A STUDY OF THE TOXICITY OF SULPHITE. I.

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The effect of sodium metabisulphite, 750 p.p.m. as SO_2, in the drinking water, has been studied through three generations of rats in experiments lasting for nearly 3 years. The metabisulphite was without effect on growth, the intake of food and fluid and the output of faeces. It did not influence fertility, the weight of the newborn or lactation: neither did it increase the frequency with which tumours developed.

IT is well known that sulphite reacts with aneurine[1] and that the sulphiting of foodstuffs reduces their aneurine activity[2,3]. This alone, however, cannot fully explain the effects observed by Fitzhugh, Knudsen and Nelson[5] in the course of the only published long-term investigation of the toxicity of a sulphited diet. These workers fed a partially synthetic diet to young rats for periods of 12 weeks or 1 year and observed the effects of mixing sodium bisulphite with the food on the growth and health of the animals. The addition of 0·1 per cent sodium bisulphite, 615 p.p.m. as SO_2, produced multiple histopathological changes, only some of which were reduced in incidence or severity by the bi-weekly injection of 100 μg. of aneurine per rat. Deficiency of aneurine was therefore not the full explanation of the findings. The present work is a first step in the search for additional causes of the long-term toxicity of sulphite. We have examined the toxicity of sodium metabisulphite itself, and have administered it in the drinking water to prevent prior interaction between the metabisulphite and the constituents of the diet. The experiments have extended over three generations of rats.

METHODS

Eleven male and 39 female newly weaned rats, not differing in age by more than 5 days, and of a uniform strain bred for cancer research by the Chester Beatty Research Institute, London, received individual earmarks for identification and were divided at random into three groups. Each group contained 13 females: there were in addition 5 males in group I and 6 males in group II. The animals of different groups and the sexes were segregated. All were kept in standard cages of Boot's type, not more than 7 in a cage, in the same room under similar conditions of lighting and humidity, at a temperature maintained within the range of 21·5° to 24·5°.

Solid diet. All rats received cube diet 41 b of Stein in wire baskets. A typical sample of this diet was assayed for us by Vitamins Ltd. and was found to contain 3·1 μg. of aneurine per g.

Fluids. All rats drank freely from graduated bottles. Those of group I were provided with tap water, those of group II received tap water containing sodium metabisulphite equivalent to 750 p.p.m. SO_2, and those of group III tap water containing metabisulphite equivalent to 375 p.p.m. SO_2. Calculated weights of sodium metabisulphite assayed

for SO_2 equivalent were added to tap water daily to yield solutions equivalent to 750 p.p.m. SO_2. The solutions containing 375 p.p.m. SO_2 were initially prepared every other day. Only later was it found that the SO_2 content of these had dropped by nearly 20 per cent in 48 hours, so that the female rats of group III had received only 90 per cent of the SO_2 intended in the period before their first mating. Thereafter all solutions of metabisulphite were prepared daily, and were found to deteriorate in SO_2 content by approximately 10 per cent in the 24 hours before a fresh solution was provided.

Estimation of growth rate. Each rat was weighed twice weekly. Curves relating body weight in g. to age in days were found to be linear from the time of weaning to the 70th to 80th day of life for both males and females. Rates of growth were therefore expressed as the slopes of these lines. Standard statistical methods, based on the normal curve of distribution were used to compare the rates of growth of rats of the same sex which had received different drinking fluids since the males and females were found to constitute two different populations in respect of growth, each homogeneous within itself.

Estimation of food intake, fluid intake and faecal output. The daily food consumption of the rats in each cage was measured as the loss in weight of the food basket in 24 hours and was expressed in g./100 g. weight of rat per day. The bottles containing the drinking fluid were graduated at 5 ml. intervals. Fluid consumption was therefore measured daily to the nearest 5 ml. and was expressed as ml. fluid drunk per 100 g. weight of rat per day.

The faeces produced in a cage were sifted from the sawdust, twice weekly, through a No. 6 sieve. The weight of faeces produced was expressed as a percentage of the weight of food eaten in the corresponding period.

Mean values for the consumption of food and fluids and for the output of faeces, and the standard errors of these means were obtained for the rats of each sex in each group, and the corresponding mean values for control and sulphite treated rats were compared by means of *t* tests.

