for up to* 2 *years. No tumours were seen in rats treated with* N-N-dinitrosopentamethylenetetramine *or* N-nitrosodiphenylamine *or with polyethylene glycol alone. Intraperitoneal sarçomas developed in two rats treated with* N-methyl-N,4-dinitrosoaniline *and in six rats treated with* polymerised N-nitroso 2,2,4-trimethyl-1,2-dihydroquinoline. *The latter were multifocal tumours which showed a number of unusual histological features. Evidence of a low-grade chronic peritonitis was found in rats in all groups.* N-methyl-N,4-dinitrosoaniline, *together with* 6-ethoxy-2,2,4-trimethyl-1,2-dihydroquinoline *and* 6-dodecyl-2,2-4-trimethyl-1,2-dihydroquinoline, *was also tested in male* C57 × DBA2 *mice in a similar fashion. A small number of intraperitoneal tumours developed in all three test groups but these results were uninterpretable as three similar tumours appeared in the solvent controls. The present observations emphasize the need for detailed investigation of potential carcinogens in rubber additives but the efficacy of the intraperitoneal route, for future testing, is questioned.*

ZUSAMMENFASSUNG

Man injizierte 120 *männlichen CB-Ratten folgende, in Polyäthylenglykol gelöste Gummizusätze intraperitonäal:* polymerisiertes N-nitroso 2,2,4-trimethyl-1,2-dihydrochinolin; N-methyl-N,4-dinitrosoanilin; N,N-dinitrosopentamethylentetramin; N-nitrosodiphenylamin. *Diese Substanzen werden während sechs Monaten einmal pro Woche injiziert und die Tiere sind während dieser Zeit und bis zu zwei Jahren unter Beobachtung. Es wurden keine Tumoren bei den Ratten festgestellt, die mit* N, N-dinitrosopentamethylentetramin *oder mit* N-nitrosodiphenylamin *oder mit* Polyethylenglykol *allein behandelt wurden. Bei zwei mit* N-methyl-N,4-dinitrosoanilin *und dei sechs mit polymerisiertem* N-nitroso-2,2,4-trimethyl-1,2-dihydrochinolin *behandelten Ratten entwickelten such intraperitonäal Sarkome. Letztere waren multifokale Tumoren mit einer Reihe ungewöhnlicher histologischer Charakteristika. Bei Ratten aller Gruppen fand man Anzeichen einer leichten chronischen Peritonitis. Versuche mit* N-methyl-N,4-dinitrosoanilin *und* 6-Aethoxy 2,2,4-trimethyl-1,2-dihydrochinolin *und* 6-Dodecyl-2,2,4-trimethyl-1,2-dihydrochinolin *wurden auch in ähnlicher Weise bei männlichen* C57 × DBA/2-*Mäusen gemacht. Einige intraperitonäale Tumoren wurden in allen drei Versuchsgruppen festgestellt, doch konnten diese Befunde nicht befriedigend interpretiert werden, da drei gleiche Tumoren in der Kontrollgruppe beobachtet wurden. Die gemachten Beobachtungen weisen einmal mehr auf die Notwendigkeit hin, Gummizusätze auf ihren Gehalt an potenziellen Karzinogenen zu untersuchen, wobei die Zweckmässigkeit der beschriebenen intraperitonäalen Methode in Frage gestellt werden darf.*

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