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PHARMACOKINETICS OF BROMIDE ION—AN OVERVIEW

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Abstract—The recent role of bromide as a residue in food and water necessitated its toxicological investigation. Although much was known already about the absorption, distribution and elimination of bromide, more data were required, especially for the rat. The pharmacokinetics in the rat were therefore investigated, with emphasis on cumulation and on the role of chloride in bromide elimination. This review recapitulates old and recent findings on the fate of bromide.

Introduction

Bromide was discovered and recognized as an element in 1826 by Balard, much to the annoyance of Liebig, who in 1825 had himself isolated it from Kreuznach mineral water but had considered it to be a compound of iodine and chlorine, thus missing the point of his own discovery (Bloch, 1929). In the second half of the last century bromide salts played an important role as anti-epileptics (Locock, 1857; Meyer & Gottlieb, 1911) and the clinical pharmacology and toxicology of bromide have been studied extensively (Goldstein, Aronow & Kalman, 1969; Maynert, 1965). By now this use is obsolete but bromide persists in many nostrums. In the course of this century bromide has been introduced increasingly into the environment as a salt-mining waste and a degradation product of fumigants. Geochemical cycles are the pharmacokinetics of Mother Earth. The very slow natural geochemical cycle of bromine has been speeded up locally by introduction of the element from the hydrosphere and lithosphere into the "technosphere" and back into the hydrosphere (Fig. 1) resulting in a redistribution towards the human environment. [The "technosphere" is the entirety of man-made constructions which are not directly derived from the biosphere but are a product of industrial processes.]

The new role of bromide as a residue in food and water necessitated its broad toxicological evaluation.

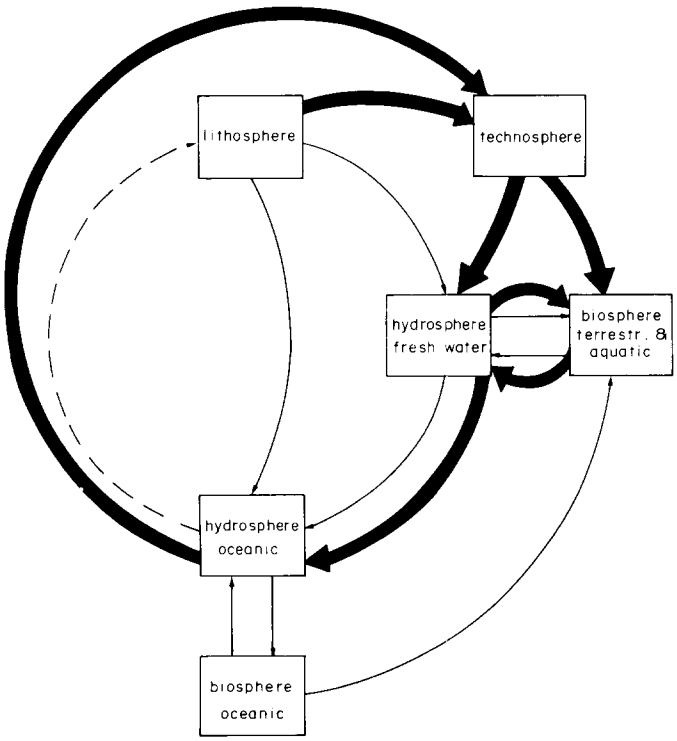

Fig. 1. Schematic representation of the geochemical cycle of bromide and its modification by industrial activities.

To reduce the uncertainty in the extrapolation from rat to man a study of the pharmacokinetics of bromide in the rat was integrated into the design of the toxicity study. In addition, parallel studies were carried out to answer questions about several details like the influence of chloride intake and the predictability of steady-state levels from single-dose kinetics.

Distribution and elimination of a single dose

Around the turn of the century many important qualitative observations about the fate of bromide in the organisms were described (Meyer & Gottlieb, 1911). In the thirties it was especially Wallace and his brilliant pupil Bernard B. Brodie who described the distribution of bromide as compared to chloride (Wallace & Brodie, 1939). Earlier Mason (1936) had recognized that bromide replaces part of the extracellular chloride, the molar sum of chloride and bromide remaining constant at approximately 110 mmol/litre. The fate of bromide in the organism may be summarized as follows:

absorption: in gastro-intestinal tract, complete
distribution: like chloride ion, mainly in the extracellular fluid; penetrates blood-brain barrier
recirculation: gastro-enteral
excretion: mainly renal; competition with chloride for tubular reabsorption

The distribution of chloride and that of bromide are indeed closely analogous (Ullberg, Appelgren, Clemedson *et al.* 1964) and almost exclusively extracellular. Exceptions are the erythrocytes and the acinar cells of the gastric wall (Pitts, 1963). The volume of distribution of bromide is approximately 0.3 litre/kg, slightly larger than that of thiocyanate. In the intestine both ions (Cl^- and Br^-) are passively absorbed by the paracellular pathway. Differences between the two ions are rather subtle. The most important one lies in their passive transport; bromide is transported more readily. This leads to larger bromide/chloride ratios in saliva and gastric acid than in plasma (Mason, 1936) and conversely to lower bromide/chloride ratios in urine (Wolf & Eadie, 1950).

The chloride ions in the extracellular fluid of the central nervous system enter it mainly via the choroid plexus by an active transport system. Chloride is transported into cerebrospinal fluid more readily than bromide. Because elimination by the CSF flow is at the same rate for both ions, bromide never reaches the same CSF/plasma ratio as chloride. For the same reasons plateau levels in the central nervous system are reached later than in plasma (Rauws, 1975).

The similarity of bromide to chloride entails an important pharmacokinetic interaction; both ions compete for tubular reabsorption. The biological half-life of bromide can be decreased by administering surplus halide (e.g. chloride) ions (Langley Czerwinski, 1958). On the other hand the already long half-life of bromide, which is 12 days in the human (Söremark, 1960) and approximately 3 days in the rat (Rauws & van Logten, 1975), may be increased considerably by a salt-deficient diet (Fig. 2). In the rat, bromide half-life was prolonged to 25 days

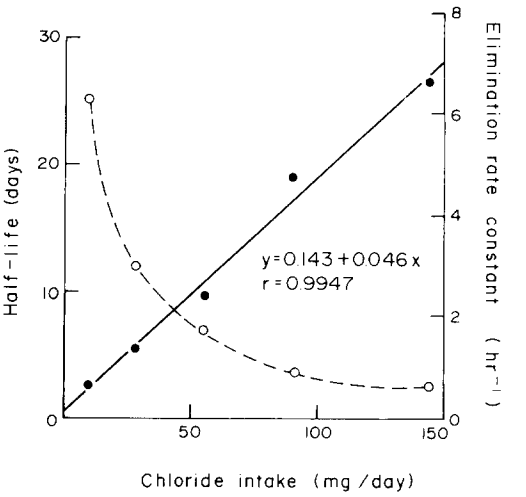

Fig. 2. Influence of dietary chloride on elimination rate of bromide in the rat: bromide half-life (○); bromide elimination rate constant(●).

on a salt-free diet with tap-water (Rauws & van Logten, 1975). Normal clearance, which was already low (2.5 ml/hr.kg), decreased under chloride depletion to approximately 0.3 ml/hr.kg.

Steady-state pharmacokinetics

In modern toxicology it is especially the effects of repeated or continuous exposure that are studied. The corresponding pharmacokinetics of the steady state has been developed by Wagner and his associates (Wagner, Northam, Alway & Carpenter, 1965) and by van Rossum (1968). The mean body load at pseudo steady state (mg/kg) is determined by dose rate D/τ (mg/kg/day) and half-life $t_{\frac{1}{2}}$ (days). In practice, the asymptotic plateau level is reached after four half-lives. Because of the long half-life of bromide, the theoretical requirement that the organism be a one-compartment system is easily satisfied. Another requirement is that the processes of absorption, distribution and elimination be linear processes. This is indeed the case over a large dosage range, as shown in the results of the 90-day toxicity study (van Logten, Wolthuis, Rauws *et al.* 1974). Only at the extremely high level of 19,200 mg/kg in the diet does a saturation of the reabsorption capacity occur, leading to a lower than proportional plasma level and an earlier plateau (Fig. 3).

Because of the linear pharmacokinetics of bromide, it is possible from the data deduced from single-dose pharmacokinetics to simulate the plasma-bromide levels at steady state on an analog computer. Although rats have less regular habits than analog computers, it has been possible to predict exactly the bromide concentration in plasma and brain at steady state, assuming a daily feeding period of 6 hours (Rauws, 1975).

In our stressful civilization a relatively large proportion of the population has to live on a diet restricted in sodium ions, which in practice also means restricted in chloride ions. As has been mentioned above, chloride depletion prolongs the elimination half-life of bromide drastically. So it was

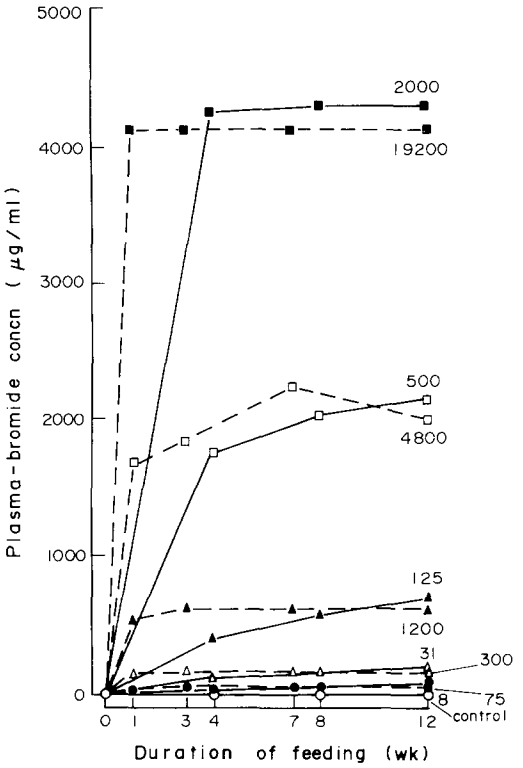

Fig. 3. Bromide accumulation in rats on a normal (---) or a chloride-depleted (——) diet containing different levels of bromide (as indicated, in mg NaBr/kg diet).

thought wise to investigate also the toxicity profile of bromide in rats on a low-chloride diet. As an integral part of the toxicity study the cumulation of bromide was followed. The results were astonishing. Under the circumstances of the experiment a ten times lower bromide level in the diet led to comparable plasma concentrations and effects (Fig. 3). One may also see that, owing to the prolonged half-life of bromide, the accumulation process takes more time, proceeding over the whole duration of the toxicity study, again with the exception of the highest dose which reached plateau levels after approximately 4 weeks (van Logten, Rauws, Kroes *et al.* 1976).

Transplacental distribution

In Ullberg's macroautoradiographic study of the distribution of halide ions in mice and rats (Ullberg *et al.* 1964), only rather vague data are given about the transplacental penetration of bromide. Transplacental distribution resulting in neonatal bromide intoxication has been described in the literature (e.g. Finken & Robertson, 1963; Pleasure & Blackburn, 1975). As an orientation, prior to embarking on studies of reproduction toxicity, the distribution of bromide in foetuses at day 20 of gestation was followed. The foetus appeared to be much more accessible to bromide than, for instance, was the mother's brain (Fig. 4). Furthermore, it seems that elimination from the foetus is much retarded in comparison with that from the mother's plasma and brain (Rauws, unpublished results 1976). Thus one

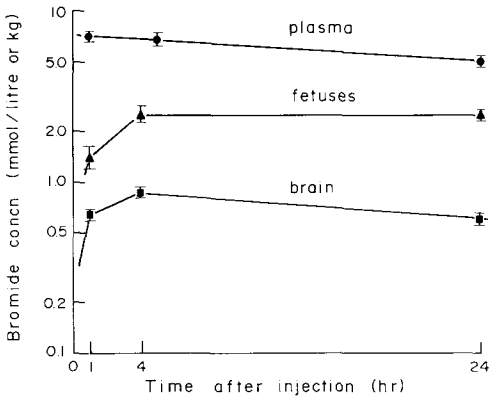

Fig. 4. Transplacental distribution of bromide in the rat, following intravenous administration of a single dose of 2.5 mmol NaBr/kg body weight on day 20 of gestation.

may expect considerable accumulation. This finding corresponds with clinical experience.

Conclusions

The integration of pharmacokinetics within the framework of the toxicological investigation has been rewarding, because it allows better interpretation and extrapolation to man. While still unusual at the start of the bromide project, in 1972, ancillary pharmacokinetics studies should be commonplace by now. Useful comments on the design and interpretation of pharmacokinetic studies in toxicology have been published in recent years (e.g. Hammer & Bozler, 1977; Hottendorf, van Harken, Madisso & Cabana, 1976; Karlog, Nielsen & Rasmussen, 1978; Smyth & Hottendorf, 1980).

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TOXICITY OF SODIUM BROMIDE IN RATS: EFFECTS ON ENDOCRINE SYSTEM AND REPRODUCTION

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Abstract—Bromide has a low acute oral toxicity, with LD_{50} values in rodents ranging from 3500 to 7000 mg/kg body weight. It is rapidly absorbed and steady-state serum levels have been reached in rats within 4 weeks. The biological half-life of bromide, and consequently the serum levels, are strongly dependent on chloride intake. Feeding of sodium bromide to rats for 90 days in concentrations of 0, 75, 300, 1200, 4800 and 19,200 mg/kg diet led to a complex of changes in the endocrine system, thyroid activation being the most prominent. Furthermore, in the highest dose groups a decrease in spermatogenesis in the testes and decreased secretory activity of the prostate or a reduction in the number of corpora lutea in the ovaries were found. A three-generation reproduction study of the same dietary concentrations showed in the two highest dose groups a decrease in fertility which appeared to be reversible upon bromide withdrawal. Macroscopically, no changes in the offspring were observed. From these studies a no-effect level for bromide ion of 240 mg/kg diet was determined, corresponding to a tentative Acceptable Daily Intake (ADI) of 0.12 mg/kg body weight. This is in good agreement with a preliminary ADI of 0.1 mg/kg established in an experiment with human volunteers, but is considerably lower than the ADI of 1 mg/kg estimated by FAO/WHO. It is suggested that bromide exerts an inhibitory effect on the thyroid, resulting in an increased hormonal stimulation of this organ by the pituitary gland.

Introduction

Bromide, a natural consitituent of plants and animals, can also be present in food commodities as a result of the use of bromine-containing compounds, like ethylene dibromide and methyl bromide, for post-harvest treatment of wheat, fruit and vegetables or as soil fumigants in intensive horticulture. Furthermore, potassium bromate is used in the baking of bread. Treatment of wheat or wheat flour (e.g. with methyl bromide) results in the formation of methylated products and bromide residue levels up to 200 mg/kg (Heuser & Scudamore, 1970; Winteringham, 1955). Studies of bromide residues in plants after soil fumigation with methyl bromide have shown that lettuce can contain bromine, present predominantly in the form of inorganic bromide, in concentrations up to 500 mg/kg produce, although the median values are considerably lower (Greve, 1983; Staarink, 1980; Wit, 1972).

The Food and Agricultural Organization and the World Health Organization have evaluated the use of bromine-containing compounds as pesticides and the toxicological data (FAO/WHO Pesticides Committees, 1965, 1967 & 1970), but no substantial toxicological information about (sub)chronic exposure to low concentrations of bromide was available. From the extensive therapeutic use of potassium bromide as a sedative and the known effects of bromism in man, an Acceptable Daily Intake (ADI) of 1 mg/kg body weight was estimated on the basis of a minimal pharmacologically effective dosage in humans of 600 mg bromide ion daily (FAO/WHO Pesticides Committees, 1967).

In our opinion, detailed toxicological information was necessary to establish a no-toxic-effect level and to evaluate the risk of bromide residues for human health. Therefore, a series of toxicity experiments has been carried out in our Institute since 1970. In this paper the results of these experiments, partly published already, and the information available in the literature are reviewed. Furthermore, additional data are presented about the effects of bromide on the endocrine and reproductive systems. Finally, the estimation of an ADI for bromide is discussed in relation to the present residue situation.

Toxicity studies

Upon oral administration, bromide exhibits a very low acute toxicity in rodent species. The oral LD_{50} has been reported as 3500 mg/kg body weight for rats (Smith & Hambourger, 1925) and as 5020 mg/kg (Voss, Haskell & Gartenberg, 1961) and 7000 mg/kg (Groff, Tripod & Meier, 1955) in mice. A short-term experiment also gave no indication of severe bromide toxicity. In this particular experiment (van Logten, Wolthuis, Rauws & Kroes, 1973), bromide was administered to Wistar rats for 4 weeks, at dietary concentrations of 300, 1200, 4800 and 19,200 mg NaBr/kg diet. Bromide appeared to be rapidly absorbed. The plasma concentration of bromide increased steeply during the first week and within 3 weeks reached a plateau level, which was directly proportional to the bromide concentration in the diet (Fig. 1). Anomalies were observed in the highest dose group only. The rats of the 19,200 mg/kg group showed signs of motor incoordination of the hind legs and depressed grooming, as a result of which the animals had a dirty appearance and a brownish fur.

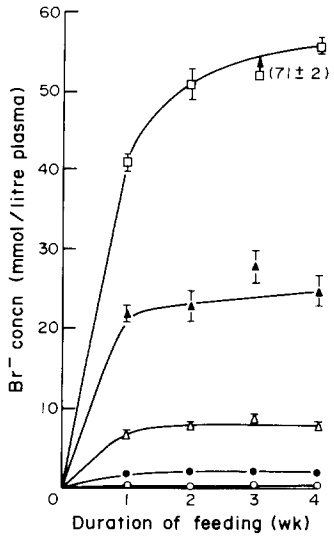

Fig. 1. Bromide concentrations in the plasma of female rats ($n = 4$) fed for 0–4 wk on diets containing 0 (control; ○), 300 (●), 1200 (△), 4800 (▲) and 19,200 (□) mg NaBr/kg diet.

Organ-weight determinations of brain, liver and kidneys at the end of the experimental period showed a significantly increased relative weight only of the kidneys. Upon histopathological examination of these organs, no evidence was found of changes attributable to the treatment. Furthermore, no changes in growth and food and water intake were observed. This was surprising since one might have expected that exposure to relatively high levels of bromide would impair the electrolyte balance, due to the replacement of 50% of the chloride in plasma, kidneys, liver and brain by bromide at the highest dose level (Fig. 2).

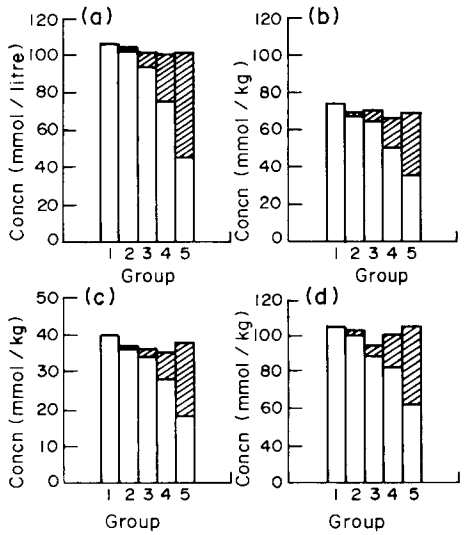

Fig. 2. Replacement of chloride □ by bromide ▨ in (a) plasma, (b) kidneys, (c) brain and (d) liver of female rats ($n = 4$) fed for 4 wk on diets containing 0 (control), 300, 1200, 4800 and 19,200 mg NaBr/kg (groups 1–5, respectively).

The results of this short-term study were largely confirmed in a 90-day experiment (van Logten, Wolthuis, Rauws *et al.* 1974) using the same dosage regimen, with the addition of a lower dose level of 75 mg NaBr/kg diet. In accordance with the previous experiment, depressed grooming and motor incoordination of the hind legs were observed only at the highest dose level. All these effects may have been due to a disturbance of the central nervous system by bromide. Histopathological examination did not reveal any evidence of changes in the musculature. In contrast to the previous short-term experiment, a significant growth retardation was observed in both sexes fed 19,200 mg NaBr/kg diet, along with a slight decrease in food conversion. Furthermore, at this dose level a slight decrease in the concentration of lymphocytes and a doubling in the concentration of neutrophilic granulocytes were found in both sexes upon haematological examination. The increase in neutrophilic granulocytes may be regarded as a stress-mediated effect, although it cannot be excluded that the effect originated from a bacterial infection as a result of the bad physical condition of the animals. The effect on the lymphocytes may be suggestive of a slight suppressive effect on the immune system by bromide, possibly indicated also by a decrease in thymus weight observed in the females.

The most prominent effects were on the thyroid and the gonads. The relative weight of the thyroid was increased in females from 1200 mg NaBr/kg onwards but in males only at the highest dose level. Males of the two highest dose groups showed a decrease in relative prostate weight. These changes were confirmed by the observation of a complex of histopathological changes in the endocrine system. In accordance with the weight change, a remarkable thyroid activation was found for both sexes in the highest dose group. In addition, a decrease in secretory activity of the prostate, a decrease in spermatogenesis and a decrease in vacuolization of the zona fasciculata in the adrenals were observed in males. Furthermore, females showed a decrease in the number of corpora lutea.

These effects, which strongly suggest an impairment of the endocrine system by bromide, have been confirmed in another 90-day experiment studying the effects of a low-chloride intake on bromide toxicity (van Logten, Rauws, Kroes *et al.* 1976). Rauws & van Logten (1975) had shown that the elimination of bromide from the circulation was strongly dependent upon chloride intake. In their study, omission of chloride from the diet caused an increase in bromide half-life by a factor of 10 compared to a normal diet. According to the steady-state concept of Wagner, Northam, Alway & Carpenter (1965) the concentration in plasma is directly proportional to the biological half-life. If exchange between plasma and target organs is rapid, the same applies for the concentration in the organs. This means that chloride withdrawal should increase the bromide concentration in plasma and tissues, and therefore the toxic action of the bromide ion. On this assumption, the bromide concentrations in the diet for the low-chloride experiment were diminished by a factor

of 10 compared to those in the normal diet study. As was expected, in this experiment bromide concentration in plasma and tissues of the corresponding groups were almost equal to those in the normal diet study. Therefore, it was not surprising that the target organs were the same and that similar toxic effects were found. In addition to the data obtained earlier, determination of the corticosterone concentration in plasma revealed a distinct decrease at the two highest dose levels. This finding fits well with the histopathological observation of less vacuolization in the zona fasciculata of the adrenals, indicative of a decreased synthesis of glucocorticosteroids. Since this process is regulated by adrenocorticotropic hormone (ACTH) released by the pituitary, the origin of the observed effects might be a dysfunction of the hypothalamus–pituitary axis. Also other morphological changes, like the decrease in corpora lutea and the impairment of spermatogenesis, and the observed growth retardation could be attributed to a decrease in pituitary function. The former might be due to a decreased secretion of gonadotropic hormones, the latter to a decreased secretion of somatotropic hormone by the pituitary gland.

However, it cannot be excluded that bromide has a direct effect on the other organs of the endocrine system. In particular, the activation of the thyroid found upon histological examination cannot be explained by a decrease in pituitary function. For a further evaluation of the effects of bromide on the endocrine system, two types of experiments have been carried out. First, as a consequence of the observed decrease in spermatogenesis and in the number of corpora lutea, the effects on reproductive performance were examined in a three-generation reproduction study, which will be reported here. Secondly, special studies of the effect of bromide on thyroid and pituitary functioning have been carried out. These studies are reported in this and the following paper (Loeber, Franken & van Leeuwen, 1983).

Reproduction study

For the reproduction study, the doses chosen were the same as those in the 90-day study (0, 75, 300, 1200, 4800 and 19,200 mg NaBr/kg diet). Male rats of proven fertility were mated with females for the first time at the age of 4 months. In three successive generations, at least two litters per female rat were raised, following the scheme depicted in Fig. 3. In the first generation a third litter was raised for the investigation of the transplacental transport of bromide. Furthermore, an additional litter was bred with parent animals of the highest dose group which were changed to the control diet in order to investigate the reversibility of the observed effects.