Tests of ability to reproduce. The 50 rats with which the experiment began were designated Generation I. Male and female rats from group II, Generation I, which had been treated with sulphite (750 p.p.m. SO_2) for 11 weeks were paired and each pair was left to run in a separate cage for a week. Males and females from control group I, Generation I, were similarly paired and mated. If no litter resulted from the first mating period, a second period of 2 weeks was allowed. The litters resulting from these matings of Generation I constituted Generation II (i). Newborn rats were weighed individually and the six heaviest were retained; this was to reduce the burden of suckling for the mother. Weaning was effected 20 to 25 days after birth, when the newly weaned were segregated according to sex. Six to 8 weeks after the birth of Generation II (i), the same pairs of rats from Generation I were again mated, allowing not more than two periods each of 2 weeks duration: the resulting litters constituted Generation II (ii).

The members of Generation II (i) were paired and mated within their dose groups when they had attained their plateau weights. Not more than two periods of 2 weeks were allowed for this purpose. The litters so produced constituted Generation III. They were weaned 21 days after birth. All the young were retained for suckling tests in Generation II (ii) and Generation III.

Postmortem and histological examinations. Weighed rats were killed with ether. The heart, lungs, liver, spleen, adrenals, testes or ovaries, seminal vesicles or uterus, and the kidneys, were removed and weighed. Parts of these, the stomach, ileum, colon, a gastrocnemius muscle and a sciatic nerve were fixed in formol saline for not less than three days before they were blocked in paraffin. By routine, all sections were stained in Ehrlich's haematoxylin and eosin except for those of sciatic nerve for which a modified Weigert-Pal technique was used with 1 per cent neutral red as the counter stain.

RESULTS

Sodium metabisulphite has been added to the drinking water of rats throughout an experiment lasting for 2½ years and extending over three generations of animals. The concentrations of metabisulphite used corresponded to 350 and 750 p.p.m. SO_2. Observation has been made of the effect of these concentrations of metabisulphite on growth, food consumption, fluid intake, faecal output, reproduction, lactation and the incidence of tumours. Water drinking rats of Generation II were derived from experimental matings between water drinking rats of Generation I. Sulphite treated rats of Generation II were the product of experimental matings between rats of Generation I which had received sulphite (750 p.p.m. as SO_2) in their drinking water from the start of the experiment, throughout mating, pregnancy and lactation, and until they were subjected to histological examination. Rats of generation III were similarly derived from Generation II.

The Effect of Metabisulphite in the Drinking Water on Growth

Curves relating body weight in g. to postnatal age in days were linear for this strain of rats from shortly after weaning until the 8th to 12th week of life; then, rather abruptly, as the animals matured sexually, the curves flattened to a plateau on which growth advanced only slowly. It was therefore possible to measure the growth rate of each animal as the slope of the steep part of this curve, and to compare the growth rates of water drinking and sulphite-treated animals in each generation by mean slopes (Table I, column 4). Males and females have been entered separately since the males grew the faster. There was no significant difference between the growth rates of the water drinking rats and those which received sulphite in their drinking water in any generation, but the growth rate of the stock as a whole accelerated from one generation to the next. This was more marked amongst the females, and was accompanied by shortening of the steep linear part of the curve relating body weight to time. Thus, whereas female rats of Generation I grew rapidly for the

