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The Importance of Looking for Further Carcinogens in Tobacco Smoke, and the Possible Role of Nitrosoanabasine

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It is necessary to look for further carcinogens in tobacco smoke because, from our present knowledge, we cannot explain its carcinogenic activity. An experiment already described in the literature *(Roe,* 1962a, 1963) demonstrates this point so clearly that I beg leave to refer to it again.

Five groups of mice were treated, respectively, with: — Group 1, Cigarette smoke condensate (CSC), Group 2, CSC to which 3,4-benzpyrene (BP) had been added so as to increase its concentration five times (X 5) above the level normally present in CSC (vide infra), Group 3, CSC with X 25 BP, Group 4, CSC with X 125 BP, Group 5, BP at the same concentration as in Group 4 but without CSC (see Table 1). All treatments were by application to the dorsal skin after removal of hair using acetone as the vehicle. Applications were made three times each week for 68 weeks. Unfortunately, as far as the simple design of the experiment is concerned, subsequent analysis showed the CSC had a higher BP content than average (2.5 μg per 4 g of CSC obtained from the smoke of 100 cigarettes instead of 1.0 μg).

Table 1

The Effect of Added 3,4-Benzpyrene (BP) on the Carcinogenicity of Cigarette smoke Condensate of Mouse-skin.

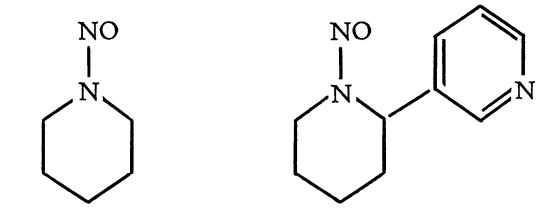
Group	Treatment	Dosage	Tumour-incidence at 68 weeks			Tumour-incidence at 84 weeks		
			Survivors	Benign tumours	Malignant† tumours	Survivors	Benign tumours	Malignant † tumours
1	Condensate with "normal" BP content*	40 mg. (as 0.2 ml. of 20% solution/ suspension in acetone) X3 weekly for 68 weeks	31	0	0	26	5	3
2	Condensate with X5 BP		21	4	0	15	5	5
3	Condensate with X25 BP		19	0	1	15	1	1
4	Condensate with X125 BP		24	17	5	14	2	9
5	BP in acetone (same concentration as in Group 4)	0.2 ml. x 3 weekly for 68 weeks	21	0	0	14	0	0

*Taken as 1 μg BP in condensate from 100 cigarettes (i. e. 4g. condensate)

†Cumulative total.

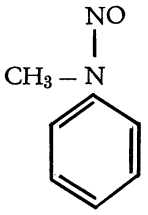
Figure 1

Formulae of substances tested and mechanism of formation of nitrosamines from amines and nitrous oxide.

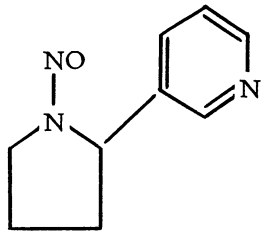


Nitrosopiperidine

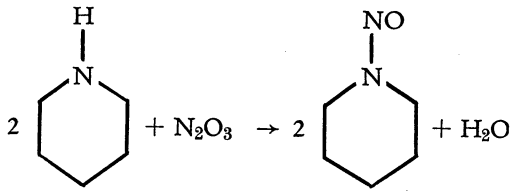
Nitrosoanabasine



Nitrosomethylaniline



Nitrosonornicotine



The results, as shown in Table 1, are remarkable in two respects:

1. CSC without added BP (Group 1) was more carcinogenic than an acetone solution containing 50 times as much BP (Group 5).
2. The carcinogenicity of X 125 BP in CSC (Group 4) was very much greater than that of the same concentration of BP in acetone (Group 5).

Many benign and a few malignant tumours had already arisen by the 68th week in the Group 4, whereas nothing more than a transitory papilloma was ever seen in Group 5.

These results indicate quite clearly that *the carcinogenicity of CSC cannot be due to its BP content.* At the same time they suggest that *constituents of CSC are capable of enhancing the carcinogenic activity of BP added to it.* This finding confirms those of others (e. g. *Gellhorn,* 1958), and gives weight to the view that tobacco smoking is more of a co-carcinogenic (tumour-promoting) influence than of a fully carcinogenic one. Previous work of our own *(Roe* et al, 1959) indicated that the phenolic fraction of CSC contains some co-carcinogenic constituents.