The results of the breeding study are given in Table 1. Since no significant differences were noted between the first and second litter per generation the results are presented as the means of both litters, unless otherwise stated. The fertility was nil in the 19,200-mg group and was markedly reduced in the 4800-mg group. In the latter group also the viability of the offspring was lower than in the other groups. A difference between the first and the second litter

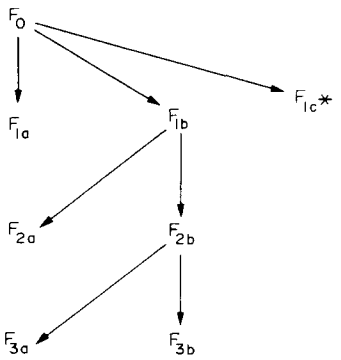

Fig. 3. Scheme of the three-generation reproduction study. F_{1a}, F_{2a} and F_{3a} litters were killed after weaning.

was found in the 4800-mg group and the results of this group are therefore given separately in Table 1. The viability of the young was greater in the second litter than in the first. Furthermore, during the lactation of the first litter all of the young alive at day 5 died before day 21. In the second litter, however, all animals alive at day 5 were still alive at day 21. Because of the diminished fertility in the two highest dose groups, second and third generations were bred only from the groups dosed with sodium bromide up to 1200 mg/kg diet. In these groups no effects related to treatment were found in the breeding results. To investigate whether infertility occurred in males or in females, a criss-cross experiment was carried out, in which untreated males and females were mated with females and males of the 19,200-mg group. Of the treated females mated with untreated males only 20% became pregnant, and none of the untreated females mated with treated males became pregnant. Therefore, the observed effects were due to infertility of

Table 1. Breeding results in reproduction study on sodium bromide fed to rats at dietary levels up to 19,200 mg/kg

Generation	Values for groups fed NaBr at dietary levels (mg/kg) of:					
	0	75	300	1200	4800	19,200
Fertility index*						
F_0	70	70	72	65	25	0
F_1	62	54	44	53	—	—
F_2	52	67	80	45	—	—
Viability index*						
F_0	90	98	96	92	32, 61†	—
F_1	92	88	80	97	—	—
F_2	96	98	93	98	—	—
Lactation index*						
F_0	95	96	95	94	0, 100†	—
F_1	93	85	72	80	—	—
F_2	99	99	99	99	—	—
Mean body weight at day 21						
F_0	40	45	43	43	—, 38†	—
F_1	41	43	40	38	—	—
F_2	36	38	38	36	—	—

*Fertility index = no. of pregnancies × 100/no. of matings; viability index = no. of pups alive at day 5 × 100/no. of pups born alive; lactation index = no. of pups alive at day 21 × 100/no. of pups alive at day 5.

†Data are given separately for first and second litter.

Table 2. Reproduction study in rats fed sodium bromide at dietary levels up to 4800 mg/kg: mean body weights and relative organ weights determined at the end of each generation

Generation	Parameter†	Values for groups fed NaBr at dietary levels (mg/kg) of: 0	75	300	1200	4800
		Males				
F_0	No./group ...	9	9	9	10	10
	Body weight (g)	422	398	383	391	362
	Adrenals	0.011	0.011	0.011	0.011	0.012
	Thyroid	0.0060	0.0057	0.0056	0.0060	0.0060
	Pituitary	0.0029	0.0029	0.0029	0.0030	0.0033
	Testes	0.680	0.745	0.776**	0.744	0.712
	Prostate	0.119	0.130	0.121	0.135	0.134
F_1	No./group ...	10	10	10	10	
	Body weight (g)	409	391	388	395	
	Adrenals	0.010	0.010	0.011	0.012	
	Thyroid	0.0063	0.0064	0.0060	0.0067	
	Pituitary	0.0026	0.0026	0.0027	0.0028	
	Testes	0.771	0.759	0.769	0.763	
	Prostate	0.077	0.093	0.093	0.102*	
F_2	No./group ...	10	10	10	10	
	Body weight (g)	438	373**	397*	378**	
	Adrenals	0.010	0.010	0.009	0.010	
	Thyroid	0.0076	0.0074	0.0079	0.0081	
	Pituitary	0.0032	0.0031	0.0027**	0.0029	
	Testes	0.787	0.821	0.679	0.793	
	Prostate	0.103	0.120	0.109	0.104	
		Females				
F_0	No./group ...	7	11	9	12	11
	Body weight (g)	254	256	249	243	249
	Adrenals	0.020	0.019	0.019	0.017*	0.017**
	Thyroid	0.0062	0.0066	0.0066	0.0073	0.0073
	Pituitary	0.0056	0.0055	0.0052	0.0052	0.0046*
	Ovaries	0.022	0.021	0.022	0.025	0.024
	Uterus	0.171	0.166	0.180	0.150	0.143
F_1	No./group ...	19	15	14	16	
	Body weight (g)	244	254	252	241	
	Adrenals	0.018	0.018	0.017	0.017	
	Thyroid	0.0073	0.0070	0.0074	0.0083	
	Pituitary	0.0047	0.0052	0.0049	0.0053*	
	Ovaries	0.026	0.029	0.027	0.027	
	Uterus	0.167	0.159	0.150	0.140*	
F_2	No./group ...	10	10	10	10	
	Body weight (g)	267	244	259	241**	
	Adrenals	0.019	0.018	0.017	0.018	
	Thyroid	0.0096	0.0083	0.0094	0.0103	
	Pituitary	0.0053	0.0048	0.0050	0.0056	
	Ovaries	0.027	0.024	0.027	0.027	
	Uterus	0.188	0.160	0.179	0.164	

†All organ weights are expressed in g/100 g body weight.
Asterisks indicate means differing significantly from that of the corresponding control group: *—$0.01 \leq P < 0.05$; **—$0.001 \leq P < 0.01$.

male as well as female rats. This conclusion is in accordance with the histopathological lesions found in the testes as well as in the ovaries in the 90-day studies.

As already mentioned, the reversibility of the effects on reproduction was studied in parent animals fed a diet containing 19,200 mg NaBr/kg for 7 months followed by a control diet for 3 months before mating. In contrast to the infertility observed earlier in these animals the breeding results for this particular experiment were as follows: fertility index, 62%; viability index, 61%; lactation index, 90%. Although the viability was lower than in the control and lower dose groups, the fertility index and lactation index were similar. From these results it is clear that the effects of bromide on reproduction are reversible.

Haematological examinations carried out 3 weeks before each mating and directly after the weaning of the last litter revealed changes similar to those observed in the 90-day study. Body- and organ-weight determinations given in Table 2 did not reveal a clear pattern of dose-related effects in the successive generations. Only the adrenals of the females of the F_0-generation showed a dose-dependent decrease in relative weight.

Macroscopic examination of all pups born during the entire experimental period provided no evidence

Table 3. Bromide concentration (corrected for control values) in plasma, tissues and 20-day foetuses of female rats fed sodium bromide at 75–4800 mg/kg diet for 7 months

Dietary concn of NaBr (mg/kg)	Maternal levels of Br^-			Br^- in foetal kidneys (mmol/kg)
	Plasma (mmol/litre)	Placenta (mmol/kg)	Kidneys (mmol/kg)	
75	0.5 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
300	2.2 ± 0.1	1.4 ± 0.1	1.4 ± 0.3	0.9 ± 0.1
1200	7.8 ± 0.9	6.3 ± 1.5	4.4 ± 1.1	3.2 ± 0.8
4800	27.6 ± 2.8	16.7 ± 1.5	15.3 ± 1.4	11.0 ± 0.6

Values are means ± SD for groups of seven animals.

of anomalies, although it is known that bromide easily crosses the placenta. The results given in Table 3, obtained during the raising of the F_{1c}-litter, show that foetuses *in utero* must have been exposed to bromide, since the concentration of bromide in the kidneys of corresponding dams and foetuses is almost equal.

Due to the sedative nature of the compound and the absence of a distinct blood–brain barrier in the developing foetus, effects on behaviour can be expected, particularly since pharmacokinetic studies have shown that the foetus is more accessible than the mother to bromide (Rauws, 1983). Therefore, it is not surprising that, as long as 40 years ago, Harned, Hamilton & Cole (1944) found a decreased learning rate in the offspring of dams dosed with 120 mg NaBr/kg body weight/day during gestation.

Special studies

Besides the effects on fertility and viability, the most prominent effect observed in the reproduction study was a decrease in thyroid hormone (T_4) concentration in the serum of the parent animals of the F_0-generation. Figure 4 shows a significant dose-related decrease, which was found for male rats in all groups after 6 weeks. Similar effects were found after 12 weeks but were less pronounced at the lower dose levels. This finding is indicative of an inhibitory action of bromide on the synthesis of thyroid hormones, resulting in a physiological feed-back mechanism of increased thyrotropic-hormone (TSH) secretion by the pituitary gland, causing an increased stimulation of the thyroid. This is in good agreement with the activation of the thyroid found by histological examination in the 90-day studies.

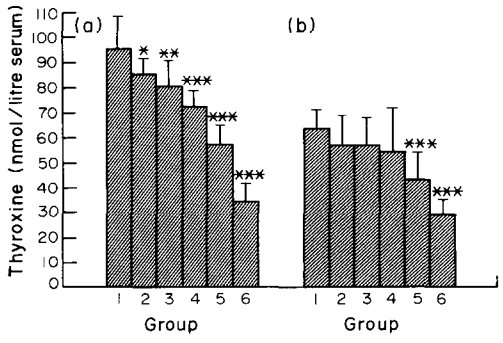

Fig. 4. Serum thyroxine concentrations in (a) male and (b) female rats ($n = 10$) fed for 6 wk on diets containing 0 (control), 75, 300, 1200, 4800 and 19,200 mg NaBr/kg (groups 1–6, respectively). Asterisks indicate means (± 1 SD) differing significantly from that of the control group: *— $0.01 \leqslant P < 0.05$; **— $0.001 \leqslant P < 0.01$; ***— $P < 0.001$.

The decrease in thyroid hormones at high dose levels was confirmed in an experiment on the time dependency of the effect of bromide on the thyroid. Using standard diets containing 4800 and 19,200 mg NaBr/kg, significantly decreased thyroxine concentrations were found in both groups. From this experiment, it appeared that after only 3 days (the first time of sampling) the thyroxine concentration in serum was significantly decreased and that it remained constant during an experimental period of 12 weeks.

The effect of bromide on thyroid function was also studied using a 'chloride-free' diet, with sodium bromide concentrations of 0, 125, 500 and 2000 mg/kg diet. The concentration of thyroxine in serum was significantly decreased in the two highest dose groups. This was in accordance with the previous experiment, in which it was shown that 500 and 2000 mg NaBr/kg added to a 'chloride-free' diet gave rise to similar serum-bromide concentrations in rats as did 4800 and 19,200 mg NaBr/kg added to normal diet (van Logten *et al.* 1976).

Furthermore, in the 'chloride-free' experiment the uptake of radiolabelled iodide by the thyroid was measured 6, 24 and 48 hours after a single intraperitoneal injection of iodine-131 (Fig. 5). At 500 mg NaBr/kg diet a significantly increased iodine uptake was observed, whereas at 125 and 2000 mg/kg the iodine uptake was only slightly increased. For an activated thyroid an increased uptake and release of iodine is generally expected. However, as can be seen from Fig. 5, the effect in this case appeared to be biphasic. At 500 mg/kg the uptake was greater than with 2000 mg/kg; in the latter group the uptake was less and the release, measured between 24 and 48 hours, seemed to be enhanced. This phenomenon can probably be explained by two opposite effects of bromide on the thyroid. Since it is known that bromide, as a halogen, competes with iodide for uptake in the thyroid and can replace iodide in thyroid hormones (Jorgensen, 1978), the synthesis of the physiological hormone thyroxine might be decreased. This would lead to an enhanced stimulation of the thyroid by the pituitary gland. At 500 mg/kg, this process causes a marked increase of iodide uptake by the thyroid. However, at 2000 mg/kg the

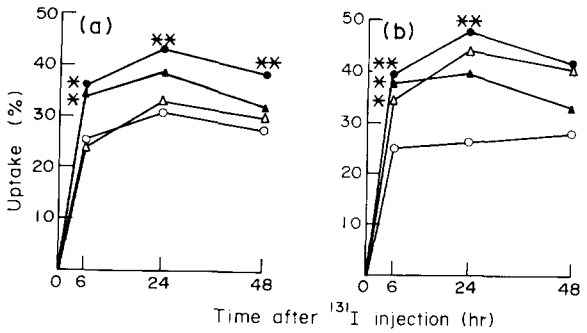

Fig. 5. Uptake of ^{131}I by the thyroid of male (a) and female (b) rats ($n = 8$) fed NaBr at dietary levels of 0 (control; ○), 125 (△), 500 (●) and 2000 (▲) mg/kg in a chloride-free diet for 90 days. Asterisks indicate values differing significantly from the corresponding control value: *— $0.01 \leqslant P < 0.05$; **— $0.001 \leqslant P < 0.01$.

bromide concentration in the serum is probably so high in relation to that of iodide that even an activated thyroid takes up relatively more bromide than iodide. Therefore, in this dose group the iodide uptake by the thyroid may well be less than in the 500-mg/kg group, although the release is faster. In this case, however, the additional possibility of a diminished stimulatory action of the pituitary at the highest dose level cannot be excluded. To elucidate this effect of bromide, detailed studies on the pituitary–thyroid axis are presented in the next paper (Loeber *et al.* 1983).

Evaluation

Although bromide has a very low acute oral toxicity, a striking complex of presumably related changes in the endocrine system was observed on subchronic administration. The most prominent alteration appeared to be the effect on the thyroid, found histopathologically and by the measurement of circulating thyroid hormones. Also in the three-generation reproduction study the decrease in thyroid hormones was the most sensitive criterion, although recent experiments (Loeber *et al.* 1983) could not confirm the effects at dietary concentrations lower than 1200 mg/kg. Therefore, on the basis of the effect of sodium bromide on the thyroid in the 90-day study, a no-effect level of 300 mg/kg diet can be determined in the rat. For the bromide ion this value corresponds to a no-effect level of 240 mg/kg diet. When a safety factor of 100 is applied to this value, a tentative ADI of 0.12 mg/kg body weight can be calculated. This is considerably less than the ADI of 1 mg/kg estimated by the FAO/WHO Pesticides Committees (1967).

However, in an experiment carried out in our Institute with human volunteers dosed with 1 mg Br^-/kg daily for 8 weeks no changes were found in haematological, biochemical or endocrinological parameters (Sangster, Kranjc, Loeber *et al.* 1982). The plasma-bromide levels in the human volunteers appeared to be about 0.9 mmol/litre. This is approximately 10% of the bromide concentration of 8 mmol/litre found to induce alterations in the thyroid of rats. This latter concentration lies within the therapeutic range of bromide (6–12 mmol/litre) in man (Wade, 1977).

On the basis of the human study in which no effect was detected, and with application of a safety factor of 10, a preliminary ADI can be determined of 0.1 mg/kg. This value is very close to the 0.12 mg/kg calculated from the rat study. This similarity in ADI estimated from the results of the two different experiments and the particular nature of the compound justify the use of the minimal safety factor of 100 which was applied to the animal data. In general, the use of this safety factor is assumed to be inadequate when no information about the chronic toxicity of a compound is available.

For a person of 60 kg, the preliminary ADI of 0.1–0.12 mg/kg will correspond to an acceptable total bromide intake of about 6–7 mg/day. Greve (1983) has already shown that the average bromide uptake in The Netherlands in the years 1976 and 1978 was 7.6 and 7.8 mg/day, respectively, with a range of 2–17 mg/day. It is not known whether recent measures to minimize the bromide residues in vegetables (restriction of the use of methyl bromide and leaching of the greenhouses after soil fumigation) have led to a lower daily intake of bromide during the last few years. The margin between the acceptable and the actual bromide intake, however, is small, particularly when the strong dependency of bromide toxicity on chloride intake is taken into account. This necessitates additional information in order to establish a definite ADI. Because of the reversibility of the observed effects of bromide in rats and the extensive clinical experience with bromide, an experiment with human volunteers seemed to be the most direct and relevant procedure to evaluate the risks of bromide for human health. Furthermore, a comparison between human experiments and the usual animal experiments facilitates the evaluation of extrapolation from animal to man and the concept of the safety factor. Therefore, an additional experiment with human volunteers has been carried out recently. The results of this are reported in one of the following papers (Sangster, Blom, Sekhuis *et al.* 1983).

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EFFECT OF SODIUM BROMIDE ON ENDOCRINE PARAMETERS IN THE RAT AS STUDIED BY IMMUNOCYTOCHEMISTRY AND RADIOIMMUNOASSAY

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Abstract—Male rats were fed a normal or sodium bromide-enriched diet for 4 or 12 weeks. Sodium bromide concentrations were 0, 20, 75, 300, 1200 and 19,200 mg/kg diet. At the end of the experiments the pituitary gland, thyroid and testes were examined by histopathological and immunocytochemical techniques, while serum hormone levels were established by radioimmunoassay. Histopathological examination revealed an activation of the thyroid and a decreased spermatogenesis in the testes in the highest dose group. Using immunocytochemical techniques a decrease was noted in the amount of thyroxine in the thyroid. No effect was found in growth hormone-producing cells in the pituitary gland, while immunoreactivity for thyroid-stimulating hormone and for adrenocorticotropic hormone was increased. The concentration of thyroxine, testosterone and corticosterone in the serum appeared to be decreased. Due to feedback regulation, the pituitary gland was stimulated to produce and release thyroid-stimulating hormone, follicle-stimulating hormone, adrenocorticotropic hormone and insulin, whereas the release of growth hormone was suppressed. Most of these changes were restricted to rats on the highest treatment level. It is concluded that sodium bromide, at least in high doses, directly disturbs the function of the thyroid, testes and adrenals.

Introduction

Bromide is a natural element of soil, plants and animals. The use of bromide-containing food additives and pesticides, however, may also contribute significantly to the bromide content of foodstuffs.

Several years ago studies were carried out in this Institute to determine the toxicity of bromide (van Logten, Rauws, Kroes *et al.* 1976; van Logten, Wolthuis, Rauws & Kroes, 1973; van Logten, Wolthuis, Rauws *et al.* 1974). From a semichronic feeding study in rats it was concluded that bromide acts primarily on the endocrine system, as evidenced by weight changes and histopathological findings. Thus, in male rats, sodium bromide, apart from retarding growth, appeared to induce an increase in the relative weights of thyroid and adrenals and a decrease in the relative prostate weight. Histopathological examination revealed an activation of the thyroid, characterized by a reduction in follicle size along with an increase in the height of the follicular epithelium and a decrease in vacuolization in the zona fasciculata of the adrenals indicative of an increase in corticosteroid release and/or diminished synthesis. In addition, spermatogenesis was decreased in the testes and less secretory activity of the prostate was observed, suggesting a diminished production of gonadotropic hormones (van Logten *et al.* 1974)

The development of immunocytochemical methods and radioimmunoassay systems for hormone determinations now facilitates the more direct localization and characterization of alterations in the endocrine system of the rat. Therefore, the present study was initiated to ascertain whether such alterations could be detected in male rats after exposure to high dietary concentrations of sodium bromide and, moreover, whether histopathological and immunocytochemical findings could be correlated with serum-hormone levels. Furthermore, a range of lower dietary concentrations of sodium bromide was studied to investigate whether a previously observed decrease in the serum-thyroxine level (van Leeuwen, den Tonkelaar & van Logten, 1983) could be confirmed.

Experimental

Experimental design

Inbred male Wistar rats (Riv: TOX[M]), weighing 60–100 g, were used throughout. They were housed in stainless-steel wire-mesh cages, two rats/cage, at $22 \pm 2°C$ and $50 \pm 5\%$ relative humidity and with a light period of 8 hours (9.00 a.m.–5.00 p.m.). The animals were fed a semi-synthetic, purified diet (Muracon SSP-Tox standard flour) obtained from Trouw and Co. (Putten).

Sodium bromide (purity 99.5%) supplied by J. T. Baker Chemicals (Deventer) was mixed with the diet to give final concentrations of 0, 20, 75, 300, 1200 and 19,200 mg/kg. Separate controls were used for the groups on the latter diet. Each test and control group for each exposure period consisted of ten rats. Diets and tap-water were available *ad lib.* During the experiments the rats were handled daily to diminish possible stress effects (Döhler, Gärtner, von zur Mühlen & Döhler, 1977). After 4 and 12 weeks, animals were decapitated and desanguinated between 9.00 and 11.00 a.m. In a separate 12-week experiment, five animals receiving 19,200 mg NaBr/kg diet

and five control animals were submitted to a release test using thyrotropin-releasing hormone (TRH; Beckman, Mijdrecht) in a dose of 1 μg/kg body weight, as described by Chihara, Kato, Ohgo *et al.* (1976). Five minutes after intravenous injection of the TRH the rats were decapitated and desanguinated. Following clotting of the trunk blood, the serum was harvested and kept at −20°C prior to use for the hormone assays.

Immunocytochemistry

After macroscopic inspection, the pituitary gland, thyroid and testes were weighed. Subsequently, the pituitary gland was fixed in 8% (w/v) formaldehyde solution containing 4.5% (w/v) mercuric chloride (sublimate) and 0.5% (w/v) sodium chloride. The thyroid and the testes were fixed in 4% (w/v) formaldehyde in 0.067 M-Sörensen buffer, pH 6.9, and in Bouin–Holland's solution, respectively. Embedding in paraplast was performed according to conventional procedures and 5-μm sections were prepared. The sublimate was removed by treatment with lugol and sodium thiosulphate. The sections were stained with haematoxylin and eosin.

Two immunoperoxidase techniques were used for the localization of the hormones. For thyroid-stimulating hormone (TSH), growth hormone (GH) and adrenocorticotropic hormone (ACTH), immunocytochemical staining was carried out in accordance with the indirect peroxidase-labelled antibody method of Nakane & Pierce (1966) using 3,3′-diaminobenzidine and hydrogen peroxide as substrates for peroxidase (Graham & Karnovsky, 1966). The antisera used were the following:

(a) Rabbit antiserum to rat TSH (A-rat TSH-S-3) obtained through the courtesy of Dr P. G. Condliffe (National Institute of Arthritis, Metabolism and Digestive Disease (NIAMDD), Bethesda, Maryland, USA; Rat Pituitary Hormone Distribution Program).
(b) A specific anti-rat-GH serum prepared in a cynomolgus monkey, using rat growth hormone (NIAMDD-rGH-B-4). In serial sections of normal rat pituitary glands, identical results were obtained with this antiserum and the A-rat GH-S-3 antiserum of NIAMDD.
(c) A specific anti-pig-ACTH serum prepared in rabbits, using purified pig ACTH (Cortrophine-Z, Organon, Oss).

Peroxidase-labelled ovine-anti-human immunoglobulins were used for the detection of monkey-anti-GH immunoglobulins and were obtained from Cappel Laboratories (Cochranville, USA). Peroxidase-labelled ovine-anti-rabbit immunoglobulins were obtained from the Pasteur Institute (Paris, France). Immunocytochemical controls were done with normal rabbit and monkey sera obtained from the local animal colonies. All sera were diluted in phosphate-buffered saline (PBS), pH 7.2.

For thyroxine (T_4) and testosterone, the unlabelled antibody method of peroxidase-antiperoxidase (PAP) according to Sternberger (1979) was applied, using Immulok Histoset Immunoperoxidase Staining Kits (Laboratorium Service Benelux, B.V., Apeldoorn-Ugchelen) with 3-amino-9-ethylcarbazole in *N,N*-dimethylformamide and hydrogen peroxide as substrates for the peroxidase reaction. Counterstaining with haematoxylin was sometimes performed.

The slides were randomized and scored blindly.

Radioimmunoassay

The hormone concentrations in the sera were measured by radioimmunoassay. For TSH, GH, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) determinations, materials kindly supplied by Dr S. Raiti (NIAMDD) were used. Iodination was carried out using the Chloramine-T technique described by Hunter & Greenwood (1962) with slight modification. For TSH, LH and FSH, separation of antibody-bound and free hormone was achieved by addition of sheep-anti-rabbit-γ-globulin coupled to a solid phase (DASP, from Organon). For GH, liquid-phase goat-anti-monkey-γ-globulin (Calbiochem, Luzern, Switzerland) was used. Counting data were evaluated using a computer programme developed by Rodbard & Lewald (1970). Results are expressed in terms of the NIAMDD RP1 preparations. Porcine insulin was purchased from Novo (Amsterdam), the antiserum from Wellcome (Weesp). Results are expressed in terms of the 4th International Standard for insulin, bovine and porcine, for bioassay. Testosterone and corticosterone were measured in ethylene glycol diethyl ether and ethylene glycol–toluene extracts, respectively. Testosterone antiserum (28% cross-reaction of 5α-dihydrotestosterone) was donated by Prof. dr. A. Vermeulen (Gent, Belgium), and the corticosterone antiserum by Prof. dr. Th. J. Benraad (Nijmegen). The steroid tracers were purchased from The Radiochemical Centre (Amersham, Bucks, UK). For T_4 determinations the Corning Immophase T_4 Kit (Gist Brocades, Delft) was used, slightly modified for rat serum.

Significance of differences between groups was analysed by Student's *t* test.