TABLE I

THE EFFECT OF SULPHITE IN THE DRINKING WATER ON THE GROWTH, THE INTAKE OF FOOD AND FLUIDS AND THE FAECES OF RATS

Generation	Sex	Treatment	Slope of growth curve Mean ± SE	Values per 100 g. rat per day Means ± SE		Faeces weight as per cent of food intake Mean ± SE
				Food intake g.	Fluid intake ml.	
I	Male	Water	5·89 ± 0·38 (5)*	7·2 ± 0·5 (22)†	9·3 ± 0·5 (22)†	22·7 ± 0·46 (22)†
		SO_2 750 p.p.m.	5·01 ± 0·39 (6)	6·7 ± 0·4 (22)	9·8 ± 0·6 (22)	22·2 ± 0·42 (22)
	Female	Water	2·45 ± 0·13 (12)	6·9 ± 0·3 (22)	10·4 ± 0·4 (22)	21·4 ± 0·42 (22)
		SO_2 375 p.p.m.	2·55 ± 0·10 (13)	7·1 ± 0·3 (22)	11·3 ± 0·5 (22)	21·3 ± 0·33 (22)
		SO_2 750 p.p.m.	2·21 ± 0·12 (13)	7·1 ± 0·3 (22)	12·0 ± 0·5 (22)	22·0 ± 0·36 (22)
II (i)	Male	Water	6·19 ± 0·42 (12)	10·1 ± 0·8 (22)	13·1 ± 0·9 (22)	19·9 ± 0·63 (20)
		SO_2 750 p.p.m.	5·18 ± 0·24 (11)	9·7 ± 0·6 (22)	15·4 ± 1·1 (22)	20·9 ± 0·67 (21)
	Female	Water	3·55 ± 0·27 (8)	11·6 ± 1·4 (17)	16·3 ± 2·3 (17)	20·3 ± 0·46 (11)
		SO_2 750 p.p.m.	3·23 ± 0·38 (6)	8·9 ± 0·6 (21)	15·0 ± 0·9 (21)	19·8 ± 0·73 (17)
II (ii)	Male	SO_2 750 p.p.m.	6·41 ± 0·57 (7)	8·6 ± 0·7 (18)	15·4 ± 1·0 (18)	24·2 ± 0·70 (18)
	Female	SO_2 750 p.p.m.	4·12 ± 0·14 (13)	9·4 ± 0·5 (15)	15·8 ± 0·9 (15)	21·2 ± 0·66 (15)
III	Male	Water	6·94 ± 0·51 (10)	13·6 ± 1·3 (9)	17·7 ± 1·2 (9)	19·9 ± 0·58 (9)
		SO_2 750 p.p.m.	6·81 ± 0·36 (5)	13·5 ± 1·3 (9)	19·8 ± 1·6 (9)	20·5 ± 0·89 (9)
	Female	Water	5·34 ± 0·15 (9)	13·9 ± 1·3 (6)	19·3 ± 1·6 (6)	21·1 ± 0·20 (6)
		SO_2 750 p.p.m.	5·16 ± 0·12 (8)	14·7 ± 1·3 (7)	23·3 ± 2·0 (7)	23·6 ± 0·92 (6)

* Number of animals.
† Number of observations.

first 80 days of life, those of the third generation reached the end of this phase in 60 days. The plateau weights of the stock (Table II) did not alter significantly. There was no significant difference between the adult weights of water drinking and sulphite drinking male rats in any generation. By contrast, the female sulphite drinking rats of Generations II (ii) and III grew at a normal rate (Table I) but matured more rapidly than the water drinking controls, so that the plateau weights of the sulphite drinking females were significantly lower than those of the water drinking females in the third generation, (t calc. = 2·5, where $n = 15$: P = $<0{\cdot}05$).

TABLE II

COMPARISON OF THE WEIGHTS OF FULL GROWN RATS OF THE FIRST, SECOND AND THIRD GENERATION TREATED AND UNTREATED WITH METABISULPHITE IN THE DRINKING WATER

Generation	Treatment	Mean body weight in g. ± S.E.	
		Male	Female
I	Water	530·0 ± 20·2 (5)*	301·7 ± 9·4 (12)*
	SO_2 375 p.p.m.		313·9 ± 8·7 (13)
	SO_2 750 p.p.m.	471·7 ± 27·1 (6)	295·4 ± 9·2 (13)
II (i)	Water	498·2 ± 28·4 (11)	320·0 ± 7·6 (8)
	SO_2 750 p.p.m.	430·5 ± 24·0 (11)	310·0 ± 10·5 (7)
(ii)	Water		353·3 ± 9·3 (3)
	SO_2 750 p.p.m.	494·3 ± 21·3 (7)	295·8 ± 7·5 (13)
III	Water	442·5 ± 10·7 (10)	287·2 ± 5·7 (9)
	SO_2 750 p.p.m.	428·0 ± 17·3 (5)	269·4 ± 5·5 (8)

* Number of animals.

The Effect of Metabisulphite in the Drinking Water on the Consumption of Food and Fluid

The spontaneous intake of food was unaffected by the addition of metabisulphite (to 750 p.p.m. as SO_2) to the drinking water (Table I, column 5) throughout three generations.