Unless the carcinogenic action of tobacco smoke can be explained by the combined action of carcinogens, such as BP, and of co-carcinogens, then clearly the most important carcinogenic constituents have yet to be found. It follows therefore that it is both rational and important that we should look for carcinogens other than polycyclic hydrocarbons in cigarette smoke. For the following theoretical reasons we chose to investigate certain specific nitrosamines: –

1. Several amines such as anabasine and nor-nicotine are known to be present in tobacco smoke.
2. Oxides of nitrogen are present in relatively high concentration in tobacco smoke.
3. Particularly in the relatively acid environment of cigarette (as opposed to cigar and pipe) smoke amines and nitrous oxide might react to form nitrosamines (see Fig. 1).

Attention was at first focussed on nitrosoanabasine and nitrosonornicotine, and the report which follows concerns the first of these.

Table 2

Experimental details

Group	Treatment (in drinking water)	Estimated daily dose (6 days per week)	Number of rats
1	Nitroso-piperidine 0.2%	5 mg.	(16 ♂ (16 ♀
2	Nitroso-methylaniline 0.2% for 7 months, then 0.1%	5 mg. then 2,5 mg.	(16 ♂ (16 ♀
3	Nitroso-anabasine 0.2%	5 mg.	(16 ♂ (16 ♀
4	None	-----	(16 ♂ (16 ♀

Biological tests of nitrosoanabasine for carcinogenic activity

Three groups of Chester Beatty albino rats, with 16 ♂ and 16 ♀ in each group were treated as shown in Table 2 with, nitrosopiperidine, nitrosomethylaniline, and nitrosoanabasine, respectively. The substances were added to the drinking water on 6 days of each week continuously throughout the experiment. A fourth group were kept under observation without treatment as controls.

Table 3 *Neoplastic Lesions.*

Group	Treatment	Oesophageal Tumours		Liver Tumours		Other Tumours
		All	Malig.	All	Malig.	
1	Nitroso-piperidine	26	16	23	10	0
2	Nitroso-methylaniline	27	18	0	—	2 (Malignant lymphoma. (Adenocarcinoma of the salivary gland.
3	Nitroso-anabasine	7	1	0	—	1 Mammary adenocarcinoma
4	None	0	—	0	—	1 Malignant lymphoma

The results in terms of tumour development are shown in Tables 3 and 4. All the tumours shown have been examined histologically. Some of the malignant liver tumours in rats of Group 1 showed metastases in the lungs. As expected from the work of Professor *Druckerey* and Dr. *Preussmann*, nitrosopiperidine gave rise to both oesophageal and liver cancer, and nitrosomethylaniline to oesophageal cancer. The only important difference is that in our experiment with nitrosopiperidine liver tumours were much more frequent. The positive results obtained with these two substances gave us confidence that our strain of rats would be susceptible to the carcinogenic action of other nitrosamines. In due course female rats treated with nitrosoanabasine

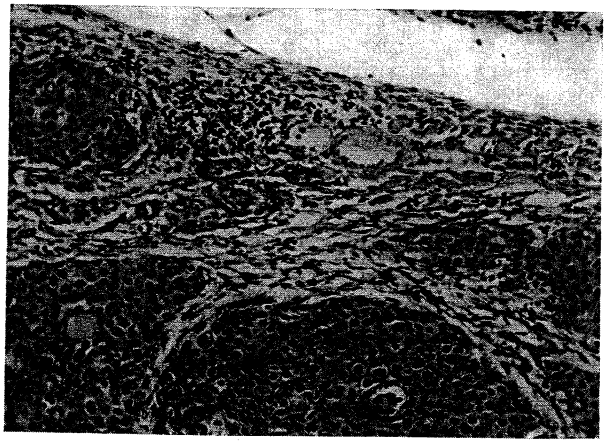


Figure 2

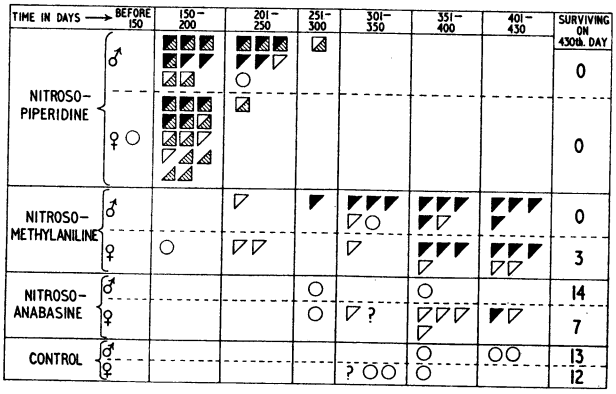
Carcinoma of the oesophagus in a female rat given nitrosoanabasine in the drinking water for 14 months. Note invasion of muscle wall. Stained H and E × 255.

began to develop oesophageal tumours. To date, six cases of multiple papilloma and one of malignant tumour of the oesophagus have been seen, all in females. Only two of the 16 males have died, both without tumours. Figure 2 illustrates one of the tumours observed in this experiment.