Results

Organ weights

In Table 1, body weights and the relative weights of the pituitary gland, thyroid and testes are listed. Compared to the control animals, the group on the 19,200 mg NaBr/kg diet showed growth retardation, which was noticeable after 4 weeks and more pronounced after 12 weeks. There was a distinct increase in the thyroid weight in the 1200-mg/kg group after 4 weeks but not after 12 weeks. In the 19,200-mg/kg group, however, a statistically significant increase was observed after both 4 and 12 weeks.

Histopathology and immunocytochemistry

Pituitary gland. No histopathological changes could be detected in the haematoxylin/eosin-stained sections of the pituitary glands of rats exposed to sodium bromide for 4 or 12 weeks. Using the immunoperoxidase techniques, selective immuno-

Table 1. Body weight and relative weights of pituitary gland, thyroid and testes of male rats fed sodium bromide in the diet for 4 or 12 weeks

Dietary concn of NaBr (mg/kg)	Body weight (g)	Relative weight (g/100 g body weight) of:		
		Pituitary gland	Thyroid	Testes
		4-wk exposure		
0	183 ± 25	0.0044 ± 0.0007	0.0065 ± 0.0015	0.967 ± 0.162
20	187 ± 22	0.0043 ± 0.0011	0.0075 ± 0.0011	0.989 ± 0.110
75	190 ± 22	0.0042 ± 0.0009	0.0074 ± 0.0014	0.989 ± 0.091
300	186 ± 17	0.0043 ± 0.0008	0.0070 ± 0.0009	0.983 ± 0.069
1200	189 ± 19	0.0042 ± 0.0007	0.0090 ± 0.0013**	0.968 ± 0.082
0	243 ± 16	0.0038 ± 0.0010	0.0086 ± 0.0018	0.921 ± 0.066
19,200	220 ± 21*	0.0044 ± 0.0013	0.0111 ± 0.0021*	0.886 ± 0.182
		12-wk exposure		
0	314 ± 25	0.0035 ± 0.0003	0.0064 ± 0.0014	0.799 ± 0.089
20	309 ± 25	0.0036 ± 0.0006	0.0071 ± 0.0014	0.835 ± 0.031
75	314 ± 30	0.0035 ± 0.0006	0.0061 ± 0.0019	0.818 ± 0.068
300	314 ± 20	0.0032 ± 0.0004	0.0067 ± 0.0019	0.824 ± 0.069
1200	309 ± 10	0.0036 ± 0.0004	0.0071 ± 0.0017	0.786 ± 0.087
0	322 ± 26	0.0033 ± 0.0005	0.0071 ± 0.0016	0.772 ± 0.094
19,200	254 ± 28***	0.0036 ± 0.0005	0.0129 ± 0.0023***	0.681 ± 0.130

Values are means ± SD for groups of ten animals and those marked with asterisks differ significantly from the control value: *—$0.01 < P < 0.05$; **—$0.001 < P < 0.01$; ***—$P < 0.001$.

cytochemical staining could be localized in different types of cell. Immunoreactive GH cells were seen to be scattered throughout the anterior pituitary gland, frequently forming small clusters or strings. The cytoplasm was strongly stained and the cells had round to oval shapes (Fig. 1). The thyrotropic cells revealed with the anti-TSH serum appeared to be polygonal, and were mainly located in the central part of the anterior pituitary gland. The staining reaction was again strong (Fig. 2a). Many stellate cells with cytoplasmic processes lying between other cells were positive for ACTH (Fig. 3a), and were preferentially localized in the lateral part of the anterior lobe. No immunocytochemical reaction was observed when the anti-hormone sera were substituted by normal sera.

Immunostaining procedures for GH, TSH and ACTH in the different types of cell in the anterior pituitary gland of the control rats and of animals treated with sodium bromide yielded different results (Table 2). The average number and size of the immunoreactive cells and the intensity of the staining

Table 2. Immunocytochemical findings in the pituitary gland of male rats fed sodium bromide in the diet for 4 or 12 weeks*

Immunocytochemical staining	Dietary concn of NaBr (mg/kg) ...	Findings† after exposure for:			
		4 wk		12 wk	
		0	19,200	0	19,200
Immunoreactive GH cells					
—decrease		4	6	4	6
—normal		6	4	6	4
Immunoreactive TSH cells‡					
—decrease		3	3	3	1
—normal		3	4	6	3
—increase (slight)		4	3	0	0
—increase (strong)		0	0	0	5
Immunoreactive ACTH cells					
—decrease		2	3	4	1
—normal		5	4	5	3
—increase (slight)		3	3	1	4
—increase (strong)		0	0	0	2

*Slides were scored blindly. No histopathological changes were observed in the haematoxylin/eosin-stained slides.

†No. of animals with stated finding out of the total examined (groups of ten except where indicated otherwise).

‡No. of animals examined for this parameter in the test and control group after the 12-wk exposure was only nine in each case.

reaction in each procedure were first ascertained to serve as a base-line against which any change could be judged. In the pituitary gland of rats treated with 19,200 mg NaBr/kg diet for 4 and 12 weeks, only a slight tendency towards less GH immunoreactivity was observed in comparison with the control animals. On the other hand, there was distinctly more immunoreactivity for TSH (Fig. 2) and ACTH (Fig. 3), but only after exposure for 12 weeks.

Thyroid. The normal thyroid is composed of an aggregation of follicles of variable size. The interfollicular regions are occupied by a highly vascular connective tissue. Each follicle is lined by the secretory epithelium consisting of a single layer of flat cells, and the lumen contains homogeneous colloid (Fig. 4a). After exposure of rats to 19,200 mg NaBr/kg diet for 4 weeks, remarkable histopathological changes were observed, characterized by an increase of follicles and a decrease in their size. The follicular epithelium was greatly heightened while the colloid was decreased in amount and more granular in appearance (Fig. 4b). This was also seen after an exposure time of 12 weeks. No marked changes in the histological picture of the thyroid were detected in the lower dosage groups, irrespective of the exposure time (Table 3).

Using the PAP method, T_4 was generally detected particularly in the follicular colloid but also in the follicular epithelium of normal rat thyroid tissue (Fig. 5a), but its distribution within the follicular colloid and the intensity of the reaction between follicles varied. In comparison with the reaction in the control animals (Fig. 5a; Table 3), the follicles of the rats treated with 19,200 mg NaBr/kg diet for 4 or 12 weeks were less intensely stained (Fig. 5b; Table 3). Moreover, in the latter groups there was less variation in the intensity of the reaction within the follicular colloid.

Testes. The normal testis is divided into several testicular lobules, each of which consists of various seminiferous tubules. Spermatogenesis within these tubules depends on the Sertoli cells. In the interstitial spaces, the testosterone-synthesizing Leydig cells are situated (Fig. 6a). A decreased spermatogenesis and a reduction of tubule diameter were observed in the rats of the highest dosage group after 12 weeks of treatment (Fig. 6b; Table 3).

No immunocytochemical staining for testosterone could be achieved by the PAP method using the Immulok Histoset Immunoperoxidase Staining Kits, whatever fixation procedures were carried out (Bouin–Holland's solution, 4% formaldehyde, 8% formaldehyde/4.5% mercuric chloride, B-5 (Lilly & Fullmer, 1976), glutaraldehyde followed by osmium tetroxide, or frozen specimens whether or not followed by 4% formaldehyde). The spontaneous loss of testosterone from the Leydig cells during the fixation and embedding procedures is well recognized (Mukai & Rosai, 1980). Nevertheless, further studies to demonstrate testosterone in the rat testis will be performed.

Radioimmunoassay

In Table 4 the levels of T_4 and TSH in the serum of rats after bromide exposure for 4 and 12 weeks have been summarized. In addition, the results of the TRH test are shown. There was a statistically significant decrease of T_4 both after the 4- and the 12-week treatment with the 19,200-mg NaBr/kg diet. Also in the 1200-mg/kg group, the T_4 level was significantly reduced after a 4-week exposure period. On the other hand, TSH levels were significantly increased in the highest dose group. TRH had no effect on the T_4 level, but as might be expected, it caused an increase in the TSH levels both in the control group and in the bromide group.

With regard to the gonadotropic hormones LH and FSH, reported in Table 5, there are a number of striking observations. In both series of experiments FSH increased significantly after exposure to a high level of bromide. This did not hold for LH. After 4 weeks a statistically significant decrease of the LH level in the highest dose group was seen, but this effect had disappeared after 12 weeks. The level of both LH and FSH was higher in the 4-week groups than in those killed at 12 weeks. Since all animals were 3 weeks old at the beginning of the experiment, the 4-week groups were younger when killed than were the 12-week groups. de Jong & Sharpe (1977) reported similar differences in LH and FSH levels in the sera of animals of these ages. With regard to the LH and FSH findings in the TRH test, it is surprising that only LH rises both in the control and in the bromide animals. Since FSH does not change, contamination of the TRH preparation with the LH/FSH-releasing hormone (LHRH) can be excluded. There may then be two explanations for this phenomenon:

(a) The observed rise of LH is due to cross-reacting TSH, although the antiserum is supposed to be specific for LH as stated by the NIAMDD (cross-reaction of TSH is 5%) and in the animals receiving the highest bromide dose (without TRH) the TSH level has increased 4–5-fold without a concomitant increase in LH. Nevertheless, pituitary hormones have been shown to exist in different molecular forms and it is possible that the TSH form released by the pituitary gland after TRH treatment is different from that released after sodium bromide treatment. This has to be investigated further.
(b) Extra handling stress is caused by the injection of TRH. It is well known that acute stress affects the level of a number of hormones, e.g. ACTH and GH (Kruhlich, Hefco, Illner & Read, 1974). LH does not seem to be very sensitive in this respect, but this possibility cannot be excluded completely. In this respect the slight elevation of the corticosterone levels in both the control and the bromide groups after TRH injection (Table 6) should be noted.

A statistically significant decrease in testosterone was observed after treatment with the 19,200-mg NaBr/kg diet for 4 and 12 weeks (Table 5).

In Table 6 the levels of GH, insulin and corticosterone in serum are shown. The results of the GH assay are difficult to interpret. The large variation within each dosage group may have been due to rapid fluctuations in GH secretion (Edén, 1979; Shin, 1982). Episodic bursts of GH secretion are responsible for 10–20-fold increases in the GH concentration in the serum within 15–30 minutes. Never-

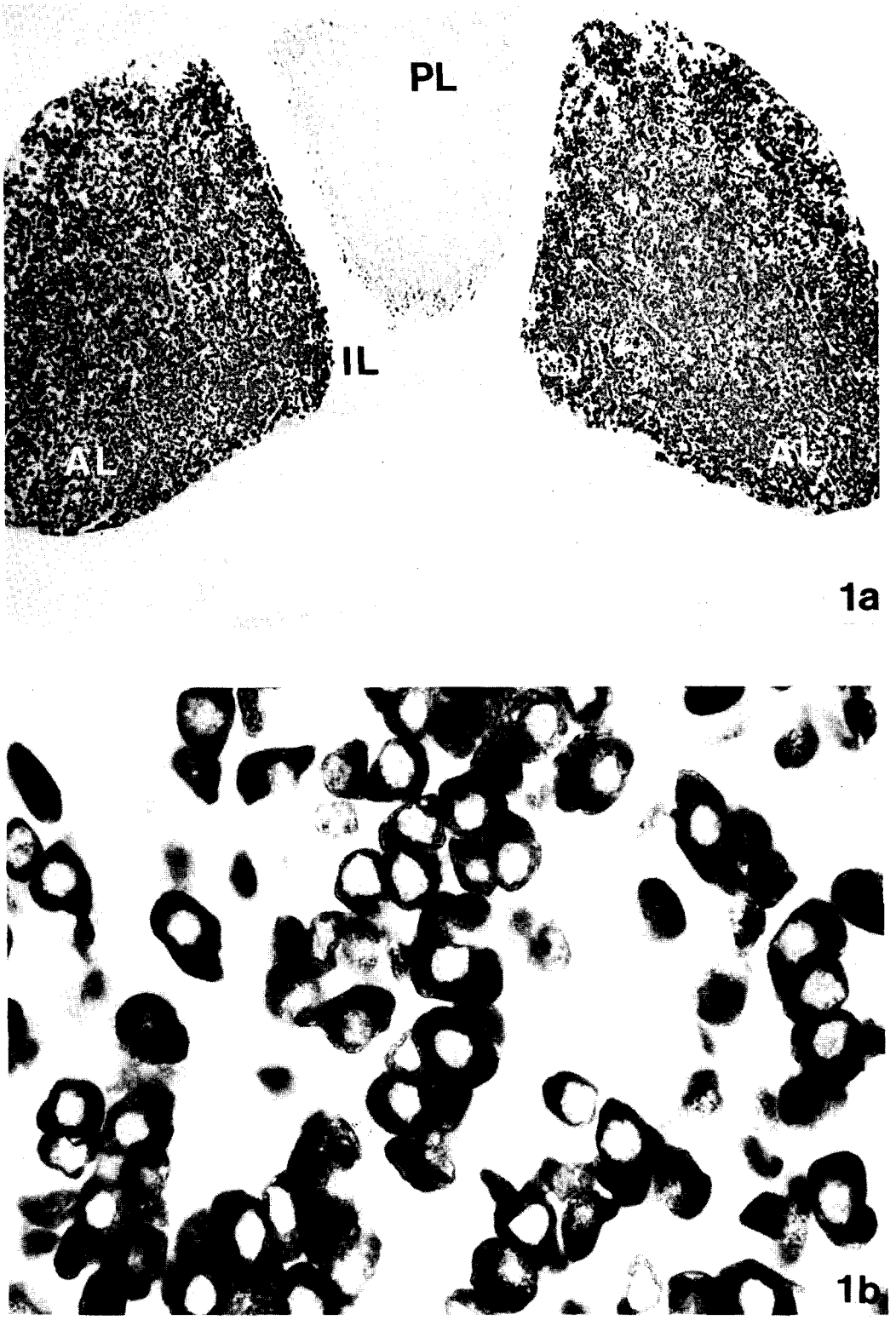


Fig. 1. Immunocytochemical staining (indirect peroxidase-labelled antibody method) for growth hormone (GH) in the pituitary gland of an adult male control rat showing (a) general view (× 37) of immunoreactive GH cells specifically localized in the anterior lobe (AL), compared with the intermediate lobe (IL) and posterior lobe (PL) and (b) detail of (a) showing the immunoreactive GH cells, round to oval in shape, × 930.

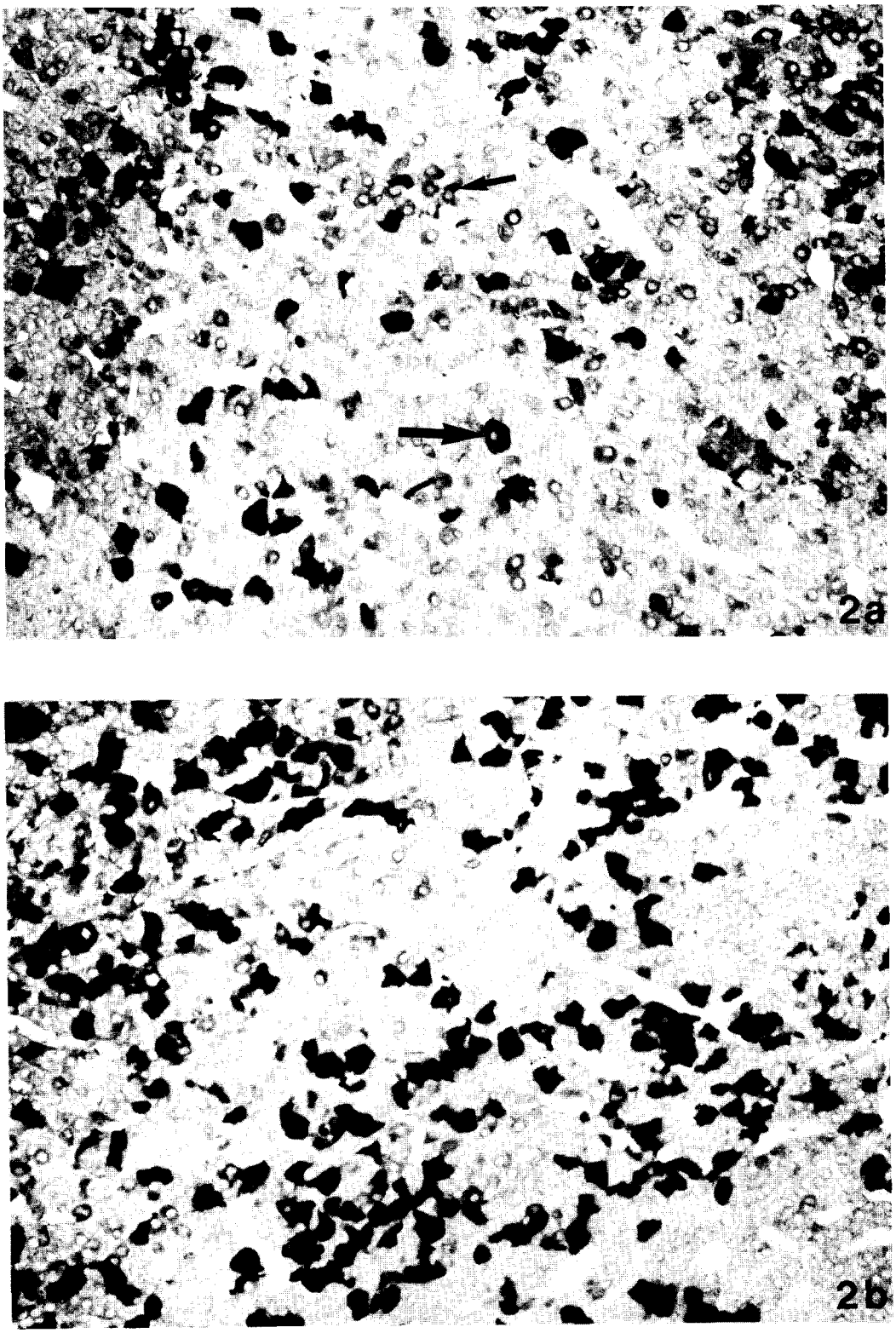
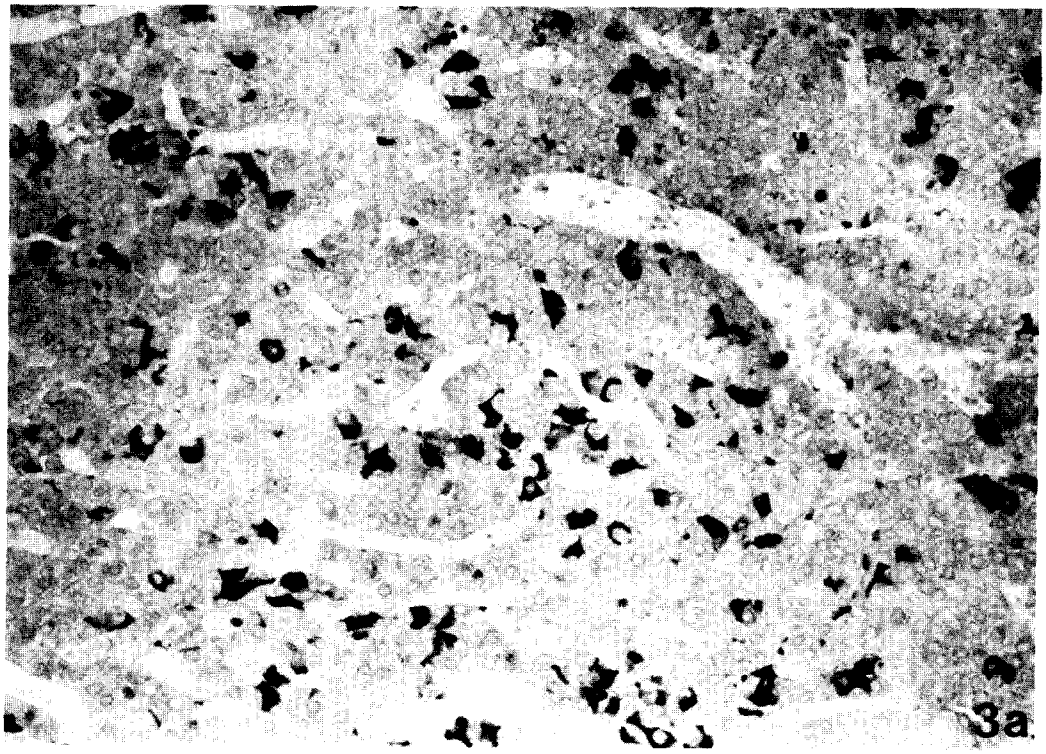


Fig. 2. Immunocytochemical staining (indirect peroxidase-labelled antibody method) for thyroid-stimulating hormone (TSH) in the pituitary gland of adult male rats: (a) from control animal showing polygonal immunoreactive TSH cells more (large arrow) or less (small arrow) intensely stained; (b) from rat fed 19,200 mg NaBr/kg diet for 12 wk, showing an increased number of strongly immunoreactive TSH cells. × 230.



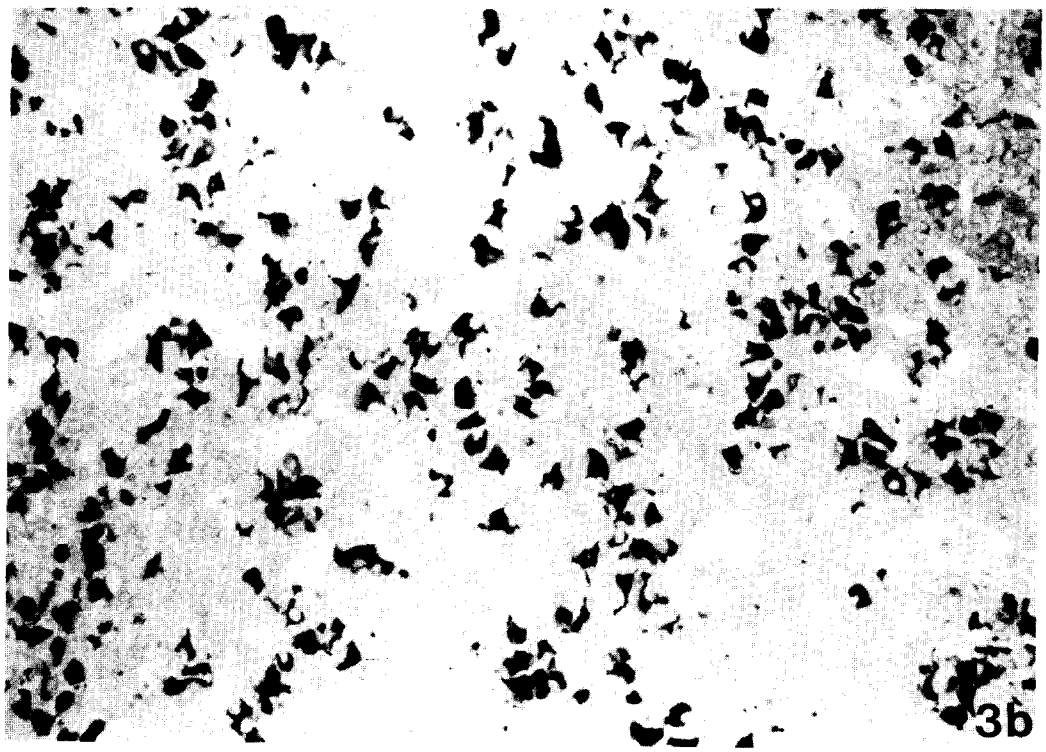


Fig. 3. Immunocytochemical staining (indirect peroxidase-labelled antibody method) for adrenocorticotropic hormone (ACTH) in the pituitary gland of adult male rats: (a) from control animal showing that immunoreactivity of ACTH appears to occur in stellate cells; (b) from rat fed 19,200 mg NaBr/kg diet for 12 wk, showing an increased number of strongly immunoreactive ACTH cells. × 230.