The female rats of the first generation which received metabisulphite (750 p.p.m. as SO_2) in their drinking water had a significantly higher fluid intake than did those rats of the same generation which drank only water (t calc. = 2·3, $n = 42$, P = $< 0{\cdot}05$), but this difference was not maintained amongst the females of later generations and was absent throughout amongst the males (Table I, column 6).

There was a progressive increase in the consumption of food and of fluid from generation to generation in the stock as a whole, so that rats of the third generation ate and drank twice as much during rapid growth as did those of the first generation. This difference can be correlated with the steady increase in the growth rate of the stock (see above).

The Effect of Metabisulphite in the Drinking Water on the Output of Faeces

The net weights of faeces excreted by each group of rats expressed as a percentage of the weight of food eaten during the period of faecal collection, followed by the standard errors of the means, are shown in Table I, column 7. There was no difference for the values for normal male and female rats, or between the generations. The only difference appearing between comparable groups of water drinking and sulphite-treated animals

was amongst the females of Generation III, where mean percentage found for the sulphite-drinking female significantly exceeded that for the group of water drinking controls (t calc. = 2·2, n = 11, P = < 0·05). This difference may have reflected a greater water content in the stools or a lesser degree of digestion or absorption of foodstuffs by the groups receiving sulphite (750 p.p.m. as SO_2) in the drinking water: in either case greater than normal speed of passage through the intestines is indicated.

The Effects of Metabisulphite in the Drinking Water on Fertility

The fertility of sulphite-treated animals was compared with that of control animals by comparing the number of periods paired animals had to be run together to produce pregnancies and the number of young per

TABLE III

THE EFFECT OF METABISULPHITE (750 P.P.M. AS SO_2) IN THE DRINKING WATER ON THE REPRODUCTION OF RATS

	Sulphite-treated rats	Control rats
Generation I		
First reproduction test		
Number of matings required/litter	1·2 ± 0·20 (5)	1·5 ± 0·07 (6)
Number of young/litter	12·6 ± 2·04 (5)	10·7 ± 1·66 (6)
Number surviving lactation/litter (six only returned to each mother)	3·6 ± 1·17 (5)	4·5 ± 1·12 (6)
Second reproduction test		
Number of matings required/litter	1·0 ± 0·00 (3)	1·0 ± 0·00 (2)
Number of young/litter	9·0 ± 0·58 (3)	10·5 ± 1·50 (2)
Number surviving lactation/litter (all the young were retained)	6·7 ± 1·50 (3)	2·5 ± 0·50 (2)
Generation II		
Single reproduction test		
Number of matings required/litter	1·3 ± 0·33 (3)	1·3 ± 0·33 (3)
Number of young/litter	8·3 ± 2·85 (3)	14·6 ± 2·19 (3)
Number surviving lactation/litter	4·3 ± 3·38 (3)	6·3 ± 3·48 (3)

All results are expressed as means ± S.E. (number of pregnancies).

litter. Table III shows that there was no difference in the numbers for the sulphite-treated and control pairs of Generation I. The young of litters from the first mating of Generation I were weighed within 24 hours of birth, and only six young were returned to each mother. Six to 8 weeks after the birth of the first litter (Generation II, (i)) some of the same pairs of rats from Generation I were remated. All the young born remained with the mothers and were not handled for the first week of life (Generation II (ii)). Reduction in the handling of the young did not increase the proportion of those surviving lactation. The chief cause of loss was cannibalism by the mother. This was most marked amongst the water drinking animals.

A similar fertility test was made using rats of Generation II (i). Again there was no significant difference for rats of these two groups. All the young were weighed within 24 hours of birth, and were returned to their mothers. The proportion of the young surviving to the end of lactation did not differ significantly between control and sulphite-treated animals. The weights of the young are compared in Table IV. A significant difference appears (P = < 0·05) between the two groups of young at birth

after the first mating of Generation I, but this does not recur in the larger samples of young born to Generation II (i).

The growth of the young during the first ten days did not differ for sulphite-treated and control animals either in the first reproduction test on Generation I or in the reproduction test on Generation II (i). An apparent difference shown in the second test on Generation I should be attributed to a failure of lactation in the control water drinking animals (Table IV).