One of the extraordinary findings in these experiments has been the almost normal appearance of the forestomach epithelium in rats dying with multiple tumours of the oesophagus. Occasional particles of epithelial hyperplasia and isolated benign papillomata of the forestomach have been the only lesions seen.

Table 4

Tumour incidence before 430 days.



The results leave no doubt concerning the carcinogenicity of all three substances, nor of their relative potency in the doses given. The effect of nitrosoanabasine was slower and weaker than that of the other two compounds. Nevertheless, the effect is already unequivocally positive and, by the time all the animals are dead, the incidence of tumours is likely to exceed 50% of the animals at risk. The apparent sex difference is puzzling as such has not been observed with other nitrosamines. We would prefer to reserve comment on this until more data from males are available (see addendum).

Attempts to detect nitrosoanabasine and nitrosonornicotine in cigarette smoke

Whilst the biological tests have been in progress attempts have been made to detect nitrosoanabasine and nitrosonornicotine in tobacco smoke. A method for detecting these substances is available. Both can be detected by either paper or thin-layer chromatography. They can be reduced with zinc and acetic acid to hydrazine derivatives, which give colours with p-dimethylaminobenzaldehyde (Ehrlich reagent) or with p-dimethylaminocinnamaldehyde.

These nitrosamines also react with an acidic solution of 2 (N-Benzyl aniline methyl) imidazoline (Antistin) to give yellow colours after 20 minutes. These gradually change to green-blue during the next 24 hours. Another more sensitive test has been developed: it is as follows: - the nitrosamines are decomposed in the presence of p-chloraniline to produce a diazonium compound, which is then coupled with N(1-naphthyl) ethylenediamine.

We have used these methods in our attempts to detect these substances in tobacco smoke, but so far without success. It appears that other constituents of the smoke

interfere with the reaction, for even when nitrosoanabasine was injected into cigarettes prior smoking, none was detected in the smoke. Moreover, nitrosoanabasine added to smoke condensate cannot at present be detected by these methods.

Conclusions

1. The precense of traces of carcinogenic polycyclic hydrocarbons such as 3,4-benzpyrene in tobacco smoke, cannot explain the carcinogenicity of the latter, unless their activity is enhanced co-carcinogenically by other constituents of the smoke, e. g. phenolic compounds.
2. It is therefore logical to look for other types of carcinogen in tobacco smoke.
3. Nitrosoanabasine, nitroso-nornicotine, and other nitrosamines may well be present in tobacco smoke, having been formed by a reaction between secondary amine precursors and oxides of nitrogen.
4. Nitrosoanabasine is carcinogenic for the oesophagus of the rat.
5. So far, attempts to detect nitrosoanabasine and nitroso-nornicotine in tobacco smoke have been unsuccessful. However, it is probable that other constituents of the smoke masked the presence of these compounds.

Zusammenfassung

Die im Tabakrauch vorhandenen Spuren von polycyclischen Kohlenwasserstoffen können für die cancerogene Wirkung der Kondensate nicht verantwortlich gemacht werden, wenn nicht anderen Rauchinhaltsstoffen, wie den phenolischen Verbindungen, eine Verstärkung der Wirkung zugeschrieben werden soll. Aus diesem Grund muß die Suche nach anderen cancerogenen Bestandteilen des Rauches fortgesetzt werden.
Nitroso-Anabasin, Nitroso-Nornikotin und andere Nitrosamine könnten aus sekundären Aminen und Stickstoffoxyden gebildet werden und deshalb im Tabakrauch vorhanden sein. Nitroso-Anabasin ist für den Oesophagus der Ratte cancerogen. Bisher gelang es nicht, Nitroso-Anabasin und Nitroso-Nornikotin im Rauch nachzuweisen. Es ist jedoch möglich, daß sie durch andere Bestandteile des Rauches maskiert werden.

References

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Addendum

Since this Symposium took place the experiments on nitrosoanabasine have been completed. Oesophageal tumours were seen in both sexes in rats of group 3. A full report has appeared (BOYLAND, E., ROE, F. J. C., GORROD, J. W., and MITCHLEY, B. C. V.: (1964) Brit. J. Cancer 18, 265—270). In another experiment mice treated with nitrosonornicotine developed multiple lung adenomata. A report of this finding has also appeared (BOYLAND, E., ROE, F. J. C., and GORROD, J. W.: [1964] Nature [Lond.] 202, 1126).