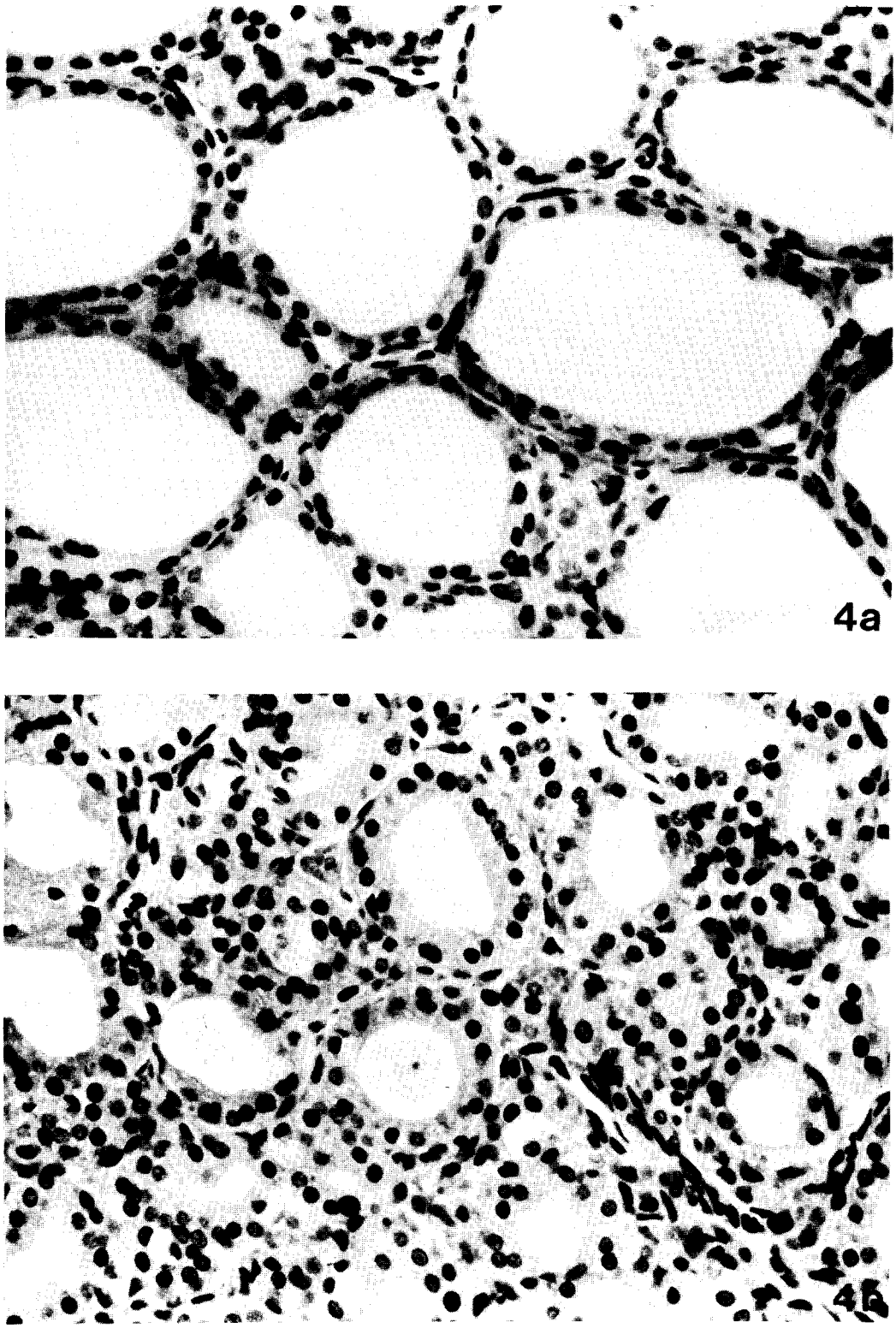
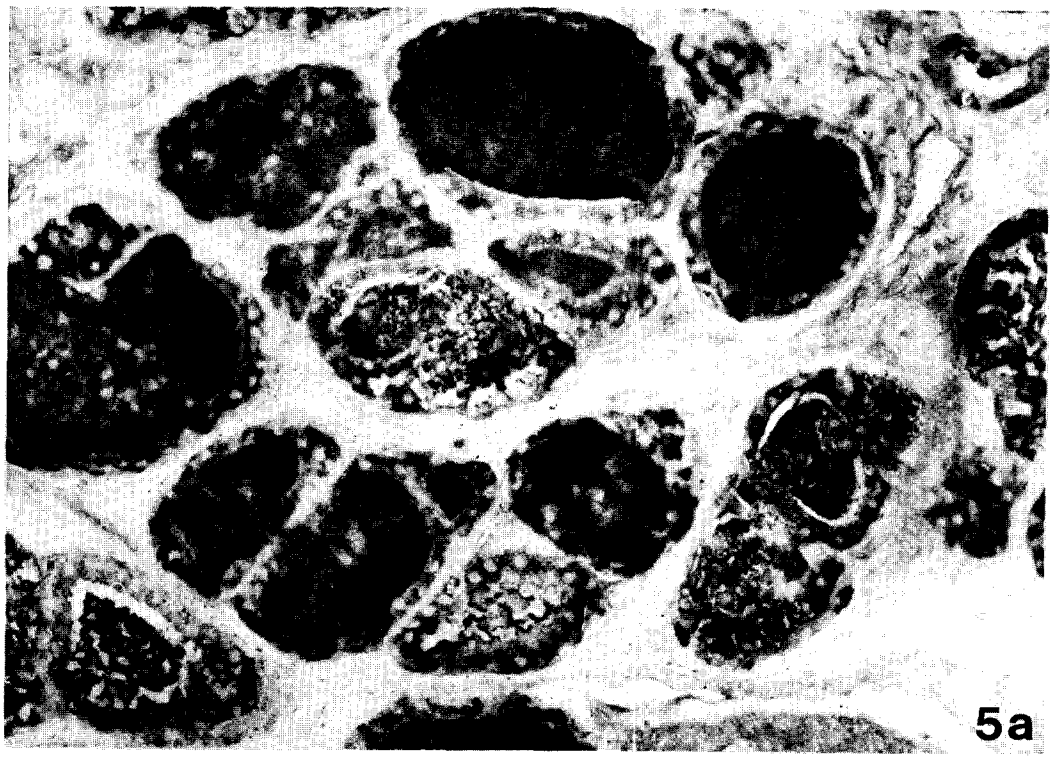


Fig. 4. Sections of thyroids of adult male rats (haematoxylin/eosin × 370); (a) from control rat, with follicles lined with flat cells surrounding a lumen filled with colloid; (b) strongly activated thyroid of rat fed 19,200 mg NaBr/kg diet for 4 wk, showing follicular epithelium composed of columnar cells, and colloid that is decreased in amount and more granular in appearance.



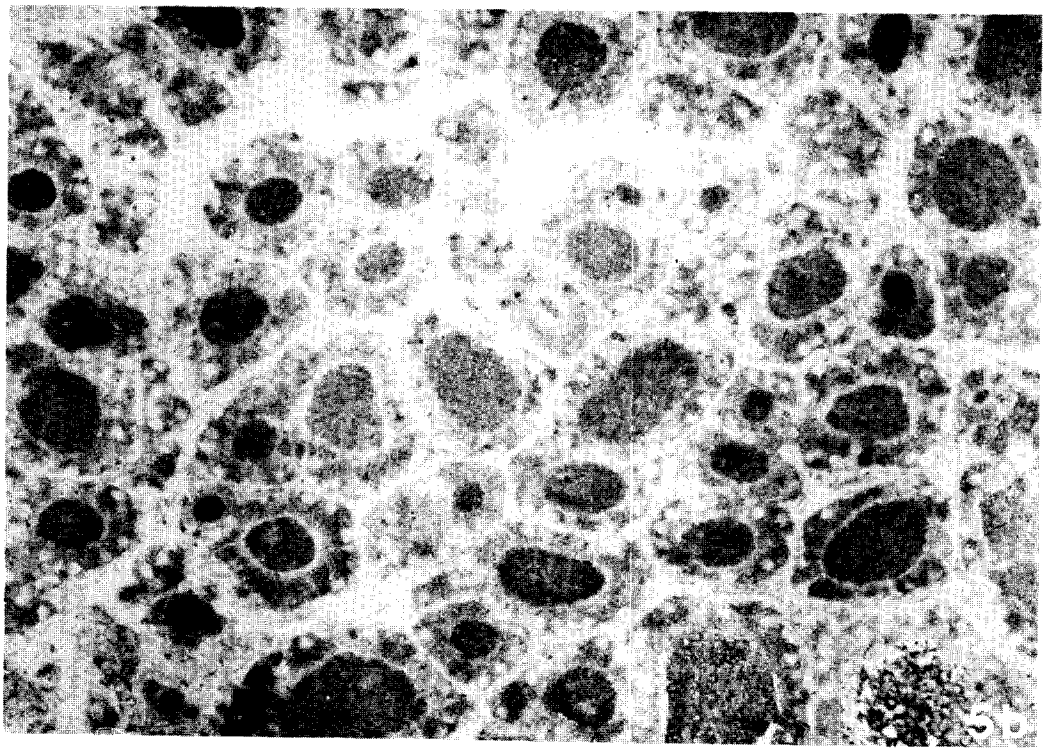


Fig. 5. Immunocytochemical staining (peroxidase-antiperoxidase antibody method) for thyroxine (T_4) in the thyroid of adult male rats: (a) from control rat, showing immunoreactivity for T_4 in the follicular colloid as well as in the cytoplasm of the follicular epithelium; (b) from rat fed 19,200 mg NaBr/kg diet for 4 wk, showing less intensely stained follicles. ×230.

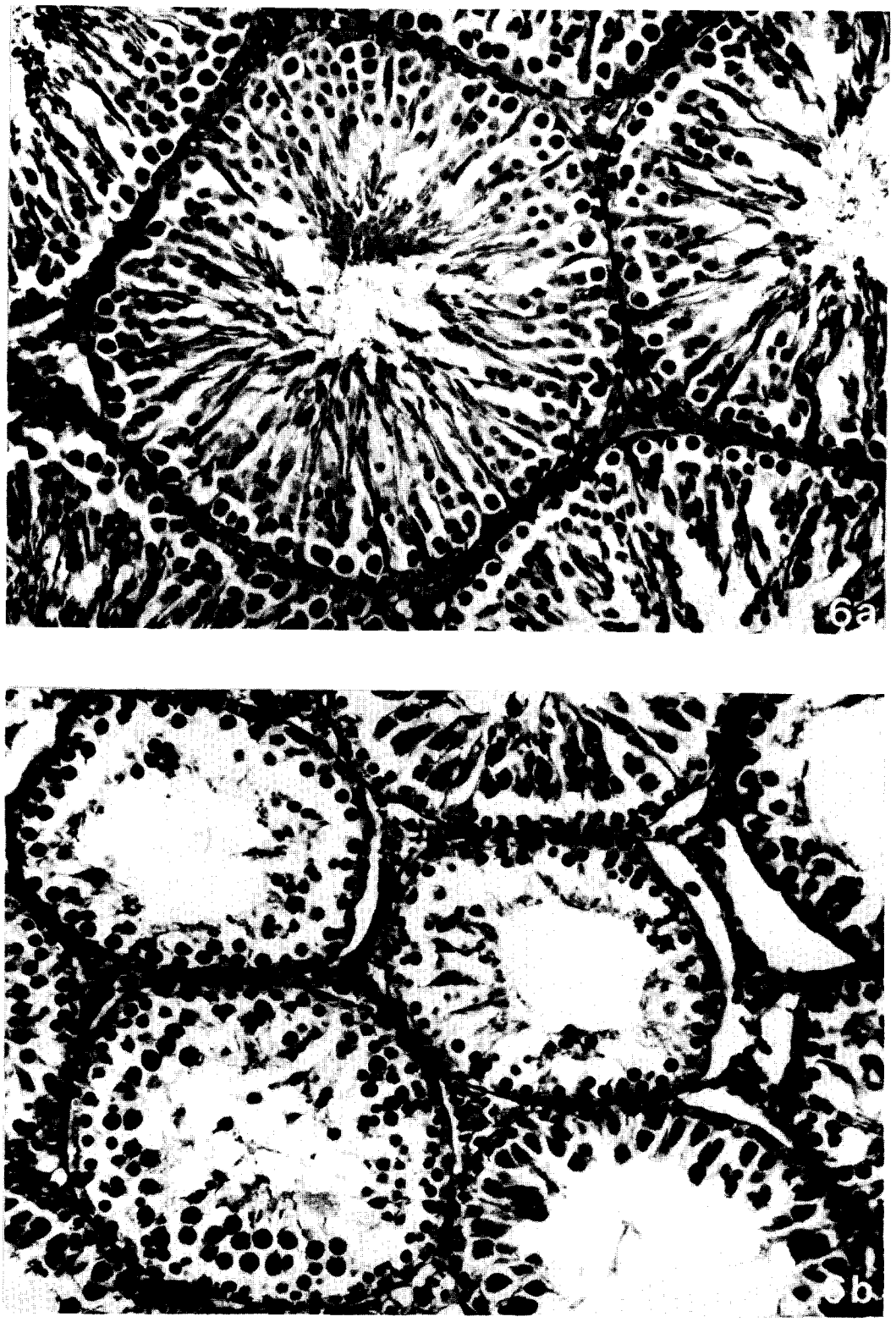


Fig. 6. Sections of testes of adult rats (haematoxylin-eosin ×230): (a) from control rat; (b) from rat fed 19,200 mg NaBr/kg diet for 12 wk, in which spermatogenesis is decreased and the seminiferous tubules are reduced in diameter compared with the normal appearance.

Table 3. Histopathological and immunocytochemical findings in the thyroid and testes of male rats fed sodium bromide in the diet for 4 or 12 weeks*

Criterion	Dietary concn of NaBr (mg/kg)...	Findings† after exposure for: 4 wk						12 wk					
		0	20	75	300	1200	19,200	0	20	75	300	1200	19,200
Thyroid													
HE staining	No. examined...	20	10	10	10	10	10	20	10	10	10	10	10
Degree of activation:													
—slight		2	1	1			1	2	2	3		2	
—moderate						1							2
—strong							9						8
Immunocytochemical staining	No. examined...	20	0	0	0	10	10	19	0	0	0	10	10
Intensity of T_4 staining:													
—decrease		1					9	2					7
—normal		19				10	1	17				10	3
Testes													
HE staining	No. examined...	20	10	10	10	10	10	20	10	10	10	10	10
Seminiferous tubules:													
—some atrophied							1						4
—several atrophied										1	1		3

HE = Haematoxylin/eosin

*Slides were scored blindly.
†No. of animals with stated finding out of total no. examined.

Table 4. Levels of thyroxine (T_4) and thyroid-stimulating hormone (TSH) in the serum of male rats fed sodium bromide in the diet for 4 or 12 weeks

Dietary concn of NaBr (mg/kg)	Serum concn after exposure for: 4 wk T_4 (nmol/litre)	4 wk TSH (μg/litre)	12 wk T_4 (nmol/litre)	12 wk TSH (μg/litre)
0	141 ± 21	142 ± 47	103 ± 12	186 ± 64
20	133 ± 24	120 ± 41	116 ± 23	176 ± 55
75	143 ± 18	155 ± 141	111 ± 9	196 ± 65
300	125 ± 14	100 ± 44	125 ± 18	265 ± 128
1200	109 ± 22**	165 ± 79	114 ± 20	259 ± 189
0	128 ± 8	193 ± 113	111 ± 14	194 ± 70
19,200	54 ± 9***	420 ± 205**	42 ± 9***	935 ± 348***
			TRH test	
0			98 ± 23	442 ± 170
19,200			45 ± 14**	1148 ± 201***

TRH = Thyrotropin-releasing hormone

Values are means ± SD for groups of ten animals (or five in the TRH test) and those marked with asterisks differ significantly from the control value: *—$0.01 < P < 0.05$; **—$0.001 < P < 0.01$; ***—$P < 0.001$.

theless, it appears that with the highest dietary level of 19,200 mg/kg there was a significant decrease after 12 weeks. Insulin levels were significantly increased by bromide treatment only in the highest dosage group but after 4 as well as after 12 weeks. Corticosterone showed a tendency to decline in the sodium bromide-treated rats, particularly in the highest dosage group.

Discussion

Bromide appears to exert dramatic changes on the endocrine status of rats, at least when fed in high doses. In the present studies attention was focused mainly on the pituitary gland, the thyroid and the testes.

Several sites of action of sodium bromide can be distinguished. One of them is a direct effect on the thyroid. After treatment with sodium bromide, T_4 production was hampered, as evidenced by immunocytochemistry and radioimmunoassay. Due to feedback regulation the pituitary gland was stimulated to produce and release TSH. In the TRH experiment the increases of TSH in the control group and the bromide group were of similar magnitude. However, only for the control group was this increase statistically significant. From this it might be concluded that in bromide-treated animals the pituitary gland has little capacity for releasing even more TSH. Marked histopathological and immunocytochemical changes in the thyroid tissue were induced, indicative of a typical hypothyroidism. Hyperplasia of the thyroid after exposure to sodium bromide was also observed in fish (Canton, Wester & Matthijssen-Spiekman, 1983). Thus, bromide appears to suppress T_4 production, probably by partly preventing the

Table 5. Levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH) and testosterone (T) in the serum of male rats fed sodium bromide in the diet for 4 or 12 weeks

Dietary concn of NaBr (mg/kg)	Serum concn after exposure for: 4 wk FSH (μg/litre)	4 wk LH (μg/litre)	4 wk T (nmol/litre)	12 wk FSH (μg/litre)	12 wk LH (μg/litre)	12 wk T (nmol/litre)
0	455 ± 80	42 ± 33	ND	228 ± 44	24 ± 8	ND
20	384 ± 72	37 ± 19	ND	266 ± 38	18 ± 7	ND
75	427 ± 60	32 ± 13	ND	256 ± 47	20 ± 6	ND
300	419 ± 107	37 ± 32	ND	250 ± 48	24 ± 10	ND
1200	438 ± 115	30 ± 22	ND	276 ± 46*	24 ± 6	ND
0	540 ± 60	32 ± 16	15.0 ± 7.1	255 ± 46	27 ± 7	10.8 ± 5.8
19,200	630 ± 100*	17 ± 11*	7.1 ± 3.4*	482 ± 117***	27 ± 12	5.0 ± 2.9*
					TRH test	
0				248 ± 31	47 ± 23	6.9 ± 3.0
19,200				469 ± 137**	56 ± 13	3.1 ± 1.2*

ND = Not determined TRH = Thyrotropin-releasing hormone

Values are means ± SD for groups of ten animals (or five in the TRH test) and those marked with asterisks differ significantly from the control value: *—$0.01 < P < 0.05$; **—$0.001 < P < 0.01$; ***—$P < 0.001$.

Table 6. Levels of growth hormone (GH), insulin and corticosterone (C) in the serum of male rats fed sodium bromide in the diet for 4 or 12 weeks

Dietary concn of NaBr (mg/kg)	Serum concn after exposure for: 4 wk GH (μg/litre)	Insulin (mIU/litre)	C (nmol/litre)	12 wk GH (μg/litre)	Insulin (mIU/litre)	C (nmol/litre)
0	557 ± 868	ND	119 ± 44	474 ± 314	11 ± 12	163 ± 135
20	445 ± 595	ND	102 ± 15	364 ± 405	14 ± 13	244 ± 278
75	596 ± 822	ND	95 ± 21	363 ± 436	10 ± 6	137 ± 99
300	673 ± 528	ND	112 ± 31	203 ± 162*	12 ± 11	121 ± 75
1200	476 ± 545	ND	118 ± 26	603 ± 794	8 ± 4	105 ± 56
0	200 ± 276	21 ± 17	208 ± 152	525 ± 645	25 ± 13	299 ± 334
19,200	368 ± 233	51 ± 15**	89 ± 32*	56 ± 62*	41 ± 9**	149 ± 187
					TRH test	
0				536 ± 487	16 ± 9	780 ± 211
19,200				33 ± 22*	36 ± 9**	530 ± 262

ND = Not determined TRH = Thyrotropin-releasing hormone
Values are means ± SD for groups of ten animals (or five in the TRH test) and those marked with asterisks differ significantly from the control value: *—$0.01 < P < 0.05$; **—$0.001 < P < 0.01$.

incorporation of iodine atoms into the thyronine ring. Several halogens, including bromide, can substitute for iodine but result in reduced hormone activity (Jorgensen, 1978). Conclusive evidence, however, may be obtained by isolation and analysis of all thyronine derivatives from the bromide-treated rats.

In a hitherto unresolved way, at least two other hormones are affected by changes in the T_4 level, namely GH and insulin. The observation that a decrease in T_4 concentration was accompanied by a decrease in GH concentration in the serum (although not confirmed by immunocytochemistry) is in agreement with similar findings reported by Coiro, Braverman, Christianson *et al.* (1979). Moreover, T_4 appears to exert a diabetogenic effect (Lenzen & Kücking, 1982). It is, therefore, conceivable that a decrease in the T_4 level results in an increase in the insulin level. The changes in serum levels of both GH and insulin are in accord with the observed growth retardation of the rats. Finally, since GH may act as a thymotropic hormone (Sorkin, Pierpaoli, Fabris & Bianchi, 1972), a decrease in GH level may also be connected with the reduction in relative thymus weight found earlier (van Logten *et al.* 1974).

The effect of sodium bromide on the adrenals seems to parallel that on the thyroid. A decrease in the corticosterone level in the serum, as assessed by radioimmunoassay, results in an increase in the production of ACTH by the pituitary gland, as found by immunocytochemical staining.

Also the testes are affected by sodium bromide. Histopathological findings now and in the past (van Logten *et al.* 1974) have shown an inhibition of spermatogenesis indicating a deterioration of the Sertoli-cell function. Recently, it has been shown that the Sertoli cell produces a substance called inhibin, which hampers FSH release by the pituitary gland (de Jong, 1979). This may be an explanation for the rise in FSH level observed in the bromide-treated rats.

In addition, the Leydig cells may be affected, resulting in a decreased production of testosterone and consequently in a lowering of the secretory activity of the prostate (van Logten *et al.* 1974). An expected rise in LH level triggered by the decreased testosterone level could not be detected. Rather, after 4 weeks a slight fall was observed. It is conceivable that the damage caused in the Leydig cells is small compared to that in the Sertoli cells, thus giving rise to changes in FSH but not in the LH levels.

In conclusion, it may be postulated that sodium bromide acts directly on certain endocrine organs such as the thyroid, adrenals and testes, thereby inducing alterations in the pituitary gland by feedback mechanisms. The absence of effects in the lower dosage groups confirms the observed no-effect level of 300 mg/kg diet found in the 90-day study, but is in contrast with the observed decrease in T_4 demonstrated in the reproduction study (van Leeuwen *et al.* 1983).

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EFFECTS OF BROMIDE ON BEHAVIOUR OF MICE

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Abstract—Groups of mice, housed individually in standard cages placed in a frame especially designed for the recording of locomotive behaviour, were fed diets containing sodium bromide at a level of 0, 400, 1200, 3600 or 10,800 ppm for 36 days. A fully automated system was used to record and process measurements of the nocturnal motility of the mice, together with three other variables (evasion time, spontaneous treadmill performance and body weight), for a total of 128 days before, during and after administration of the test diets. The data obtained, analysed statistically by univariate and multivariate analysis of variance, indicated that for sodium bromide, the 'effect limit' based on behavioural variables and body weight lay for mice between 400 and 1200 ppm in the diet. The fact that the overall effect of the 10,800 ppm dietary level was not completely reversible seemed to be largely attributable to the effect of this level of sodium bromide administration on body weight.

Introduction

In human bromide intoxication, mental and neurological disturbances are the most common and prominent features. They represent a serious and early aspect of the intoxication. The earliest symptoms (Goodman & Gilman, 1956) are impaired thought and memory, drowsiness, dizziness, disturbed consciousness, disorientation, irritability and emotional disturbances. The signs of neurological disturbance include tremors of the hands, lips and tongue, thick speech, weakness, sluggishness of movements, motor incoordination, staggering gait and changes in reflexes.

There is no objective and quantitative method for recording these effects in standard toxicological feeding studies. It may be possible to observe this type of effect but only at doses that are far from correlating with the therapeutic level in man. But only low doses are of interest in making decisions relating to residue levels.

In our experiment, bromide was used to look for the specific effects of a substance without the complication of metabolic problems and to test the basic usefulness of the method. One aim was therefore to record, in feeding experiments, signs interpretable as indicators of mental or neurological effects. For this purpose we recorded the activity and times of inactivity of the mice during the night. Further methods were used to obtain correlates for mental or neurological disturbances in the daytime.

It can be assumed that the effects of substances containing neurotoxic components are mainly dynamic; i.e. they produce in organ systems reactions that may be functionally compensated with time. Therefore it is necessary to record the events continuously. Thus it is possible to recognize time dependence (e.g. phasic courses) and other states of reaction (e.g. new balances) not only during but also after the administration of the test substances, when the new balances are disturbed and the organism has to react again in a specific manner.