The Effect of Metabisulphite in the Drinking Water on the General Health of Rats

The general health of the control and sulphite-treated animals remained excellent throughout the first 9 months of the experiment. Occasional

TABLE IV

THE EFFECT OF METABISULPHITE IN THE DRINKING WATER SUPPLIED TO RATS ON THE YOUNG BORN TO THEM

	Treated rats 750 p.p.m. as SO_2	Control rats
Generation I		
First test		
Weight of newborn in g.	7·2 ± 0·23 (23)	5·8 ± 0·14 (14)
Weight of young at 10 days in g.	21·2 ± 1·97 (11)	19·6 ± 1·64 (6)
Second test		
Weight of young at 10 days in g.	20·2 ± 0·50 (24)	14·1 ± 1·46 (6)
Generation II (i)		
Weight of newborn in g.	6·7 ± 0·12 (25)	6·4 ± 0·11 (44)
Weight of young at 10 days in g.	19·8 ± 0·92 (15)	20·2 ± 1·27 (23)

All results are means ± S.E. (number of animals).

snuffles and sticky eyes developed, but these were as frequent amongst the controls as the experimental animals.

An epidemic respiratory infection broke out in the tenth month of the trial. This killed some of the rats of the first generation, but nearly all of those of the second and third generations recovered. Incidence of infection was spread evenly between the control and sulphite-treated animals. The latter could not, therefore, be claimed to show either increased or decreased susceptibility to the infection. There were as many deaths among the control as among the experimental animals.

Postmortem and histological findings. Four or five rats were selected at random from each group in Generation I for postmortem and histological examination of their organs a few days after the onset of the respiratory infection in the tenth month of the experiment. Metabisulphite treatment was maintained throughout the infection and thereafter. All survivors of the epidemic were kept until they had either reached 2 years of age or developed a well established tumour, then they were killed and examined.

There were no significant differences between the body weights of rats killed at 10 months and at 2 years of age. Therefore the weights of all animals subjected to postmortem examination are shown together in Table V combined according to the treatment they had been given. The

drinking of sodium metabisulphite solution (750 p.p.m. SO_2) throughout life affected neither the weights of the rats (Table V) nor the percentage of the weight contributed by the various organs (Table VI).

There was no evidence of clinical polyneuritis before death, nor were spectacle eyes, blanching of the incisor teeth, or browning of the uteri evident in animals which had received 750 p.p.m. SO_2 in their drinking water. These changes were described by Fitzhugh, Knudsen and Nelson[5] in rats fed solid diets containing sulphite.

TABLE V

POSTMORTEM WEIGHTS OF ADULT RATS IN G.

Sex	Drinking fluid supplied throughout life		
	Water	Solution of sodium metabisulphite	
		375 p.p.m. SO_2	750 p.p.m. SO_2
Females	409 ± 13·1 (20)*	360 ± 25·5 (10)	408 ± 10·0 (22)
Males	636 ± 26·1 (13)	—	599 ± 23·3 (18)

* Number of animals

TABLE VI

POSTMORTEM WEIGHTS OF THE ORGANS OF ADULT RATS EXPRESSED AS MEAN PERCENTAGE BODY WEIGHT ± S.E.

Organ	Drinking fluid supplied throughout life		
	Water	Solution of sodium metabisulphate	
		375 p.p.m. SO_2	750 p.p.m. SO_2
FEMALES			
Liver	4·1 ± 0·15 (20)*	3·8 ± 0·23 (10)*	3·8 ± 0·13 (22)*
Heart	0·45 ± 0·06 (20)	0·52 ± 0·05 (10)	0·48 ± 0·02 (22)
Spleen	0·45 ± 0·08 (20)	0·38 ± 0·05 (10)	0·31 ± 0·02 (22)
Kidneys ..	0·91 ± 0·08 (20)	0·99 ± 0·64 (10)	0·86 ± 0·03 (22)
Lungs	1·48 ± 0·28 (20)	2·08 ± 0·84 (10)	1·12 ± 0·19 (22)
Adrenals ..	0·03 ± 0·002 (20)	0·04 ± 0·004 (10)	0·03 ± 0·03 (22)
Uterus	0·34 ± 0·03 (20)	0·21 ± 0·15 (10)	0·38 ± 0·08 (22)
MALES			
Liver	3·40 ± 0·02 (13)	—	3·30 ± 0·09 (18)
Heart	0·40 ± 0·03 (13)	—	0·49 ± 0·04 (18)
Spleen	0·27 ± 0·01 (13)	—	0·23 ± 0·01 (18)
Kidneys	0·92 ± 0·08 (13)	—	0·84 ± 0·02 (18)
Lungs	0·84 ± 0·09 (13)	—	1·02 ± 0·01 (18)
Adrenals ..	0·02 ± 0·002 (13)	—	0·014 ± 0·001 (18)
Testes	0·73 ± 0·06 (13)	—	0·69 ± 0·06 (18)