Diskussion

Preussmann: Dr. *Kriek* erwähnte die Methylierung von Phosphat-Gruppen als Reaktion mit Diazomethan. Ich möchte die Aufmerksamkeit auf die Tatsache lenken, daß die Methylester der Phosphorsäure starke alkylierende Mittel sind. So ist z. B. Trimethylphosphat ein starkes methylierendes Mittel, das fast so stark wirkt wie Dimethylsulfat. Vielleicht hat auch dies etwas mit dem Wirkungsmechanismus zu tun.

Schmähl: Dr. *Kriek* sagte uns, daß die Cancerogenität der Nitrosamine um so geringer wird, je länger die Kette ist. Das Diäthylnitrosamin zeigt aber beispielsweise eine stärkere carcinogene Wirkung als das Dimethylnitrosamin. Dr. *Magee* und wir fanden nach Applikation von Dimethylnitrosamin Lebertumoren in gleicher Häufigkeit. Wir haben keinen Unterschied der biologischen Aktivität zwischen Dimethyl- und Diäthylnitrosamin gefunden. Die akut toxische Dosis von Dimethylnitrosamin liegt bei ungefähr 30 mg/kg, die des Diäthylnitrosamins ist bei ungefähr 210 mg/kg anzunehmen, sie ist also ungefähr siebenmal so hoch. In gleicher Weise unterscheiden sich die Dosierungen, mit denen Tumoren hervorgerufen werden können.

Druckrey: Wir sollten hier zwischen Tumorausbeute, Dosis und Induktionszeit unterscheiden. Die Menge von Diäthylnitrosamin ist im Vergleich zu Dimethylnitrosamin sehr schwer zu schätzen, weil wir wissen, daß jede Dosis schließlich Tumoren erzeugen kann. Es ist mehr die Induktionszeit, durch die sich die Verbindungen unterscheiden. Andererseits kann ich aus meiner Erfahrung mit Dimethylnitrosamin sagen, daß wir nur ungefähr 60 bis 70% Lebertumoren erhielten, während wir mit der Diäthylverbindung fast bei allen Tieren Tumoren erzeugen konnten.

Die Methyl-alkyl-Verbindungen zeigen die gleiche Aktivität. Die akute letale Dosis von Methyl-äthyl- und Methyl-amyl-nitrosamin ist ungefähr gleich. Sie liegt bei etwa 90 bis 100 mg/kg Körpergewicht, obgleich das Molekulargewicht beim Methyl-amylnitrosamin fast doppelt so hoch ist. Die carcinogene Dosis dieser Methyl-alkyl-Derivate ist ebenfalls gleich. Dies ist aber bei den Äthyl-alkyl-Verbindungen aus unbekannten Gründen anders.

Ich glaube daher, daß (1.) die Dealkylierung und (2.) die Labilität oder Stabilität des entstehenden Diazoalkans in Betracht gezogen werden sollten.

Magee: The nitrosamines seem to be more carcinogenic than the simple alkylating compounds such as dimethylsulfate. We ourselves are doing experiments with dimethylsulfate, but we have not gone very far.

Druckrey: Ich möchte noch einige Worte zum Vortrag von Dr. *Roe* sagen. Der Versuch, Cigarettenrauchkondensat und das darin enthaltene Benzpyren mit Benzpyren-Lösungen zu vergleichen, ist sehr interessant, denn er zeigt, daß das Benzpyren allein die Carcinogenität des Tabakrauches nicht erklärt. Glauben Sie, daß hier ein cocarcinogener Effekt vorliegt oder könnte die Wirkung einem anderen synergistischen Carcinogen zugeschrieben werden?

Roe: Abundant quantitative data are necessary before initiating activity, promoting activity, and complete carcinogenic activity can be clearly distinguished. In the past the distinction has been made in respect of particular substances on the basis of insufficient data.

von Euler: In any of these experiments with carcinogenic substances, were there any tumours observed in the adrenal medulla? I ask this, because *Eränkö* (Finland), a colleague of mine, injected rats with relatively large doses of nicotine for about two months. He observed tumor-like growth in the adrenal medulla.

Schmähl: Wir haben in Freiburg niemals solche Tumoren gesehen, obgleich wir über die gesamte Lebenszeit Nikotin in subtoxischen Dosen wöchentlich gegeben haben.

Magee: I would like to ask Prof. *Druckrey* another question. In the case of nitrosomethyl-aniline, is it absolutely necessary to postulate another mechanism? Do you think that the phenyl-group might be split away and this leads to traces of the monomethylnitrosamine and that we might be dealing with methylation as perhaps we are with the other asymmetrical nitrosamines?

Druckrey: Wir können noch nicht genau sagen, was geschieht. Man sollte aber dem Phenyldiazonium Interesse zuwenden.