Experimental

NMRI male albino mice were housed individually in plastic cages (Makrolon, type 1) in five groups each of 20 animals. They were adapted to a fixed quantity (6 g) of Altromin standard diet (Altromin GmbH, Lippe) and given water *ad lib.* Sodium bromide, 99.5% pure and obtained from E. Merck AG (Darmstadt), was dissolved in water and added to the diet at levels of 0, 400, 1200, 3600 and 10,800 ppm (mg NaBr/kg diet). The duration of the experiment was 128 days, the test diets being administered from day 43 to day 78.

The equipment used to measure spontaneous motility can record movements simultaneously for a maximum of 120 individually caged mice. Each cage is controlled by four infra-red light barriers (Fig. 1) placed independently in the cage. Light transmitters and receivers are arranged with part of the electronics on printed circuits so that normal animal maintenance and grooming is possible without any restriction. The light barriers work with short light impulses, thus producing a substantially higher light intensity to prevent failures in measurement caused by dust and dirt and to reduce the expenditure on electronics. The frequency of the impulse sequence is

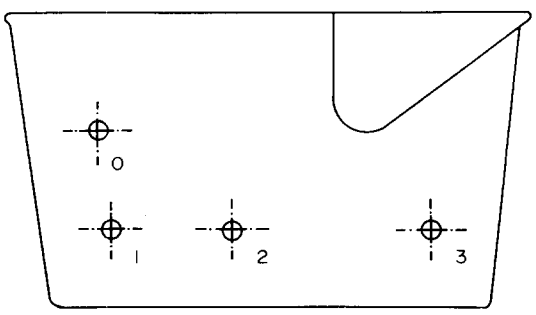

Fig. 1. Arrangement of infra-red light barriers (0–3) in a cage for measurement of motility in mice.

fixed so that interruptions of the light barriers are detectable if their duration is not less than 10 msec. Since the signals of the light barriers have to indicate motility, their outputs are logically connected. This is established by counting an impulse of a particular light barrier only if another light barrier has detected a signal before. Thus all movements of an animal that are not motility will be ignored. If they meet this pre-condition, the signals (assigned to animal and light-barrier number) are registered on a data file, where they are accessible to the computer in a preset time period.

From the values obtained in this experiment the following variables were processed:

(1) Variables 0–3: the impulses from measurement positions 0–3 totalled over 12 hours;
(2) Variables 4–7: the numbers of periods of 5 minutes in which any movement occurs over 12 hours;
(3) Variables 8–10: in sequence, the evasion time, the spontaneous performance on the treadmill in 10 minutes, and the body weight as a general basic value.

The evasion time is the time an animal takes to leave a small isolated area where it has been placed. The running behaviour on the treadmill is measured by counting the revolutions of the wheel in 10 minutes. Every working day these variables were recorded—the first seven overnight from 6 pm to 6 am and the last three in the morning at fixed times.

From the begining it was planned to automate the measurements, protocol, and management of the data using electronic and data processing. Further development and improvement of the monitoring of these and related variables was undertaken to minimize the rate of error, to reduce the work load and to increase efficiency.

The statistical analysis was mainly accomplished by multivariate analysis of variance (MANOVA; Ahrens & Läuter, 1974; Morrison, 1967) and additionally by univariate analysis of variance (ANOVA). The multivariate analysis was chosen because a multidimensional approach to the variables better illustrates their interdependency.

Results

An example of the motility of the mice over one night (from 6 pm to 6 am) is given in Fig. 2. In this and the following figures the traces have been smoothed for clarity. Figure 2 shows the recording on day 3 after the start of administration of the test diet. The control group has two characteristic periods of higher activity at about 7–8.30 pm and in the morning from 4 am to the end of the recording at 6 am. The two low-dosage groups follow nearly the same course. The 3600-ppm group takes a similar course, but the first period is elevated in magnitude and prolonged. In the group on the 10,800-ppm diet, this typical behaviour is absent. The animals move more continuously, without noticeable resting time, but the total amount of movement is lower.

The activity measured in impulses at the position of light barrier 2 and totalled over 12 hours daily for

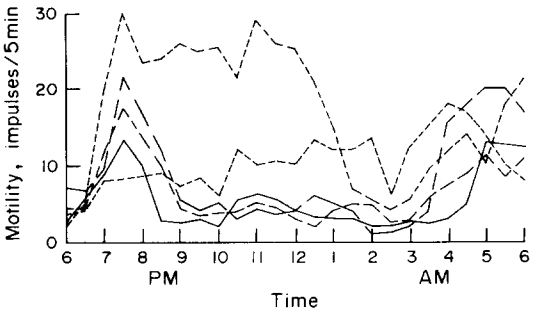

Fig. 2. Motility of mice measured overnight at position 0, 3 days after the start of administration of sodium bromide at dietary levels of 0 (——), 400 (— — —), 1200 (– – –), 3600 (- - -) and 10,800 (····) ppm.

the whole experiment of 128 days is shown in Fig. 3. The upper part shows the mean values of activity in logarithmic transformation. From these data an analysis of variance was performed every day, and the results in F values are shown in the lower part of the figure. The motility in the two high-dosage groups rose after the beginning of sodium bromide administration, passed a maximum and developed a plateau. A sudden decrease in motility followed the end of treatment but the group on the highest dosage did not return to the original level of activity. The F values demonstrate this immediate effect and the significance of bromide administration at these two dosages. The two lower dosages did not have visible effects.

In Fig. 4 the influence of bromide on the evasion time is represented. Again the means and corresponding F values are plotted. It can be seen that all the

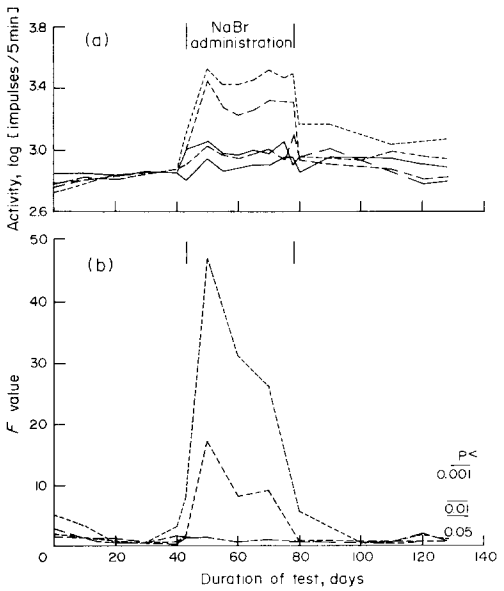

Fig. 3. Mean activity of mice at position 2 before, during and after the feeding of sodium bromide-containing diets (identified as in Fig. 2): (a) activity in logarithmic transformation; (b) result of univariate analysis of variance.

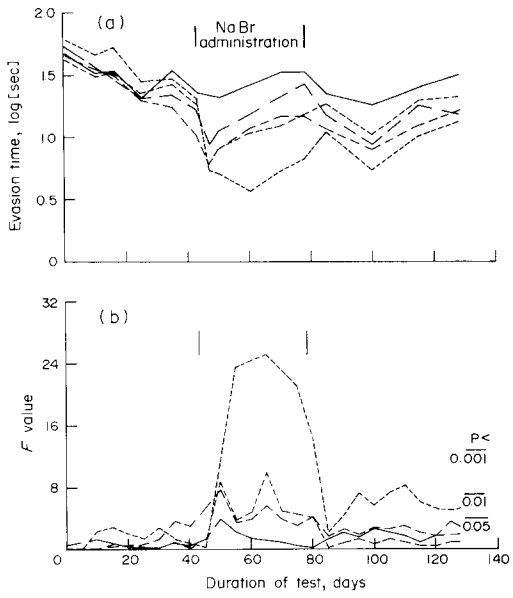

Fig. 4. Evasion time of mice before, during and after the feeding of sodium bromide-containing diets (identified as in Fig. 2): (a) evasion time in logarithmic transformation; (b) results of univariate analyse of variance.

bromide diets caused a decrease in evasion time. The high-dosage group shows the most obvious change. All the other groups, with the exception of that on the lowest bromide level, more or less reach the range of significance.

An effect on behaviour on the treadmill (Fig. 5) was evident only in the group on the 10,800-ppm diet. The F values show two marked peaks after the beginning and the end of bromide administration.

To correlate these behavioural effects with the usual feeding study the body weight of the animals

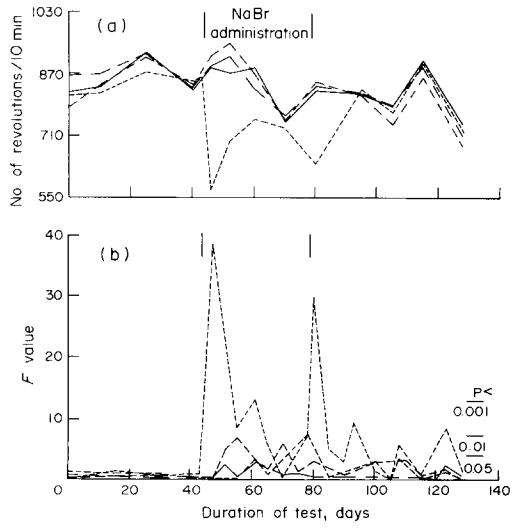

Fig. 5. Treadmill behaviour of mice before, during and after the feeding of sodium bromide-containing diets (identified as in Fig. 2): (a) number of revolutions; (b) result of univariate analysis of variance.

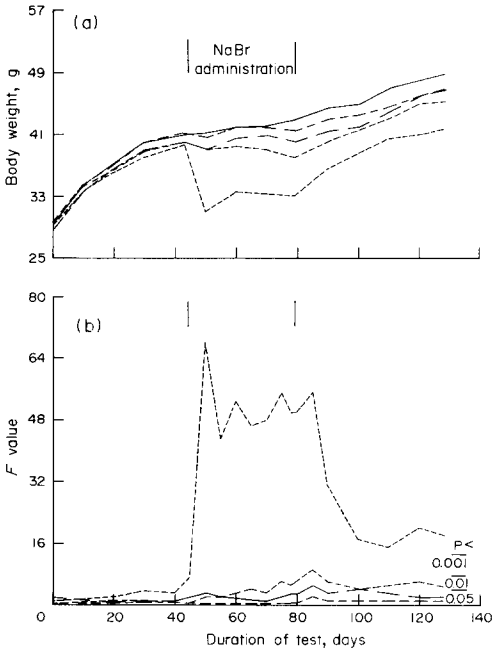

Fig. 6. (a) Body weight of mice before, during and after the feeding of sodium bromide-containing diets (identified as in Fig. 2) and (b) the result of univariate analysis of variance.

was recorded every day along with the other variables. In Fig. 6 the development of body weight and the results of the analysis of variance are represented. The most prominent impact was again associated with the highest dietary level, with which a high degree of statistical significance was maintained until the end of the experiment. The effects of the other test diets were statistically less significant.

Figure 7 shows the results of a multivariate analysis of variance performed daily on the 11 measured variables. The effects of the lowest level of 400 ppm were obviously not significant. The significance of the effects of 1200 ppm NaBr seen in only some variables could be established by this method, and demonstrates that the 'effect limit' based on behavioural variables and body weight lies between 400 and 1200 ppm NaBr in mice. It can also be seen that the effects of the 10,800-ppm diet are not completely

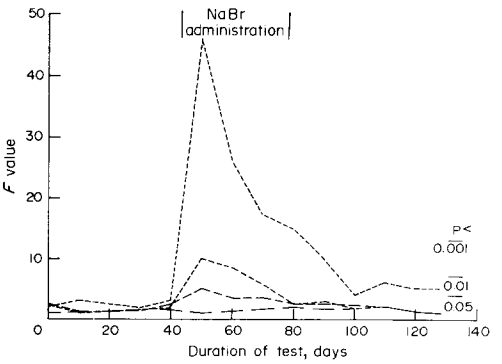

Fig. 7. Results of multivariate analysis of variance of 11 measured variables (diet identification as in Fig. 2).

reversible but, on the basis of the results of the statistics, this seems to be largely due to the influence of the retardation in body weight.

Discussion

In this experiment we tried to find out whether behavioural variables are applicable in a standard feeding study. For this purpose it is necessary to be able to quantify these variables. This requires the exact description and performance of an adequate measuring technique that can produce processable data. One major consideration is the behavioural characteristics typical of each single animal; these react together very individually but also very sensitively, producing wide variations in a group. Furthermore the time dependency of the behavioural manifestations extends this variation. Therefore the sensitivity of the method is a question of an adequate data analysis.

In spite of knowing that our simple measuring technique does not make allowance for all the difficult aspects, especially of locomotive behaviour, we interpret the substance-related effects found as being in a way equivalent to mental and neurological disturbances. The reduction in evasion time and the disturbance of the normal nocturnal rhythm of motility indicate a disinhibition. The obvious difference between the two highest dosage groups shows another aspect of bromide intoxication.

On the basis of the results of van Logten, Wolthuis, Rauws *et al.* (1974) a comparison of the effects found in our study with those known to occur in man is made possible by a consideration of the concentrations in the experimental diets. The dietary level of chloride of about 0.8 g/kg (van Logten, Rauws, Kroes *et al.* 1976) corresponds to that of our mouse diets. It can be assumed that in mice, as in the rat (van Logten *et al.* 1974), the plasma concentration of bromide is proportional to the dietary concentration. The threshold of effects in mice between 400 and 1200 ppm in our study would therefore correspond to about 200–500 mg/litre plasma on the basis of the data of van Logten *et al.* (1974). This concentration lies below that of the plasma levels—750–1000 mg/litre (Goodman & Gilman, 1956)—that in man are associated with mental and neurological disturbances.

This approximation provides in our opinion a satisfactory agreement between the effects in mice and the findings in man. The bromide content of food can be evaluated on the basis of known effects in man (FAO/WHO Pesticides Committees, 1967) and there is no necessity for more precise calculations on the basis of animal studies. In any case human data should be used to evaluate the special risk associated with low-salt diets.

With the method used in this study, behavioural effects in mice have been shown to correspond with findings in man, using bromide as a model substance. It may be concluded that the method can be useful for evaluating risks of substances that cannot be tested in man or for studying substances with unknown behavioural effects.

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THE INFLUENCE OF SODIUM BROMIDE IN MAN: A STUDY IN HUMAN VOLUNTEERS WITH SPECIAL EMPHASIS ON THE ENDOCRINE AND THE CENTRAL NERVOUS SYSTEM

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Abstract—Sodium bromide was administered orally in capsules to healthy volunteers in doses of 0, 4 or 9 mg Br^-/kg/day using a double-blind design. Each treatment was given to seven males for 12 weeks and to seven non-pregnant females (not using oral contraceptives) over three full cycles. Special attention was paid to possible effects on the endocrine and central nervous systems. At the start and end of the study, a full medical history, the results of a physical examination, haematological studies and standard clinical chemistry and urine analyses were recorded for each subject. These showed no changes for individuals following treatment, except for some incidence of nausea associated with bromide-capsule ingestion. Mean plasma-bromide concentrations at the end of treatment were 0.08, 2.14 and 4.30 mmol/litre for males and 0.07, 3.05 and 4.93 mmol/litre for females of the 0-, 4- and 9-mg Br^-/kg/day groups, respectively. Plasma half-life was about 10 days. In the females taking 9 mg Br^-/kg/day (but in no other group) there was a significant ($P < 0.01$) increase in serum thyroxine and triiodothyronine between the start and end of the study but all concentrations remained within normal limits. No changes were observed in serum concentrations of free thyroxine, thyroxine-binding globulin, cortisol, oestradiol, progesterone or testosterone, or of thyrotropin, prolactin, luteinizing hormone (LH) and follicle-stimulating hormone before or after the administration of thyrotropin-releasing hormone and LH-releasing hormone. Analysis of neurophysiological data (EEG and visual evoked response) showed a decrease in δ_1- and δ_2-activities and increases in β-activities and in mean frequency (Mobility parameter) in the groups on 9 mg Br^-/kg/day, but all the findings were within normal limits.

Introduction

Inorganic bromide has been widely used in medicine for more than 100 years as an antiepileptic, hypnotic or sedative medicine. During the last 20 years its use has decreased considerably due to the availability of more selective and more effective compounds. The toxicity of bromide in therapeutic dosages is relatively low. Side effects are limited to the gastrointestinal tract, the central nervous system (CNS) and the skin. Nausea and vomiting may occur immediately after ingestion of a therapeutic dose. Side effects originating in the CNS are called 'bromism'. Symptoms include mental dullness, apathy and drowsiness. Bromide intoxication is manifested by restlessness, ataxia and hallucinations, depending on the dose administered. In severe cases coma occurs. As early as 1936, electroencephalographic changes were reported in bromide intoxication (Lennox, Gibbs & Gibbs, 1936). Chronic intoxication may be accompanied by an acneiform dermatitis sometimes called 'bromoderma'. Changes in the anatomy or function of endocrine organs during the use of bromide as a medicine or in bromide intoxication have not been reported, but neither have there been any systematic studies of this aspect.

The effect of inorganic bromide in the rat has been extensively studied by van Logten, Wolthuis, Rauws *et al.* (1974). Dose-dependent changes in all endocrine organs were demonstrated. The most sensitive organ was the thyroid gland in female animals. At plasma-bromide concentrations comparable to therapeutic levels in man, an increased activity of the thyroid was demonstrated. Experiments in male rats on higher bromide doses led to the conclusion that this effect is accompanied by a decrease in serum-thyroxine concentration (Loeber, Franken & van Leeuwen, 1982).

Because of the apparent differences between rat and man in the effects of bromide, it was decided that the results from animal experiments might have only a limited relevance to man when used as a basis for an 'acceptable daily intake' for bromide. Because of a lack of human data an investigation was performed in volunteers. For 8 weeks the acceptable daily intake of 1 mg Br^-/kg (FAO/WHO Pesticides Committees, 1967) was administered. At a plasma-bromide concentration of about 10% of the therapeutic plasma concentration, no effects were observed even in the endocrine system, to which particular attention was paid (Sangster, Krajnc, Loeber *et al.* 1982).

Because of the long plasma half-life of bromide in

man (10–12 days), the administration period of 8 weeks was considered to be relatively short. Moreover the study did not include objective parameters measuring changes in the function of the CNS. Therefore the need was felt to perform a more comprehensive second study. Its design was similar to the first study and the same variables were measured. However, two different doses of bromide were administered to two groups of volunteers, equally divided between the sexes, over a period of 12 weeks or during three menstrual cycles. Also a quantitative analysis of the EEG and the evoked response at the start and end of the investigation was performed to obtain information on the functioning of the CNS.

Experimental

Volunteers. The investigation was performed in healthy volunteers aged between 19 and 31 years. They were selected after compilation of a full medical history, a physical examination and the following haematological and biochemical analyses: in blood—haemoglobin concentration, haematocrit, MCV, MCH, MCHC, leucocyte concentration, differential white cell count and thrombocyte concentration; in serum—urea, creatinine, sodium, potassium, chloride, alkaline phosphatase, γ-glutamyl transpeptidase, glutamic–oxalacetic and glutamic–pyruvic transaminases, lactic dehydrogenase and protein; in urine—glucose and albumin. For all analyses, standard clinical chemical methods were used. From all volunteers, informed consent was obtained.

Sodium bromide administration. During the administration period of 12 weeks all volunteers maintained their usual diets. In females the test duration depended on their menstrual cycles, always lasting three full cycles. All volunteers were subdivided into weight classes of 10 kg. Female and male subjects were randomly divided into groups receiving 0, 4 or 9 mg Br^-/kg/day administered in a double-blind design.

Persons within the same weight class and dosage group received the same amount of bromide (Table 1). The Hospital Pharmacy of the University Hospital, Utrecht, performed the randomization and provided the capsules containing either cellulose or the appropriate amount of bromide in the form of sodium bromide.

Final examinations. At the end of the investigation a full medical history was obtained, a physical examination was carried out and the haematological and biochemical analyses carried out for the selection were repeated.

Bromide and other electrolyte determinations. At the start, after every 14 days and at the end of the experiment, serum-electrolyte and plasma-bromide concentrations were determined. At the start and at the end of the experiment, sodium, chloride and bromide concentrations were determined in a 24-hour urine sample, together with the creatinine concentration. Bromide was determined using the colorimetric method of Hunter (1955).

Endocrine system studies

At the start and at the end of the investigation, the serum concentrations of the following hormones were determined by radioimmunoassay (RIA): thyroxine (T_4), free thyroxine (FT_4), thyroxine-binding globulin (TBG), triiodothyronine (T_3), cortisol, oestradiol, progesterone, testosterone, thyrotropin (TSH), prolactin, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Also, 20 and 60 minutes after a combined iv injection of 200 μg thyrotropin-releasing hormone (TRH) and 100 μg LH-releasing hormone (LHRH), TSH, prolactin, LH and FSH were determined. Blood sampling was always done between 9 and 10 am. Female volunteers were examined as far as possible in the same follicular phase of their cycles.

Table 2 shows the age, sex and weight of all volunteers and the bromide doses they received. For the females, duration of cycle and menstruation are given and the numbers of days after the first day of menstruation at which the experiment started and ended.

Statistical analyses with respect to the separate endocrine variables were performed by application of Student's t test in each group to the logarithm of the individual ratios Ce/Cs, where Ce stands for concentration at the end and Cs for concentration at the start of the investigation. For each group of female or male volunteers the Student's test statistic was calculated combining the standard deviation for the three groups into one value. Only when log(Ce/Cs) for the control group did not differ significantly from zero were the values of log(Ce/Cs) in the groups receiving 4 or 9 mg Br^-/kg/day considered to carry any conclusive weight as evidence for an effect of bromide.

To evaluate the increase of the TSH, prolactin, LH and FSH concentrations after administration of

Table 1. Distribution of volunteers according to weight class and amounts of sodium bromide administered daily in capsules

Weight class (kg)	Volunteers and NaBr intakes in groups on:								
	0 mg Br^-/kg/day			4 mg Br^-/kg/day			9 mg Br^-/kg/day		
	No.		NaBr	No.		NaBr	No.		NaBr
	M	F	(mg/day)	M	F	(mg/day)	M	F	(mg/day)
50–59	—	2	0	—	2	284	—	2	638
60–69	2	3	0	1	3	336	1	3	754
70–79	4	2	0	5	2	386	4	2	870
80–89	1	—	0	1	—	438	2	—	986

TRH and LHRH, a '40-minute concentration', the geometrical mean of the concentrations measured 20 minutes and 60 minutes after the administration, was introduced. Subsequently this 40-minute concentration was expressed as a multiple of the concentration before the administration of TRH and LHRH. The multiple obtained in this way quantifies for each individual person the increase in the hormone concentration in response to TRH and LHRH. Eventually Student's t test was applied to the logarithm of the individual ratios, "the multiple calculated from the concentrations at the end of the investigation divided by the multiple calculated from the concentrations at the start of the investigation".

For each endocrine organ a maximum of four variables was assessed. When for each of four variables a statistical test is performed with a 1% probability of falsely rejecting the null hypothesis of no effect, the significance probability in simultaneous testing will certainly be less than 5%. It was therefore decided to fix the significance level at 1% when testing separate endocrine variables.

Table 2. Details of individual volunteers and duration and timing of dosing with 0–9 mg Br^-/kg/day

Volunteers				Dosing		
					Timing of experiment (days after day 1 of menstruation)	
Sex	Age	Weight	Duration of menstruation cycle (days)	Duration of experiment (days)	Start	End
			0 mg Br^-/kg/day			
M	25	75	—	84		
	20	79	—	84		
	23	77	—	84		
	22	77	—	84		
	26	69	—	84		
	21	66	—	84		
	23	75	—	84		
F	22	71	5/25	76	8	11
	19	50	5/30	81	12	10
	23	60	5/28	88	11	12
	22	75	4/27	84	12	12
	30	63	5/28	78	10	11
	23	58	5/28	81	12	12
	21	67	5/30	101	14	13
			4 mg Br^-/kg/day			
M	19	74	—	84		
	21	82	—	84		
	24	74	—	84		
	25	75	—	84		
	28	77	—	84		
	24	79	—	84		
	21	67	—	84		
F	25	54	5/28	98	11	13
	24	52	7/30	87	12	11
	19	61	5/28	91	11	12
	20	64	5/27	80	12	11
	22	62	7/28	105	12	17
	25	70	4/30	99	12	12
	24	70	7/28	81	10	10
			9 mg Br^-/kg/day			
M	29	79	—	84		
	21	84	—	84		
	22	82	—	84		
	31	67	—	84		
	20	75	—	84		
	24	70	—	84		
	23	75	—	84		
F	20	73	4/28	79	12	12
	22	63	5/30	85	14	12
	22	57	5/27	86	10	11
	19	58	5/35	92	15	13
	20	73	4/28	73	12	11
	19	61	6/28	—	11	—
	19	63	5/28	93	10	10

Central nervous system studies

To assess the activity of the central nervous system, the spontaneous EEG and evoked cerebral activity of each subject were recorded and analysed quantitatively at the start and end of the investigation at the same time of the day. As it was not possible to perform the endocrinological tests and neurophysiological recordings on the same day, the EEG at the start was recorded not more than 4 weeks before the beginning of the administration period. At the end of the investigation, recording was done not more than 3 days before or after cessation of the ingestion of bromide.