* Number of animals.

Histological examination was made of the tissues of thirteen females and four males from each group, water drinking and sulphite drinking (750 p.p.m. as SO_2), 10 months after the beginning of treatment. No abnormalities were found in the spleens, adrenal glands, stomachs, ileums, colons, gastrocnemius muscles, sciatic nerves, uteri or testes or in the seminal vesicles of any of these animals. The nine cases of focal nephtitis and two of cloudy swelling of the liver were almost evenly distributed between sulphite treated and control animals. These changes should most probably be ascribed to the infection prevalent at that time because

there was evidence of acute infection in the lungs of every one of these animals irrespective of their treatment group. None had developed tumours.

Thirty-seven per cent of the 54 animals kept for 2 years developed tumours. The incidence was higher among the females (44 per cent) than among the males (27 per cent), and was unaffected by the addition of sodium metabisulphite (750 p.p.m. as SO_2) to the drinking water. Of 16 females drinking water, one developed a lipoma, two had tumours of lymphoid type, and three tumours which had arisen from the duct of a sebaceous gland. Among the 16 females drinking sulphite in their water, three developed lymphoid tumours, four had tumours arising from sebaceous ducts, and one a fibroid. There were two lymphoid tumours, one duct tumour and one lipoma among nine untreated males; two lymphoid tumours arose in a group of 13 sulphite drinking males.

The full histological examination of the tissues of the 54 animals kept for 2 years under test revealed no abnormalities of the spleens, adrenal glands, ileums, colons, gastrocnemius muscles, sciatic nerves, ovaries or testes, or of the uteri or seminal vesicles, except when these were involved in a growth. Twenty-seven cases of excess fat, three of cloudy swelling and seven of infiltration of the portal tracts in liver were evenly distributed among the males and females of both control and treatment groups. So were the 28 cases of chronic and seven of acute infection of lung. Renal scarring with old nephron damage was found in nineteen animals of either sex, spread evenly over control and treatment groups. Hyaline degeneration of heart muscle was confined to two treated and two untreated males. Two small acute gastric ulcers were found in water drinking males and one in a sulphite drinking male. Evidence of hyperplasia of the gastric mucosa, which occurred when rats were fed a solid diet containing 650 p.p.m. SO_2[5], was absent.

Discussion

The toxicity of sodium bisulphite (615 p.p.m. as SO_2) mixed in a solid diet was only in small part prevented by biweekly injection of 100 μg. aneurine to each rat[5]. The residual toxicity could have been due to the sulphite itself or to the products of its interaction with constituents of the solid food. The first of these two possibilities has now been excluded because sodium metabisulphite (750 p.p.m. as SO_2) in drinking water has proved non-toxic and the weight of fluid drunk has exceeded that of diet eaten by each generation of rats.

This work was undertaken during the tenure, by I. L. Natoff, of a studentship for training in research, presented by Carter and Co. Ltd., who also defrayed the expenses of the work.

References

1. Williams, Waterman, Keresztesy and Buchman, *J. Amer. chem. Soc.*, 1935, **57**, 536.
2. Morgen, Kimmel, Field and Nichols, *J. Nutrition*, 1935, **9**, 369.
3. Morgen, *Amer. J. Public Health*, 1935, **25**, 328.
4. Paveck, *Industr. Engng Chem.*, 1946, **38**, 835.
5. Fitzhugh, Knudsen and Nelson, *J. Pharmacol.*, 1946, **86**, 37.