For the spontaneous EEG, three different montages were used (Fig. 1), each consisting of 12 channels. For each montage 150 seconds were recorded. The visual evoked response (VER) was recorded with eight channels (stimulus parameters: duration 5 msec; intensity 3000 Lux at 25 cm; interval 1.7–2.7 sec random; no. of stimuli 100). Use of a special electrode cap (Blom & Anneveldt, 1982) for the recordings appreciably diminished the discomfort to the subject and permitted the recording of many subjects in a short time. The signals were digitized and stored on magnetic tape for off-line processing. On-line Fourier analysis facilitated control of signal quality during recording. The VER was digitized and the averaged response was stored in the same way as the EEG.

Off-line processing consisted of the removal of artefacts to acquire 100 seconds of artefact-free EEG. Thereafter a spectral analysis was performed on 10×10 seconds and the whole was averaged. The power spectrum of each channel was subdivided into frequency bands (Blom, 1980) and the power in each of the following bands was used as a variable: power δ_1-band (0.1–0.4 Hz), power δ_2-band (1.5–3.4 Hz), power θ-band (3.5–7.4 Hz), power α_1-band (7.5–9.4 Hz), power α_2-band (9.5–12.4 Hz), power β_1-band (12.5–17.4 Hz), power β_2-band (17.5–24.4 Hz) and power β_3-band (24.5–30 Hz). From the same signal were calculated the Hjorth parameters (Hjorth, 1970) which are time-domain parameters indicating mean power (Activity), mean frequency (Mobility) and band width (Complexity). For statistical analysis of the results the logarithmically transformed absolute and relative power in the different bands were used. The latter is the logarithm of the power in a frequency band relative to the total power in that channel: $\log(P_i/P_{tot})$, where P_i = power in frequency i and P_{tot} = total power in that channel. The actual variables were the differences between the logarithmically transformed power values before and at the end of the whole treatment period. This amounts for the relative power to:

$$\log(P_i/P_{tot})_s - \log(P_i/P_{tot})_e = \log([P_i(s) \times P_{tot}(e)]/[P_i(e) \times P_{tot}(s)]),$$

and for the absolute power to: $\log(P_s/P_e)_i$, where $P_{s(i)}$ = power in frequency band i before the administration period and $P_{e(i)}$ = power in frequency band i at the end of the investigation.

As this expresses the ratio between two powers it can be dimensioned in dB. If the total power (P_{tot}) in the same subject is used in a multivariate analysis of variance as variables correlate between channels while also correlation exists between the different variables. When these correlations are not taken into account effects may be mainly caused by the effect of correlation. Multivariate analysis of variance takes these correlations into account. The results given are calculated for montage 2 (Fig. 1). Since it was found that there was no difference between men and women regarding the effects of bromide, the results for both sexes were combined for each dose group to attain more stable results.

Because little or no effect was to be expected, and in view of the rather large non-systematic variance of the EEG and the relatively small number of subjects per group, testing was done on a 5 and a 10% level of significance (two-sided).

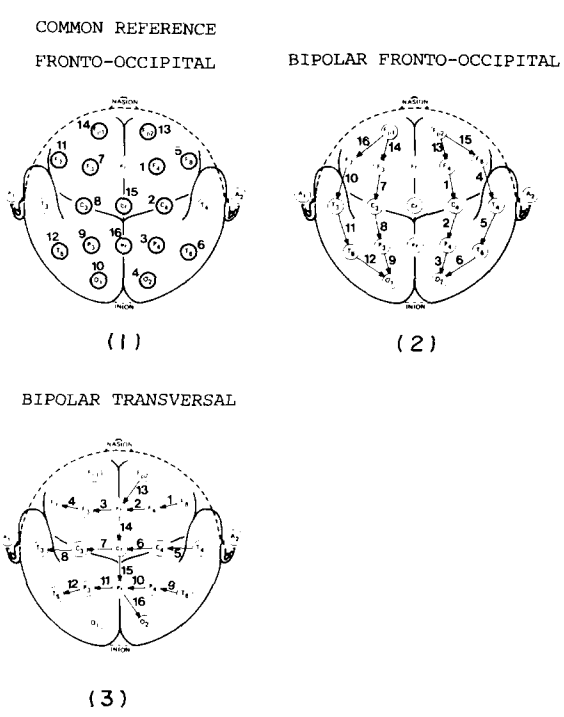

Fig. 1. The three montages of each of 12 channels used for the EEG. The figures denote the channel numbers. In the monopolar montage the references are indicated by thickening of the electrodes.

Results

General aspects

The medical histories obtained and the physical examinations made during the selection revealed no findings relevant to the investigation. Eight females had used oral contraceptives in the past, but this use had been stopped at least 6 months before the start of the study.

Of the female volunteers, one dropped out because of an intercurrent illness while another appeared at the end of the investigation in the postovulatory phase of her cycle because of a misunderstanding. Results of two other female volunteers were excluded because of the development of intercurrent gynaecological pathology not related to the investigation.

Therefore, the results of only four females in the group receiving 4 mg Br^-/kg/day and of only six in the group receiving 9 mg Br^-/kg/day have been evaluated.

The results of the medical histories and physical examinations at the end of the investigation were identical with those at the time of selection of the volunteers except for the occurrence of nausea and mention of a decrease in mental concentration and an increased need of sleep. Two subjects receiving 4 mg Br^-/kg/day noticed some nausea on several consecutive days after ingestion of the capsules. The symptom disappeared when the capsules were taken during the meal. This occurred in five persons in the group ingesting 9 mg Br^-/kg/day but in none of the control group. A decreased ability to concentrate and an increase in sleepiness was mentioned by no females but by five males receiving 4 mg Br^-/kg/day and by one female and one male receiving 9 mg Br^-/kg/day.

In contrast to the examination at the selection one male volunteer showed a granulocytopenia at the end of the investigation. The performance of several additional measurements suggested that he had a cyclic neutropenia. No changes in the haematological variables were demonstrated during the investigation in the other volunteers.

The results of the serum biochemical and urine analyses at the start and end of the study were within normal limits and no significant changes were observed except that three subjects showed an increase in the concentration of γ-glutamyl transpeptidase. In two this appeared to be related to the consumption of ethanol-containing beverages.

Bromide

The mean plasma-bromide concentration in all subjects ranged between 0.06 ± 0.01 and 0.08 ± 0.01 mmol/litre (mean $\pm$ SD) at the start of the investigation. At the end the levels were 0.07 ± 0.01 mmol/litre for the females and 0.08 ± 0.02 mmol/litre for the males in the control group. The mean values for the groups receiving bromide are given in Table 3. No changes occurred in the mean plasma bromide concentrations of the control groups during the investigation. The two groups receiving sodium bromide showed bromide concentrations that gradually increased during the first 6 weeks of the experiment and then remained stable except in the males receiving 9 mg Br^-/kg/day. Here, plasma concentrations increased until week 8 after which a non-significant decrease was observed. No significant differences were found between females and males at either dose level. The mean plasma-bromide concentrations for the two dose levels of bromide differed significantly from each other both for the females ($0.02 > P > 0.01$) and for the males ($P < 0.001$; Student's t test).

Considering the fact that a substance, when regularly administered, usually reaches a steady state in plasma after four times the plasma half-life and that in this study a steady state for bromide was reached after 6 weeks, it may be concluded that in these volunteers the plasma half-life of bromide was about 10 days.

Table 4 shows the bromide, chloride and creatinine excretion in 24-hour urine samples at the start and

Table 3. Plasma-bromide levels in volunteers given capsules containing 0, 4 or 9 mg Br^-/kg/day as sodium bromide for 12 weeks or over three menstrual cycles

	Bromide concn (mmol Br^-/litre plasma)					
	Males			Females		
Dose						
Time* (mg Br^-/kg/day)...	0	4	9	0	4	9
(wk) No./group...	7	7	7	7	4	6
0	0.07 ± 0.01	0.07 ± 0.01	0.08 ± 0.01	0.06 ± 0.01	0.07 ± 0.02	0.07 ± 0.01
2	0.07 ± 0.01	1.43 ± 0.20	2.88 ± 0.56	0.07 ± 0.01	1.51 ± 0.31	3.02 ± 0.71
4	0.06 ± 0.02	1.60 ± 0.44	3.98 ± 0.32	0.07 ± 0.02	1.97 ± 0.26	3.87 ± 0.72
6	0.08 ± 0.02	2.22 ± 0.78	4.39 ± 0.40	0.07 ± 0.02	3.04 ± 0.38	4.68 ± 0.80
8	0.07 ± 0.02	2.06 ± 0.43	4.99 ± 0.77	0.07 ± 0.02	2.72 ± 0.41	4.70 ± 0.94
10	0.07 ± 0.02	2.16 ± 0.36	4.89 ± 0.64	0.06 ± 0.01	2.95 ± 0.42	4.94 ± 1.68
12†	0.08 ± 0.02	2.14 ± 0.72	4.30 ± 0.71	0.07 ± 0.01	3.05 ± 0.82	4.93 ± 1.07

*Time from start of test (wk 0).
†End of study: wk 12 (males) or the end of three menstrual cycles.
Values are means $\pm$ SD for the numbers of volunteers shown.

Table 4. Bromide, chloride and creatinine excretion in 24-hour urine samples from volunteers before and immediately after ingestion of capsules containing 0, 4 or 9 mg Br^-/kg/day for 12 weeks (males) or over three menstrual cycles

Dose (mg Br^-/kg/day)	No./group	Excretion (mmol/24 hr) at: Start of test Bromide	Chloride	Creatinine	End of test Bromide	Chloride	Creatinine
				Males			
0	7	0.07 ± 0.03	152 ± 91	14.9 ± 5.5	0.11 ± 0.04	202 ± 75	13.3 ± 3.4
4	7	0.07 ± 0.04	126 ± 68	15.2 ± 4.6	2.41 ± 1.62	177 ± 81	13.8 ± 2.1
9	7	0.09 ± 0.03	176 ± 71	15.3 ± 1.6	6.17 ± 3.31	208 ± 82	14.8 ± 1.0
				Females			
0	7	0.06 ± 0.03	85 ± 35	9.6 ± 2.3	0.06 ± 0.03	118 ± 46	10.0 ± 5.0
4	4	0.08 ± 0.04	145 ± 54	13.8 ± 4.3	2.61 ± 0.45	109 ± 33	10.8 ± 4.0
9	6	0.08 ± 0.03	141 ± 53	11.6 ± 2.4	2.69 ± 1.61	103 ± 27	9.6 ± 2.8

Values are means ± SD for the numbers of volunteers shown.

end of the investigation for the different groups. The mean bromide excretion at the start ranged from 0.06 ± 0.03 to 0.09 ± 0.03 mmol/24 hr (mean ± SD). Assuming that the bromide excretion in a 24-hour urine sample reflects the average daily bromide consumption there is no difference from the mean bromide content of 0.10 mmol Br^- (range 0.035–0.182 mmol) in 24-hour duplicate meals (Greve & Verschraagen, 1978).

In the control group the mean bromide excretion did not change. At the end of the investigation that in the females receiving 4 or 9 mg Br^-/kg/day was 2.61 ± 0.45 and 2.69 ± 1.61 mmol/24 hr, respectively, whereas the daily administered dose of bromide was 1.76 ± 0.14 and 3.66 ± 0.50 mmol, respectively. The mean excretion was higher ($0.02 > P > 0.01$) in the 4-mg group and lower (but not significantly) in the 9-mg group than the daily administered dose of Br^-. The mean bromide excretion in the two groups did not differ significantly.

In the male groups receiving 4 and 9 mg Br^-/kg/day the mean bromide excretion was 2.41 ± 1.62 and 6.07 ± 3.31 mmol/24 hr, respectively. The mean dose administered daily was 1.88 ± 0.14

Table 5. Serum concentrations of thyroxine (T_4), free thyroxine (FT_4), thyroxine-binding globulin (TBG) and triiodothyronine (T_3) in volunteers before and immediately after ingestion of 0, 4 or 9 mg Br^-/kg/day for 12 weeks (males) or over three menstrual cycles

Determination	Dose (mg/kg/day)	Serum concentrations: Start of test	End of test	log (end/start)
		Males		
T_4 (nmol/litre)	0	104 ± 24	111 ± 15	0.035 ± 0.072
	4	114 ± 14	120 ± 20	0.018 ± 0.057
	9	108 ± 16	108 ± 12	0.002 ± 0.036
FT_4 (pmol/litre)	0	23 ± 5	24 ± 4	0.024 ± 0.060
	4	26 ± 2	27 ± 3	0.008 ± 0.042
	9	24 ± 3	24 ± 2	0.004 ± 0.030
TBG (mg/litre)	0	18.6 ± 3.1	18.9 ± 2.7	0.010 ± 0.055
	4	18.5 ± 3.5	19.4 ± 4.2	0.017 ± 0.069
	9	17.3 ± 2.9	18.0 ± 4.6	0.014 ± 0.032
T_3 (nmol/litre)	0	1.7 ± 0.3	1.9 ± 0.2	0.049 ± 0.060
	4	1.9 ± 0.4	1.9 ± 0.4	− 0.003 ± 0.084
	9	1.8 ± 0.2	1.9 ± 0.2	0.028 ± 0.055
		Females		
T_4 (nmol/litre)	0	119 ± 25	117 ± 22	− 0.005 ± 0.043
	4	123 ± 14	122 ± 12	− 0.002 ± 0.027
	9	115 ± 11	131 ± 15	0.056 ± 0.046**
FT_4 (pmol/litre)	0	25 ± 3	24 ± 3	− 0.021 ± 0.056
	4	26 ± 3	25 ± 4	− 0.015 ± 0.052
	9	22 ± 2	25 ± 2	0.051 ± 0.055*
TBG (mg/litre)	0	23.9 ± 4.1	22.7 ± 2.5	− 0.017 ± 0.038
	4	22.3 ± 1.4	23.5 ± 1.8	0.024 ± 0.024
	9	24.5 ± 4.5	23.6 ± 3.8	− 0.014 ± 0.048
T_3 (nmol/litre)	0	1.6 ± 0.2	1.8 ± 0.3	0.048 ± 0.056*
	4	1.8 ± 0.2	1.8 ± 0.2	0.001 ± 0.075
	9	1.8 ± 0.2	2.1 ± 0.2	0.078 ± 0.021**

Values are means ± SD for groups of seven volunteers, apart from the groups of females on 4 and 9 mg Br^-/kg/day which consisted of four and six volunteers respectively. Statistical analysis on log (end/start): *—0.01 < two-sided $P < 0.05$; **—0.001 < two sided $P < 0.01$.

and 4.31 ± 0.39 mmol, respectively. In neither group was the mean excretion significantly higher than the daily administered dose.

The mean chloride excretion in the 24-hour urines of the different groups did not differ significantly and reflected a sodium chloride consumption ranging from 4.9 to 10.2 g, which is normal for The Netherlands. However, large individual differences were observed in the chloride excretion at the start and end of the investigation. The same applies to the individual creatinine excretion which under physiological cirumstances is relatively stable (Mautalen & Casco, 1970). No differences were found in the mean creatinine excretion of the different groups at the start and end of the investigation.

Endocrine system studies

In Table 5 the variables relating to the function of the thyroid (T_4, FT_4, TBG and T_3) are summarized. The log (Ce/Cs) for each of the control groups did not differ significantly (at $P < 0.01$) from zero. The log (Ce/Cs) of T_4 and T_3 in the female volunteers receiving 9 mg Br^-/kg/day differed significantly from zero ($0.01 > P > 0.001$). In these six females, the change in T_4 and T_3 concentration was on average + 14 and + 20% (for T_4 the 99% confidence interval for the mean percentage increase includes values ranging from 1 to 28%; for T_3 the interval includes values ranging from 3 to 38%). No significant differences (at the 0.01 significance level) were recorded for FT_4 and TBG concentrations. No significant differences in any of the four variables were found in the females receiving 4 mg Br^-/kg/day or in either group of male volunteers receiving bromide.

For none of the variables related to the function of the gonads and adrenals (oestradiol, progesterone, testosterone and cortisol) did log (Ce/Cs) differ significantly from zero in any of the groups. Similarly, no significant values for log (Ce/Cs) were recorded in respect of the TSH, prolactin, LH and FSH values in either sex of any of the three groups before administration of TRH and LHRH. The changes in these four variables after TRH/LHRH administration to each individual were within physiological limits (Table 6); no significant differences from zero were demonstrated in the '40-minute concentration' values expressed as log (Me/Ms), quantifying the response to bromide after TRH/LHRH administration in terms of multiples of the concentration before this treatment (see Experimental). [Tables providing details of these results are available from the authors.]

Table 6. Serum concentrations of thyrotropin (TSH) and prolactin in volunteers before and immediately after ingestion of 0, 4 or 9 mg Br^-/kg/day for 12 weeks (males) or over three menstrual cycles, determined in each case on samples taken before and after administration of thyrotropin-releasing hormone (TRH) and luteinizing hormone-releasing hormone (LHRH)

Hormone	Dose (mg Br / kg/day)	No./ group	Serum concentration (mean ± SD)		
			Start of test	End of test	log (end/start)
Without TRH/LHRH injection					
Males					
TSH (mIU/litre)	0	7	1.8 ± 0.6	2.1 ± 0.7	0.043 ± 0.153
	4	7	2.0 ± 0.2	2.2 ± 0.8	− 0.001 ± 0.204
	9	7	2.0 ± 0.7	2.1 ± 0.4	0.026 ± 0.191
Prolactin (IU/litre)	0	7	0.35 ± 0.12	0.40 ± 0.13	0.064 ± 0.114
	4	7	0.25 ± 0.08	0.24 ± 0.09	− 0.036 ± 0.184
	9	7	0.18 ± 0.09	0.24 ± 0.12	0.127 ± 0.254
Females					
TSH (mIU/litre)	0	7	1.9 ± 0.8	2.2 ± 1.0	0.060 ± 0.112
	4	4	2.8 ± 0.5	2.1 ± 0.9	− 0.152 ± 0.127
	9	6	1.8 ± 0.7	2.0 ± 1.0	0.045 ± 0.217
Prolactin (IU/litre)	0	7	0.35 ± 0.21	0.28 ± 0.14	− 0.071 ± 0.194
	4	4	0.46 ± 0.17	0.36 ± 0.17	− 0.109 ± 0.110
	9	6	0.27 ± 0.14	0.28 ± 0.19	− 0.020 ± 0.132
After TRH/LHRH injection					
Males					
TSH†	0	7	3.8 ± 0.9	3.1 ± 1-0	− 0.10 ± 0.16
	4	7	3.2 ± 0.8	3.3 ± 0.9	0.01 ± 0.14
	9	7	3.4 ± 2.1	2.8 ± 0.4	− 0.02 ± 0.23
Prolactin†	0	7	2.8 ± 0.9	2.8 ± 1.0	0.00 ± 0.11
	4	7	2.7 ± 0.9	3.0 ± 1.7	0.03 ± 0.18
	9	7	3.0 ± 1.1	2.4 ± 0.8	− 0.10 ± 0.21
Females					
TSH†	0	7	5.2 ± 2.8	5.6 ± 1.7	0.07 ± 0.14
	4	4	6.0 ± 1.7	6.8 ± 3.4	0.03 ± 0.09
	9	6	10.1 ± 5.7	6.6 ± 2.3	− 0.16 ± 0.37
Prolactin†	0	7	4.2 ± 1.6	4.2 ± 1.4	0.08 ± 0.17
	4	4	3.3 ± 0.6	4.4 ± 1.2	0.12 ± 0.10
	9	6	4.1 ± 1.3	3.9 ± 1.2	− 0.01 ± 0.13

†Expressed as a multiple of the concentration before TRH/LHRH injection.
Statistical analyses on log(end/start) ratios (see Experimental) showed no significant findings.

Table 7. Significant variables of different cortical areas with respect to the differences in the absolute powers of montage 2 (Fig. 1)

		Significant variables											
	Area...	F–C		F–T		C–P		T–T_p		P–O		T_p–O	
Parameter	Hemisphere...	L	R	L	R	L	R	L	R	L	R	L	R
Log power δ_1-band		0.04	0.02	0.06	0.08	0.06	0.07	0.10	—	—	—	—	—
Log power δ_2-band		—	0.05	0.04	—	0.02	0.09	0.07	—	—	—	—	—
Log power θ-band		—	—	—	—	0.04	0.10	0.07	—	—	—	—	—
Log power α_1-band		—	0.03	0.02	0.09	0.04	0.01	0.07	0.02	0.09	—	0.09	0.03
Log power β_2-band		0.04	0.08	0.07	0.01	—	—	—	0.06	—	—	—	—
Log power β_3-band		—	—	0.07	0.02	—	—	—	0.08	—	—	—	—
Log total power		—	0.01	—	0.10	—	—	—	—	—	—	—	—
Quotient θ/α_1		—	0.07	0.10	—	0.04	0.02	—	0.04	—	—	0.04	0.07
Activity		0.10	0.01	—	—	—	—	—	—	—	—	—	—
Mobility		0.03	0.02	0.08	0.02	0.02	—	—	0.02	—	—	—	—
Complexity		—	—	0.10	—	—	—	—	—	—	—	—	—

F = Frontal C = Central T = Temporal P = Parietal O = Occipital

Central nervous system studies

Visual inspection of the EEG records did not reveal overt differences caused by bromide. A slight increase in sleep patterns was detectable in the second recording. In Table 7 the statistically significant variables are grouped for the different cortical areas. They represent those variables that showed a significant effect of bromide. Examination reveals that in the temporo-occipital and parieto-occipital areas practically no effect was visible.

Regarding the total power, a moderate effect was detectable in the frontal areas. In other parts only a trend in that direction could be seen. For the three dosage groups alike, a slight decrease in overall power indicated a general effect attributable to small attenuation differences. The most important changes were found in the δ-bands, the α_2-bands and the β-bands. Regarding scalp distribution, these effects were most pronounced over the temporal and central areas. The occipital and parietal areas showed practically no significantly changes.

The observed effects were symmetrical. Apparent asymmetries in significance were due to the rather small changes and the relatively small number of subjects. Although in the normal EEG asymmetries between the left and right hemispheres exist because of the difference in hemispheric dominance (Butler & Glass, 1974), these are irrelevant here as only the differences between the start and the end of the investigation were analysed.

Correlation analysis shows that the effects were generalized. This means that all the different areas displayed the same direction of change due to bromide for the different parameters of the EEG. Only the magnitudes differed. In Table 8 the mean values and standard deviations are given for the five most important EEG variables for the different cortical areas. In Fig. 2, the effects for the three different test groups are depicted. For one cortical area, Fig. 2a shows the δ_1-band course in which the decrease in power is visible. The same effect is visible for the δ_2-band (Fig. 2b). The mean decrease induced by bromide as 3 dB for the fronto-central and fronto-temporal areas and 2 dB for the temporal and central parts. In contrast, the β-bands showed an increase in power amounting to about 3 dB (Fig. 2d). Although visible in this graph, the effect is much better observed when the relative instead of the absolute power is used, because of the statistically better properties of the former.

A remarkable course is seen for the α_1-band in Fig. 2c. All areas showed a significant decrease with the lower dose group but, in some areas, the effect disappeared with the higher dose. The significance of this finding is not clear. It may have been fortuitous or the expression of the start of a shift in power between frequency bands.

The Mobility parameter (Fig. 2e) clearly indicated a general increase in the mean frequency, which varied between 1–2 Hz for the fronto-central and 3–4 Hz for the temporal areas. It was calculated directly from the initial EEG in quite a different way and confirmed the shift in power from the lower frequency bands to the higher already demonstrated in the individual bands of the power spectrum.

The VERs were analysed in exactly the same way for the differences between the three groups. Here the latencies and amplitudes of the six main components were used. The only effect visible was a relatively small decrease in the latency of wave IV and a slight increase in the amplitude of waves II and IV in the channels that referred to temporal or frontal electrodes, in the group on the highest bromide dosage. As the VER is generated in the occipital lobes and the spontaneous EEG analysis showed that the effects were located mainly in the more anterior cortical areas, these rather moderate changes were to be expected.

Discussion

The purpose of this study was to determine whether administration of 4 or 9 mg Br^-/kg/day for 3 months in males or for three full cycles in females might induce any effect on healthy human volunteers taking their normal diet. Special attention was paid to the endocrine system and the central nervous system—the endocrine system because endocrine effects were induced in rats by the administration of sodium bromide, the central nervous system because the therapeutic use of sodium bromide suggested that an effect might be expected.

The plasma-bromide concentration at the start of

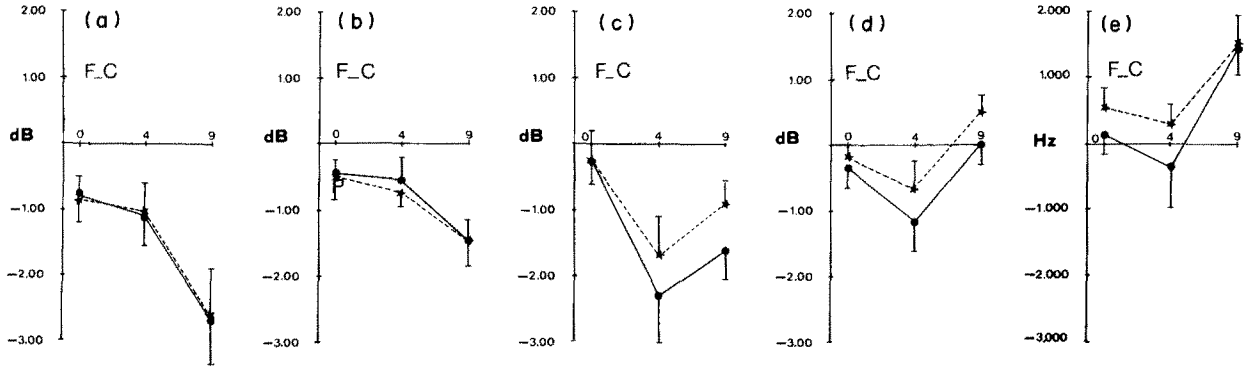

Fig. 2. Course of the main spectral parameters over the left and right fronto-central area for the three dosage groups: (a) $\log(P_e/P_s)$ δ_1-band; (b) $\log(P_e/P_s)$ δ_2-band; (c) $\log(P_e/P_s)$ α_1-band; (d) $\log(P_e/P_s)$ β_2-band; (e) $M_e - M_s$ (M = Mobility). Right hemisphere, *——*; left hemisphere, ●——●.

Table 8. Means of variables for each cortical area of montage 2 (Fig. 1) for groups of 14, 13 and 11 volunteers taking, respectively, 0, 4 and 9 mg Br^-/kg/day for 12 weeks (males) or during three full cycles

Parameter	Br^- dose (mg/kg/day)	Mean values ± SD for areas: F–C L	F–C R	F–T L	F–T R	$T–T_p$ L	$T–T_p$ R	C–P L	C–P R
Log power δ_1-band	0	0.078 ± 0.105	0.083 ± 0.131	0.180 ± 0.152	0.113 ± 0.161	0.154 ± 0.160	0.100 ± 0.107	0.058 ± 0.090	0.090 ± 0.079
	4	0.110 ± 0.162	0.106 ± 0.154	0.225 ± 0.211	0.131 ± 0.330	0.092 ± 0.132	0.105 ± 0.112	0.086 ± 0.159	0.094 ± 0.120
	9	0.268 ± 0.240	0.266 ± 0.266	0.363 ± 0.266	0.398 ± 0.436	0.233 ± 0.216	0.228 ± 0.246	0.162 ± 0.097	0.182 ± 0.121
Log power δ_2-band	0	0.047 ± 0.082	0.048 ± 0.140	0.097 ± 0.161	−0.004 ± 0.182	0.001 ± 0.129	0.015 ± 0.105	0.057 ± 0.081	0.060 ± 0.061
	4	0.057 ± 0.128	0.073 ± 0.062	0.093 ± 0.108	0.051 ± 0.114	0.104 ± 0.118	0.094 ± 0.051	0.076 ± 0.145	0.087 ± 0.094
	9	0.145 ± 0.117	0.144 ± 0.143	0.176 ± 0.111	0.160 ± 0.173	0.146 ± 0.149	0.071 ± 0.094	0.150 ± 0.105	0.155 ± 0.095
Log power α_1-band	0	0.027 ± 0.139	0.026 ± 0.160	−0.036 ± 0.213	−0.006 ± 0.218	−0.013 ± 0.267	0.014 ± 0.281	0.011 ± 0.208	0.035 ± 0.248
	4	0.228 ± 0.244	0.170 ± 0.216	0.170 ± 0.274	0.210 ± 0.246	0.287 ± 0.270	0.250 ± 0.260	0.293 ± 0.256	0.273 ± 0.245
	9	0.160 ± 0.161	0.088 ± 0.118	0.072 ± 0.205	0.023 ± 0.117	0.167 ± 0.255	0.121 ± 0.211	0.177 ± 0.216	0.174 ± 0.199
Log power β_2-band	0	0.034 ± 0.110	0.018 ± 0.080	−0.035 ± 0.197	0.074 ± 0.290	0.092 ± 0.220	0.133 ± 0.302	0.008 ± 0.094	0.047 ± 0.077
	4	0.113 ± 0.156	0.063 ± 0.141	0.159 ± 0.388	0.223 ± 0.458	0.164 ± 0.357	0.155 ± 0.313	0.079 ± 0.161	0.071 ± 0.139
	9	0.001 ± 0.108	0.049 ± 0.096	−0.215 ± 0.289	−0.132 ± 0.279	−0.098 ± 0.242	−0.066 ± 0.349	0.019 ± 0.098	−0.010 ± 0.099
Mobility	0	−0.158 ± 1.074	−0.537 ± 1.031	−0.847 ± 2.457	−0.222 ± 2.934	0.342 ± 2.442	0.338 ± 2.442	−0.057 ± 0.679	−0.168 ± 0.524
	4	0.342 ± 2.064	−0.297 ± 0.984	−0.515 ± 5.193	0.706 ± 5.678	0.290 ± 3.445	0.202 ± 3.689	−0.133 ± 1.210	−0.069 ± 0.828
	9	−1.455 ± 1.450	−1.513 ± 1.620	−5.063 ± 4.670	−3.352 ± 6.431	−2.435 ± 2.285	−1.273 ± 4.162	−0.665 ± 0.631	−0.795 ± 0.550

F = Frontal C = Central T = Temporal P = Parietal

the investigation in all volunteers was of the same magnitude as in draftees in whom a mean concentration of 0.06 ± 0.02 mmol/litre was measured (Krajnc, den Tonkelaar & van Logten, 1979). In the control group of volunteers the plasma-bromide levels did not change during the investigation. The therapeutic plasma-bromide concentration ranges from 6 to 12 mmol/litre (Wade, 1977). In rats an effect was measured at plasma concentrations of 7.7 ± 1.1 mmol/litre and higher (van Logten *et al.* 1974). At the end of our investigation the mean plasma concentration in females and males receiving 9 mg Br^-/kg/day was therefore 66% of the lower therapeutic plasma concentration and over 50% of the plasma concentrations at which effects were observed in rats.

In the four groups receiving sodium bromide, the plasma-bromide concentration reached a steady state after 6 weeks. The estimated plasma half-life of bromide of about 10 days corresponds very well with the literature (Wade, 1977). In the male volunteers taking 9 mg Br^-/kg/day the plasma bromide concentration decreased slightly from week 8 to week 12. Whether this decrease was induced by changes in chloride intake, by physiological variation or by the inaccurate taking of the capsules could not be established.

The mean bromide and chloride excretion measured in urine collected at the start over 24 hours for all subjects reflected the usual bromide and chloride content of the daily diet in The Netherlands. It is well known that collecting 24-hour urine samples other than in hospital, though it seems easy, is in effect rather difficult. The individual creatinine excretion figures at the start and end of the investigation led to the conclusion that unfortunately not all subjects were as accurate as possible in urine collection. Therefore, the results of the urine analyses were not used for further kinetic interpretation.

The results of three female subjects could not be included in the evaluation for reasons not connected with the investigation. In one male subject a haematological abnormality not related to the ingestion of capsules was discovered accidentally. In the 38 subjects remaining, gastric discomfort (nausea) associated with the ingestion of bromide-containing capsules was recorded. In subjects taking sodium bromide a relation between the dose administered and the incidence of nausea was present, but this was absent in subjects taking placebos. Gastric discomfort is a known side effect of sodium bromide. Since the investigation was performed to establish a no-effect level in man with respect to food while the bromide dose was ingested once a day, this phenomenon may not be considered to be relevant to the study.

Several subjects mentioned a decrease in mental concentration and an increase in sleepiness, a known possible effect. However, on consideration of the relation between the mentioning of this symptom and the dose administered, association with the bromide ingested seemed unlikely. The results of the medical histories, the physical examinations and the haematological and biochemical analyses revealed no other effects attributable to bromide.

In the female subjects taking 9 mg Br^-/kg/day a significant increase in T_4 and T_3 was observed ($P < 0.01$), although the individual concentrations of T_4 and T_3 in this group were within normal limits at the start and the end of the investigation. FT_4 and TBG concentrations did not change. The slight increase in T_4 and T_3 did not induce a measurable decrease or an increase of TSH. The reaction of the pituitary to the administration of TRH did not change either. No such changes in the endocrinological variables relating to the function of the thyroid were observed in male subjects receiving the same dosage of sodium bromide or in males or females taking 4 mg Br^-/kg/day.

No changes were observed in measurements of hormones produced by the adrenals, the gonads and the pituitary gland.

It is striking that in man an effect is found in the female thyroid, because the female thyroid is also the most sensitive endocrine organ in rats. However, in contrast to man, rats showed a decrease in thyroid function (Loeber *et al.* 1982). Therefore confirmation of these results in another study would seem to be necessary.

Statistically significant changes were found in the EEG. These were apparent as shifts in power in spectral bands and a shift in mean frequency. These shifts never exceeded normal limits but reflected a shift in background EEG activity.

An increase in β-activity was described by Greenblatt, Levin & Schegloff (1945) who studied the EEG in patients with varying degrees of bromide intoxication and reported that with higher plasma levels (> 100 mg/100 ml or 15 mmol/litre) the EEG is characterized by diffuse slow activity, while with lower plasma levels (< 12.5 mmol/litre) faster rhythms can be seen. The results from our investigation show a decrease in the δ-activity, an increase in the β-activity and an increase in mean frequency, expressed in the Mobility. The rather conspicuous course of the α_1-band may have been fortuitous and due to the small number of subjects and the small shift in power. It may, however, also have reflected the fact that the arbitrary choice of limits of the power bands may obscure effects. If the effect of bromide at low doses consists of a slight decrease in power in the middle frequency range (around 7–9 Hz), this will be visible. When however, with higher plasma concentrations, the power distribution is shifted to higher frequencies, the former effect disappears. Such a differential effect of drugs in low doses is not uncommon (e.g. Herman & Kubicki, 1981; Saletu, Grunberger, Rajna & Karobath, 1980). Another result is that not all cortical areas are involved to the same extent. The occipital and parietal areas show only minor changes, while the changes are most pronouced in the fronto-temporal and central areas. The effects are strictly symmetrical. The asymmetrical occurrence of significant variables apparent in Table 7 may only reflect the small differences between the two hemispheres that may be expected in a small number of subjects.

Neurophysiological variables differ from biochemical entities in that they express dynamic rather than static values. Differences in patterns and different cortical areas, particularly between right and left, form the basis of the evaluation of EEG parameters.

Provisionally the effects found in the group receiving 4 mg Br^-/kg/day must be considered to be fortuitous. However, before regarding the changes in the higher dose group as an effect and 4 mg Br^-/kg/day as a no-effect level in man, it will be necessary to perform another study confirming the observed endocrine and neurophysiological results.

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INTERNATIONAL SYMPOSIUM ON RESIDUES AND TOXICITY OF BROMIDE: SUMMARY AND CONCLUSIONS

In the first paper of this symposium Dr Greve discussed the advantages and disadvantages of methods for the analysis of bromide. Moreover, data were given on residues of bromide in food and feedstuffs and on the daily intake of bromide residues by humans. The average daily intake in The Netherlands was estimated in two total-diet studies in the summer of 1976 and the winter of 1978. The average bromide content in the total diet was 3.6 and 3.2 mg/kg for the first and second study, respectively. Based on these levels and the food intakes, the estimates of daily bromide intake were 7.8 (range 3–15) and 7.6 (1.8–17.2) mg/person/day. These data showed a good agreement between summer and winter diets.

The paper further gave an account of residue data from classified food items and feedstuff samples. In general, the residues were either low ($\leqslant 1$ mg/kg food) or medium (*c.* 5 mg/kg food), but occasionally high residues ($\geqslant 200$ mg/kg food) were found in certain leafy vegetables or herbs. The main source of the high residues was treatment of soils with methyl bromide.

The following paper, by Drs Wegman, Hamaker and de Heer, was concerned with studies on the bromide-ion balance of a polder district where there was large-scale use of methyl bromide for soil fumigation. The concentration of bromide ion was determined in precipitation, surface water and ground water, in which the maximum concentrations were found to be 0.98, 41 and 17 g/m^3, respectively. The highest concentrations of bromide in surface water were found during the main fumigation season in September–October.

During a one-year period (September 1979–August 1980) a bromide balance was computed for the polder district, Delfland, based on the supply and discharge of bromide from the polder area. The use of methyl bromide contributed 222 Mg (Mg = 1000 kg) to the input of bromide ion in the polder district. This corresponded to 68% of the total input of bromide ion (325 Mg). On average over a year about 15% of the applied methyl bromide (1500 Mg, calculated as bromide) was converted to bromide ion. Corrective measures have since been introduced. The former use of low-density polyethylene (LDPE) sheeting has been banned and from 1981 a more gas-tight sheeting has had to be used. This allows for the use of a third of the earlier dose of methyl bromide.

The paper dealing with ecotoxicological studies on sodium bromide, presented by Drs Canton and Wester and Mrs Mathijssen-Spiekman, was an example of the broad nature of the papers presented at this symposium. The acute toxicity of bromide for fresh-water organisms was studied in four species: an alga, a crustacean species (*Daphnia magna*) and two species of fish, an Oryzias and a Poecilia. In other studies an effect on reproduction was found both in Daphnia and in the fish Poecilia, the guppy. Histopathological examination in the long-term studies revealed no effect in the Oryzias. In the Poecilia several histological changes were found, notably concentration-related thyroid hyperplasia, myopathy and regressive changes in the female reproductive tract. The thyroid changes are of special interest in view of the subsequently discussed findings in the rat.

The NOEC × (LC(EC)25/LC(EC)50) value, suggested earlier as an index for water quality based on ecotoxicity, was estimated to be 1 mg bromide/litre using as the criterion reproduction in the test with Poecilia. Concentrations found in surface water frequently exceed this value and it is claimed that levels can be so high that acute effects might be expected. As a general toxicologist, I have the feeling that the fact that the ecotoxicological studies were performed in closed vessels should be taken into consideration when interpreting the environmental importance of the studies.

Dr Rauws entered the field of mammalian and human toxicology. His overview of the pharmacokinetics of the bromide ion included a general discussion of the use and usefulness of pharmacokinetic studies in toxicology. The similarity of bromide to chloride gives rise to an important pharmacokinetic interaction. Both ions compete for reabsorption in the kidney tubules. Because of this competition, high chloride reabsorption will lead to higher bromide excretion and *vice versa*. This has been experimentally confirmed. The biological half-life of bromide can be decreased by administration of chloride. Conversely, the normal half-life of bromide, in man 12 days and in the rat 3 days, may be increased by a salt-restricted diet. In experiments by Rauws and the late van Logten the bromide half-life in the rat could thus be prolonged to 25 days by ingestion of a salt-free diet with tap water. It was found that the plasma bromide level could be influenced considerably by a low-chloride diet.

Two further findings should be mentioned. First the effect of chloride depletion can be mirrored in that the same plasma-bromide level can be obtained with much less bromide in the diet; secondly the foetus appears to be more accessible to bromide than the mother and elimination from the foetus is retarded.

The next three papers discussed, in detail, the toxic effects of bromide ion in the rat. The first paper by Drs van Leeuwen and den Tonkelaar and the late Dr van Logten dealt primarily with effects on the endocrine system and reproduction. This contribution provided a comprehensive discussion of the effect of bromide ion in the rat. In a 90-day oral study the dose levels used were 0, 75, 300, 1200, 4800 and 19,200 mg/kg in the diet and a complex of changes in

the endocrine system was observed. The effect on the thyroid, an activation, was the most prominent. In the highest dose groups, different effects were noted; e.g. atrophy of the testes and an effect on the prostate occurred in male rats, while in females a reduced number of corpora lutea were found. A three-generation reproduction study carried out with the same dose levels revealed a decrease in fertility in the two highest dose groups. This effect was, however, reversible on withdrawal of the bromide.

On the basis of the effect on the thyroid in the 90-day study (an increase of relative organ weight down to 1200 mg/kg diet), a no-effect level of 300 mg sodium bromide/kg was established. This corresponded to 240 mg bromide ion/kg diet, equivalent to 12 mg/kg body weight. By application of a safety factor of 100, a tentative ADI of 0.12 mg/kg is suggested. This figure will be considered again in connection with the study on human volunteers.

In the 90-day rat study reported here and in previous studies there were indications of a more general effect on the endocrine system. Further studies in the rat were performed to elucidate the effect of bromide on the thyroid, including studies on a chloride-depleted diet. The effect on thyroid function was studied using several parameters, including thyroxine concentration in the serum and uptake of radiolabelled iodine by the thyroid. The effect was complex and will not be discussed further in this summary.

A study was undertaken by Drs Loeber, Franken and van Leeuwen to elucidate the effect of sodium bromide in the rat. Sophisticated techniques of histopathology and clinical chemistry were employed, including immunocytochemistry (Franken) and radioimmunoassay (Loeber). Male rats were fed 0, 20, 75, 300, 1200 or 19,200 mg/kg diet for 4 or 12 weeks. At the end of the experiments the pituitary gland, thyroid and testes were examined by histopathological and immunocytochemical techniques. Serum hormone levels were estimated by radioimmunoassay. Through the application of these techniques it can be concluded that sodium bromide, at least in high doses, disturbs the function of the thyroid and the testes directly, thereby indirectly affecting the pituitary gland. There are indications from the results that other endocrine organs, such as the pancreas and adrenal, are involved.

The study by Drs Hansen and Hübner from the BGA in Berlin elucidated the effect of bromide on the behaviour of mice. Using a comparatively simple model in mice, behavioural effects were studied in an objective way. With the help of a computer, it was possible to cope with the great amount of data that comes from such an experiment. The threshold effect was between 400 and 200 mg bromide/kg diet in the short-term study. Using the plasma levels of bromide found in the 90-day rat study it could be estimated that behavioural effects in mice may appear at somewhat lower levels than the levels related to mental and neurological disturbances in man.

The paper by Drs Sangster, Blom, Sekhuis, Loeber, Rauws, Koedam and Krajnc and the late Dr van Logten indicated a considerable team effort. The authors come from six departments of the National Institute of Public Health and from the TNO—Netherlands Institute for Preventive Care. This was a follow-up of an earlier study in human volunteers. In the first study the volunteers were administered an oral daily dose equal to the FAO/WHO JMPR-recommended ADI of 1 mg bromide/kg body weight for 8 weeks. No effects, particularly on the endocrine system, were observed. In the study reported at this symposium clinical observations were supplemented with the use of advanced techniques, especially for studying the effects on the endocrine system and the central nervous system. Healthy young male and female volunteers were administered a higher dose of bromide than in the first study. The doses in the new study were 0, 4 and 9 mg bromide/kg body weight. The higher dose had an effect on thyroid hormones in female subjects, while no effect was found at 4 mg/kg. It is remarkable that in man, as in the rat, the thyroid was the most sensitive endocrine organ, although in man—in contrast to rats—an increase in thyroid function was found.

Statistically significant effects were found in the EEG, the changes being expressed as shifts in power in the spectral bands and a shift in mean frequency. This effect was found in the 9-mg bromide/kg body weight group, while the provisional conclusion was that no effect was apparent in the 4-mg/kg group. It is considered necessary to perform a further study to confirm these preliminary results.

Comments and conclusions in relation to the toxicological evaluation

The results of a further study will be important in many respects, but mainly in relation to the evaluation of the average daily intake compared to the provisional ADI based on the rat study. An ADI of 0.12 mg/kg would, in a 60-kg person, lead to an acceptable bromide intake of 7.2 mg daily. This is nearly equal to the average intakes of 7.8 and 7.6 mg mentioned in the first paper today. It is not an acceptable situation that the average intake is about the same as or even exceeds the ADI. The rat-based ADI could be taken to be supported by the first human study, in which no effect was found at 1 mg/kg body weight. It is generally acceptable to apply a safety factor of 10 to such human data and this would give an ADI of 0.1 mg/kg body weight, approximately the same value as the rat-based ADI.

At this time I should like to add a further comment. Even if the results of the second comprehensive human study are not final, it seems satisfactory from a health point of view that the study indicates that the no-effect level in humans might be 4 mg bromide/kg body weight/day. If this is confirmed in further studies it would allow for the calculation, from these data, of a tentative ADI of 0.4 mg/kg body weight, which would correspond to an acceptable intake, for a 60-kg man, of 24 mg/day. Compared with this daily intake the average bromide intakes found in the two Dutch total diet studies (7.6 and 7.8 mg/day) would be much more acceptable from a toxicological point of view.

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Research Section

SHORT-TERM TOXICITY STUDY IN RATS OF CHLORINATED CAKE FLOUR

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Abstract—Male and female Wistar rats were fed for 28 days on a diet containing either chlorinated (1257 or 2506 ppm chlorine) or unchlorinated flour. No significant differences between groups in body weight were observed in the males. A significant inverse correlation between body weight and treatment level, attributable to a corresponding trend in food intakes, was found for the females only. No significant differences between absolute organ weights were found, but when the weights were adjusted for covariance with body weight, dose-related increases in kidney weight (males) and liver weight (both sexes) were found. Histopathological examination revealed no pathological tissue changes attributable to the chlorination of the flour.

INTRODUCTION

Chlorinated flour has been reported to be toxic to rats (Cunningham & Lawrence, 1978; Cunningham, Lawrence & Tryphonas, 1977). The evidence for this conclusion is based partly on feeding chlorinated flour to male rats for only 2 wk, after which time reduced growth rates, increased liver and kidney weights, and changes in the histology of the liver were observed. The flour treatment levels used in this work were 2000 ppm and 10,000 ppm. When the treatments were carried out for 10 wk similar effects were seen (although histological studies were not made) and the lower dose did not affect liver weight.

In earlier work from this laboratory (Fisher, Hutchinson, Berry *et al.* 1979a) rats were fed with cake made from flour treated with up to 10,700 ppm chlorine (the "saturation" level for this flour) for periods of up to 19 months. The differences in liver and kidney weights then observed were not associated with any pathological changes in these tissues, in contrast to the changes reported in the much shorter trials of Cunningham *et al.* (1977). The following trial was therefore carried out to determine the effects of feeding chlorinated flour to rats for 4 wk.

EXPERIMENTAL

Flour. Flour containing the statutory vitamin and mineral supplements, including chalk, was supplied by Spillers Ltd; aliquots were chlorinated at their Technological Research Centre, Cambridge at levels of 1250 and 2500 ppm respectively, using chlorine of 99.99% purity. The control flour contained 581 ppm total chlorine. The flours contained 11.5% protein, and 13.3% moisture, and their colour grade was 2.9.

Diets. The three diets, designated 0, 1250 and 2500, differed only in the level of chlorine in the flour, and consisted of (% by weight): flour 80, casein 10.7, corn oil 4, mineral mixture (Jones & Foster, 1942) 4, vitamin mixture (Fisher, Hutchinson, Berry *et al.* 1979b) 1, L-lysine hydrochloride 0.3.

Animals. Weanling Wistar-derived Porton strain rats, nine males and nine females per diet group, were bred at FMBRA from stock (MRC category 3) supplied by Olac Southern Ltd, Bicester, Oxfordshire and housed in groups of three in conditions described previously (Fisher *et al.* 1979b). Food and water were available *ad lib.* Food intakes, corrected for spillage, were recorded thrice weekly and animals were weighed daily. Rats were killed with an overdose of ether and their kidneys, livers, hearts and brains were removed and weighed. Tissues were preserved in buffered formalin for histological examination.

Histopathology. Sections (5 μm) of livers and kidneys were stained with haematoxylin and eosin for examination.

Statistical analysis. This was carried out as described previously (Fisher *et al.* 1979b) using a Texas 980B computer.

RESULTS

No evidence of abnormality was observed in the appearance or behaviour of the animals during the

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Table 1. Mean body weights of rats fed unchlorinated or chlorinated cake flours in their diet for one month

Chlorine level*	Day...	Mean body weight (g) 0	7	14	21	28†
Males						
0		86	133	184	236	270
1250		89	135	183	238	273
2500		88	130	180	231	264
Females						
0		78	112	139	161	176
1250		75	112	134	158	173
2500		75	109	131	153	166

*The flours designated 0, 1250 and 2500 contained 0, 1257 and 2506 ppm of added chlorine, respectively.
†The SEM terminal body weights were 5.3 g for males and 3.2 g for females.
Values are means for groups of nine animals. Terminal body weights of females were inversely related to the chlorine treatment level ($P < 0.05$).

Table 2. Mean food intakes of rats fed unchlorinated or chlorinated cake flours in their diet for one month

Chlorine level*	Week...	Mean food intake (g/day) 1	2	3	4†
Males					
0		14.5	18.1	19.9	22.2
1250		14.7	18.0	20.2	22.1
2500		13.8	18.0	20.1	21.1
Females					
0		12.5	14.7	14.9	17.1
1250		12.7	13.5	14.1	16.0
2500		12.4	13.3	14.1	15.1

*The flours designated 0, 1250 and 2500 contained 0, 1257 and 2506 ppm of added chlorine, respectively.
†The SEM was 2.4 g/day for males and 1.5 g/day for females.
Values are means for groups of nine animals. Food intakes of females were inversely related to chlorine treatment level in wk 4 ($P < 0.05$).

trial. There were no significant differences in terminal body weights in the males (Table 1). In the females, terminal body weight was inversely correlated with chlorine treatment level. The food intakes of the females, but not the males, showed a significant inverse correlation at week 4 with chlorine treatment level (Table 2); the corresponding trend in terminal body weights of the females disappeared after adjustment for covariance with the food intakes. Both absolute organ weights and weights after adjustment for covariance with body weight (Shirley, 1977; Takizawa, 1978) are shown in Table 3. There were no significant differences in absolute organ weights between treatment groups. However the weights adjusted for covariance with body weight showed significant dose-related increases in kidney weights in males, and in liver weights in both sexes. Adjusted brain and heart weights showed no correlation with chlorine treatment level.

Liver and kidney sections showed the features listed in Table 4. Only minor histopathological findings were noted in the livers. Cell vacuolation was

Table 3. Mean organ weights of rats fed unchlorinated or chlorinated cake flours in their diet for one month

Treatment group†	Mean organ weight (g) Brain	Kidneys	Heart	Liver
Absolute weight				
Males				
0	1.71	2.04	1.06	13.81
1250	1.83	2.19	0.99	14.81
2500	1.84	2.14	1.00	14.74
SEM	0.065	0.071	0.050	0.427
Females				
0	1.78	1.47	0.74	8.18
1250	1.64	1.46	0.74	8.56
2500	1.64	1.44	0.73	9.38
SEM	0.065	0.038	0.035	0.431
Weight adjusted for covariance with body weight				
Males				
0	1.71	* 2.04	1.06	* 13.77
1250	1.81	2.15	0.98	14.59
2500	1.86	2.19	1.01	15.00
SEM	0.061	0.047	0.050	0.317
Females				
0	1.80	1.45	0.74	*** 7.81
1250	1.64	1.45	0.74	8.49
2500	1.63	1.47	0.72	** 9.81
SEM	0.065	0.035	0.035	0.360

†The flours designated 0, 1250 and 2500 contained 0, 1257 and 2506 ppm of added chlorine, respectively.
Values are means for groups of nine animals. Those marked with asterisks differ significantly (ANOVA and Student's t test) from those for the corresponding cake-fed control group (**$P < 0.01$). Vertical lines joining values indicate a statistically significant dose relationship (*$P < 0.05$; ***$P < 0.001$) but the slopes of the dose-response relationships only differ significantly from zero for diet 2500.

Table 4. Frequency of histopathological features in rats fed unchlorinated or chlorinated cake flours in their diet for one month

		No. of rats showing the feature listed*					
	Chlorine level†...	0		1250		2500	
Histopathological feature	Sex...	M	F	M	F	M	F
Liver							
Cell vacuolation		8	1	3	1	7	3
Cells in mitosis		4	2	4	3	3	1
Cells with large nuclei		1	2	2	1	2	2
Leucocytic and haemopoietic foci		1	3	3	4	1	3
Kidney							
Swollen cells with some vacuolation in distal tubules		2	2	0	3	1	1
Calculi in collecting tubules		1	6	1	2	0	6
Leucocytic foci		0	0	0	0	1	0
Small cortical cyst		0	0	0	0	1	0

M = Male F = Female

*Each group comprised nine rats.

†The flours designated 0, 1250 and 2500 contained 0, 1257 and 2506 ppm of added chlorine, respectively.

of the 'glycogen' type rather than fat vacuolation. Cells in mitosis are common in young animals, as are haemopoietic foci. Some of the leucocytic foci observed in the liver were minute and not readily distinguished from haemopoietic foci.

The presence at such an early age (mainly in the females) of calculi or minute calcareous deposits in the collecting tubules of the kidneys is remarkable. Some had only solitary calculi, others had several, but the chlorine treatment level had no effect on their incidence.

DISCUSSION

In their 14-day study of rats given chlorinated cake flour Cunningham *et al.* (1977) found that growth rates were significantly reduced, by 20.7 and 85.7% respectively for rats given flour chlorinated at 0.2% and 1%. However, in the present study after 28 days on chlorinated flour diets, rats of both sexes showed only small differences between groups in terminal body weights (deficits in males of 2% (NS) and in females of 5.8% ($P < 0.05$) for the high-dose groups). The significant trend to lower terminal body weight with increasing chlorine treatment level noted in females, but not in males, was statistically completely attributable to the associated reduction in food intake by these animals. Moreover, the growth rates of the male control Wistar rats in the present trial of 6.6 g/day contrast sharply with the exceptionally poor growth rates of 2.4 g/day of the control group fed unchlorinated wheat flour by Cunningham *et al.* (1977), suggesting either a gross deficiency in the basal diet fed to their animals, or their use of abnormal rats. Although their weanling rats were smaller than ours, the higher growth rates of controls in other experiments in that paper, 4.6 g/day (for rats fed wheat gluten in their diet) and 5.6 g/day (for pair-fed rats fed wheat lipids in their diet), suggest that the rats in the first trial were in very poor condition, and the results must therefore be open to some doubt.

The organ weight data obtained in the present work accord with our earlier work (Fisher *et al.* 1979a), in which feeding of rats with diets containing 93% cake made from heavily chlorinated flour (10,700 ppm) caused significant ($P < 0.001$) elevations of liver and kidney weights relative to body weight, compared with controls. The absolute weights of these organs in treated animals were also significantly different from those of controls in the earlier trial but not in the present work. These findings are in agreement with those of Cunningham *et al.* (1977). However, no pathological changes were found in the livers and kidneys of the rats either in our earlier trial (Fisher *et al.* 1979a) or in the present work, contrary to the findings of the Canadian workers. The animals in our earlier study were fed with a highly unpalatable diet (food intakes were consistently lower than for controls in a series of experiments) for 19 months, and it is conceivable that the organ weight changes represented a physiological adaptation to the nature of the diet, e.g. an increase in liver metabolism in order to dechlorinate the chlorinated fatty acids ingested. The absence of pathological changes attributable to the chlorination of flour in the present trial confirms our earlier findings and strengthens this conclusion. Furthermore, in earlier work (Fisher *et al.* 1979a) evidence was obtained that the effects of chlorination were not simply related to treatment level, since growth was inhibited by cake made from highly-chlorinated flour that had been diluted with unchlorinated flour, but was not inhibited by cake made from flour that had been chlorinated directly to the same level.

Certain features of the evidence of Cunningham *et al.* (1977) and Cunningham & Lawrence (1978) detract from the acceptability of concluding on the basis of their results that chlorinated flour is toxic to rats:

(i) Food intakes are not reported, and thus proper assessment of the body-weight gain data is not possible.

(ii) Flour, rather than cake, was fed in all but one of the published experiments. It has become widely accepted in recent years that toxicity testing of

food additives should be carried out using a food product in the form that is consumed by the public, so that changes in the test compound or its products induced during food processing are taken into account. This attitude has been adopted officially in the UK, where tests on flour are no longer regarded as acceptable (Committee on Medical Aspects of Food Policy, Pharmacology Sub-Committee, 1974) for the evaluation of additives used in making baked products such as bread or cake.

(iii) A low-protein flour was treated with exceptionally high levels of chlorine in the studies of Cunningham and his colleagues. In commercial practice the highest level used, 2500 ppm, is usually reserved for the high-protein flours used in making fruit cake.

(iv) Levels of 2000 or 10,000 ppm chlorine were used in flour and flour lipids, and even 50,000 ppm chlorine in gluten; this approach is highly questionable. It can neither be assumed that results obtained with high levels of chlorine can be meaningfully extrapolated to low levels, nor that chlorination of a subfraction of flour or dough produces similar products to those found when whole flour is chlorinated. The fallacy is seen clearly in the case of gluten, since flour chlorinated to 50,000 ppm would not form a gluten (Ewart, 1968). Indeed, whole flour would not be capable of taking up anything like this amount of chlorine, the maximum uptake being of the order of 11,000 ppm. Where a vast excess of a highly reactive element like chlorine is available, the likelihood of secondary reactions is obviously greatly increased. Cunningham *et al.* (1977) and Cunningham & Lawrence (1978) do not seem to have taken into account the fact that about half the chlorine used in flour treatment is recoverable as inorganic chloride (Sollars, 1961), and overlook the implications of differences in the partition of the chlorine between the various components of flour, which must vary with chlorination level.

Long term studies have been carried out since that trial was completed (Fisher *et al.* 1979a; Fisher, Hutchinson, Berry *et al.* 1983a,b). These do not substantiate the claim that chlorination of flour for use in cake-making is hazardous to the health of the consumer, and indeed the Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment (UK) has recommended that chlorine be included in Class A, those additives that the available evidence suggests are acceptable for use in food.

Acknowledgement—We are grateful to the staff of Spillers Ltd (now Dalgety-Spillers Ltd) for supply, chlorination and analysis of the flours.

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Research Section

SHORT-TERM TOXICITY STUDY IN RATS OF CHLORINATED CAKE FLOUR

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(*Received* 10 *August* 1982)

Abstract—Male and female Wistar rats were fed for 28 days on a diet containing either chlorinated (1257 or 2506 ppm chlorine) or unchlorinated flour. No significant differences between groups in body weight were observed in the males. A significant inverse correlation between body weight and treatment level, attributable to a corresponding trend in food intakes, was found for the females only. No significant differences between absolute organ weights were found, but when the weights were adjusted for covariance with body weight, dose-related increases in kidney weight (males) and liver weight (both sexes) were found. Histopathological examination revealed no pathological tissue changes attributable to the chlorination of the flour.

INTRODUCTION

Chlorinated flour has been reported to be toxic to rats (Cunningham & Lawrence, 1978; Cunningham, Lawrence & Tryphonas, 1977). The evidence for this conclusion is based partly on feeding chlorinated flour to male rats for only 2 wk, after which time reduced growth rates, increased liver and kidney weights, and changes in the histology of the liver were observed. The flour treatment levels used in this work were 2000 ppm and 10,000 ppm. When the treatments were carried out for 10 wk similar effects were seen (although histological studies were not made) and the lower dose did not affect liver weight.

In earlier work from this laboratory (Fisher, Hutchinson, Berry *et al.* 1979a) rats were fed with cake made from flour treated with up to 10,700 ppm chlorine (the "saturation" level for this flour) for periods of up to 19 months. The differences in liver and kidney weights then observed were not associated with any pathological changes in these tissues, in contrast to the changes reported in the much shorter trials of Cunningham *et al.* (1977). The following trial was therefore carried out to determine the effects of feeding chlorinated flour to rats for 4 wk.

EXPERIMENTAL

Flour. Flour containing the statutory vitamin and mineral supplements, including chalk, was supplied by Spillers Ltd; aliquots were chlorinated at their Technological Research Centre, Cambridge at levels of 1250 and 2500 ppm respectively, using chlorine of 99.99% purity. The control flour contained 581 ppm total chlorine. The flours contained 11.5% protein, and 13.3% moisture, and their colour grade was 2.9.

Diets. The three diets, designated 0, 1250 and 2500, differed only in the level of chlorine in the flour, and consisted of (% by weight): flour 80, casein 10.7, corn oil 4, mineral mixture (Jones & Foster, 1942) 4, vitamin mixture (Fisher, Hutchinson, Berry *et al.* 1979b) 1, L-lysine hydrochloride 0.3.

Animals. Weanling Wistar-derived Porton strain rats, nine males and nine females per diet group, were bred at FMBRA from stock (MRC category 3) supplied by Olac Southern Ltd, Bicester, Oxfordshire and housed in groups of three in conditions described previously (Fisher *et al.* 1979b). Food and water were available *ad lib.* Food intakes, corrected for spillage, were recorded thrice weekly and animals were weighed daily. Rats were killed with an overdose of ether and their kidneys, livers, hearts and brains were removed and weighed. Tissues were preserved in buffered formalin for histological examination.

Histopathology. Sections (5 μm) of livers and kidneys were stained with haematoxylin and eosin for examination.

Statistical analysis. This was carried out as described previously (Fisher *et al.* 1979b) using a Texas 980B computer.

RESULTS

No evidence of abnormality was observed in the appearance or behaviour of the animals during the

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Table 1. Mean body weights of rats fed unchlorinated or chlorinated cake flours in their diet for one month

Chlorine level*	Day...	Mean body weight (g) 0	7	14	21	28†
Males						
0		86	133	184	236	270
1250		89	135	183	238	273
2500		88	130	180	231	264
Females						
0		78	112	139	161	176
1250		75	112	134	158	173
2500		75	109	131	153	166

*The flours designated 0, 1250 and 2500 contained 0, 1257 and 2506 ppm of added chlorine, respectively.
†The SEM terminal body weights were 5.3 g for males and 3.2 g for females.
Values are means for groups of nine animals. Terminal body weights of females were inversely related to the chlorine treatment level ($P < 0.05$).

Table 2. Mean food intakes of rats fed unchlorinated or chlorinated cake flours in their diet for one month

Chlorine level*	Week...	Mean food intake (g/day) 1	2	3	4†
Males					
0		14.5	18.1	19.9	22.2
1250		14.7	18.0	20.2	22.1
2500		13.8	18.0	20.1	21.1
Females					
0		12.5	14.7	14.9	17.1
1250		12.7	13.5	14.1	16.0
2500		12.4	13.3	14.1	15.1

*The flours designated 0, 1250 and 2500 contained 0, 1257 and 2506 ppm of added chlorine, respectively.
†The SEM was 2.4 g/day for males and 1.5 g/day for females.
Values are means for groups of nine animals. Food intakes of females were inversely related to chlorine treatment level in wk 4 ($P < 0.05$).

trial. There were no significant differences in terminal body weights in the males (Table 1). In the females, terminal body weight was inversely correlated with chlorine treatment level. The food intakes of the females, but not the males, showed a significant inverse correlation at week 4 with chlorine treatment level (Table 2); the corresponding trend in terminal body weights of the females disappeared after adjustment for covariance with the food intakes. Both absolute organ weights and weights after adjustment for covariance with body weight (Shirley, 1977; Takizawa, 1978) are shown in Table 3. There were no significant differences in absolute organ weights between treatment groups. However the weights adjusted for covariance with body weight showed significant dose-related increases in kidney weights in males, and in liver weights in both sexes. Adjusted brain and heart weights showed no correlation with chlorine treatment level.

Liver and kidney sections showed the features listed in Table 4. Only minor histopathological findings were noted in the livers. Cell vacuolation was

Table 3. Mean organ weights of rats fed unchlorinated or chlorinated cake flours in their diet for one month

Treatment group†	Mean organ weight (g) Brain	Kidneys	Heart	Liver
Absolute weight				
Males				
0	1.71	2.04	1.06	13.81
1250	1.83	2.19	0.99	14.81
2500	1.84	2.14	1.00	14.74
SEM	0.065	0.071	0.050	0.427
Females				
0	1.78	1.47	0.74	8.18
1250	1.64	1.46	0.74	8.56
2500	1.64	1.44	0.73	9.38
SEM	0.065	0.038	0.035	0.431
Weight adjusted for covariance with body weight				
Males				
0	1.71	* 2.04	1.06	* 13.77
1250	1.81	2.15	0.98	14.59
2500	1.86	2.19	1.01	15.00
SEM	0.061	0.047	0.050	0.317
Females				
0	1.80	1.45	0.74	*** 7.81
1250	1.64	1.45	0.74	8.49
2500	1.63	1.47	0.72	** 9.81
SEM	0.065	0.035	0.035	0.360

†The flours designated 0, 1250 and 2500 contained 0, 1257 and 2506 ppm of added chlorine, respectively.
Values are means for groups of nine animals. Those marked with asterisks differ significantly (ANOVA and Student's t test) from those for the corresponding cake-fed control group (**$P < 0.01$). Vertical lines joining values indicate a statistically significant dose relationship (*$P < 0.05$; ***$P < 0.001$) but the slopes of the dose-response relationships only differ significantly from zero for diet 2500.

Table 4. Frequency of histopathological features in rats fed unchlorinated or chlorinated cake flours in their diet for one month

		No. of rats showing the feature listed*					
	Chlorine level†...	0		1250		2500	
Histopathological feature	Sex...	M	F	M	F	M	F
Liver							
Cell vacuolation		8	1	3	1	7	3
Cells in mitosis		4	2	4	3	3	1
Cells with large nuclei		1	2	2	1	2	2
Leucocytic and haemopoietic foci		1	3	3	4	1	3
Kidney							
Swollen cells with some vacuolation in distal tubules		2	2	0	3	1	1
Calculi in collecting tubules		1	6	1	2	0	6
Leucocytic foci		0	0	0	0	1	0
Small cortical cyst		0	0	0	0	1	0

M = Male F = Female

*Each group comprised nine rats.

†The flours designated 0, 1250 and 2500 contained 0, 1257 and 2506 ppm of added chlorine, respectively.

of the 'glycogen' type rather than fat vacuolation. Cells in mitosis are common in young animals, as are haemopoietic foci. Some of the leucocytic foci observed in the liver were minute and not readily distinguished from haemopoietic foci.

The presence at such an early age (mainly in the females) of calculi or minute calcareous deposits in the collecting tubules of the kidneys is remarkable. Some had only solitary calculi, others had several, but the chlorine treatment level had no effect on their incidence.

DISCUSSION

In their 14-day study of rats given chlorinated cake flour Cunningham *et al.* (1977) found that growth rates were significantly reduced, by 20.7 and 85.7% respectively for rats given flour chlorinated at 0.2% and 1%. However, in the present study after 28 days on chlorinated flour diets, rats of both sexes showed only small differences between groups in terminal body weights (deficits in males of 2% (NS) and in females of 5.8% ($P < 0.05$) for the high-dose groups). The significant trend to lower terminal body weight with increasing chlorine treatment level noted in females, but not in males, was statistically completely attributable to the associated reduction in food intake by these animals. Moreover, the growth rates of the male control Wistar rats in the present trial of 6.6 g/day contrast sharply with the exceptionally poor growth rates of 2.4 g/day of the control group fed unchlorinated wheat flour by Cunningham *et al.* (1977), suggesting either a gross deficiency in the basal diet fed to their animals, or their use of abnormal rats. Although their weanling rats were smaller than ours, the higher growth rates of controls in other experiments in that paper, 4.6 g/day (for rats fed wheat gluten in their diet) and 5.6 g/day (for pair-fed rats fed wheat lipids in their diet), suggest that the rats in the first trial were in very poor condition, and the results must therefore be open to some doubt.

The organ weight data obtained in the present work accord with our earlier work (Fisher *et al.* 1979a), in which feeding of rats with diets containing 93% cake made from heavily chlorinated flour (10,700 ppm) caused significant ($P < 0.001$) elevations of liver and kidney weights relative to body weight, compared with controls. The absolute weights of these organs in treated animals were also significantly different from those of controls in the earlier trial but not in the present work. These findings are in agreement with those of Cunningham *et al.* (1977). However, no pathological changes were found in the livers and kidneys of the rats either in our earlier trial (Fisher *et al.* 1979a) or in the present work, contrary to the findings of the Canadian workers. The animals in our earlier study were fed with a highly unpalatable diet (food intakes were consistently lower than for controls in a series of experiments) for 19 months, and it is conceivable that the organ weight changes represented a physiological adaptation to the nature of the diet, e.g. an increase in liver metabolism in order to dechlorinate the chlorinated fatty acids ingested. The absence of pathological changes attributable to the chlorination of flour in the present trial confirms our earlier findings and strengthens this conclusion. Furthermore, in earlier work (Fisher *et al.* 1979a) evidence was obtained that the effects of chlorination were not simply related to treatment level, since growth was inhibited by cake made from highly-chlorinated flour that had been diluted with unchlorinated flour, but was not inhibited by cake made from flour that had been chlorinated directly to the same level.

Certain features of the evidence of Cunningham *et al.* (1977) and Cunningham & Lawrence (1978) detract from the acceptability of concluding on the basis of their results that chlorinated flour is toxic to rats:

(i) Food intakes are not reported, and thus proper assessment of the body-weight gain data is not possible.

(ii) Flour, rather than cake, was fed in all but one of the published experiments. It has become widely accepted in recent years that toxicity testing of

food additives should be carried out using a food product in the form that is consumed by the public, so that changes in the test compound or its products induced during food processing are taken into account. This attitude has been adopted officially in the UK, where tests on flour are no longer regarded as acceptable (Committee on Medical Aspects of Food Policy, Pharmacology Sub-Committee, 1974) for the evaluation of additives used in making baked products such as bread or cake.

(iii) A low-protein flour was treated with exceptionally high levels of chlorine in the studies of Cunningham and his colleagues. In commercial practice the highest level used, 2500 ppm, is usually reserved for the high-protein flours used in making fruit cake.

(iv) Levels of 2000 or 10,000 ppm chlorine were used in flour and flour lipids, and even 50,000 ppm chlorine in gluten; this approach is highly questionable. It can neither be assumed that results obtained with high levels of chlorine can be meaningfully extrapolated to low levels, nor that chlorination of a subfraction of flour or dough produces similar products to those found when whole flour is chlorinated. The fallacy is seen clearly in the case of gluten, since flour chlorinated to 50,000 ppm would not form a gluten (Ewart, 1968). Indeed, whole flour would not be capable of taking up anything like this amount of chlorine, the maximum uptake being of the order of 11,000 ppm. Where a vast excess of a highly reactive element like chlorine is available, the likelihood of secondary reactions is obviously greatly increased. Cunningham *et al.* (1977) and Cunningham & Lawrence (1978) do not seem to have taken into account the fact that about half the chlorine used in flour treatment is recoverable as inorganic chloride (Sollars, 1961), and overlook the implications of differences in the partition of the chlorine between the various components of flour, which must vary with chlorination level.

Long term studies have been carried out since that trial was completed (Fisher *et al.* 1979a; Fisher, Hutchinson, Berry *et al.* 1983a,b). These do not substantiate the claim that chlorination of flour for use in cake-making is hazardous to the health of the consumer, and indeed the Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment (UK) has recommended that chlorine be included in Class A, those additives that the available evidence suggests are acceptable for use in food.

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LONG-TERM TOXICITY AND CARCINOGENICITY STUDIES OF CAKE MADE FROM CHLORINATED FLOUR

1. STUDIES IN RATS*

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Abstract—Wistar rats were fed for 104 wk on cake-based diets in which the cake, prepared from unchlorinated flour, or flour treated with 1250 or 2500 ppm chlorine, formed 79% of the diet on a 12.6% moisture basis. A fourth group was fed stock diet 41B. No differences in appearance, health, behaviour or mortalities attributable to the flour treatment were observed. Female but not male mortalities were significantly higher for cake-fed rats than for those fed diet 41B. Dose-related haematological effects were seen at various stages in cake-fed rats. Dose-related increases in plasma alanine and aspartate aminotransferases were noted at 12 months in males but not in females, for whom all the values were elevated. A dose-related diminution in blood sugar at 12 months was seen only in females. A dose-related increase in urinary aspartate aminotransferase was seen only in males. Urinary *N*-acetylglucosaminidase activity per mg creatinine did not differ significantly between groups. At *post mortem* a dose-related reduction in spleen weight was found in the females only. The lesions found were those expected in ageing rats, but were observed earlier in rats fed cake. Glomerulonephrosis affected rats fed cake more than those fed diet 41B. Cake diets promoted nephrocalcinosis, unrelated to flour treatment. Increased splenic haematopoiesis occurred in about half of the females in the cake diet groups but less frequently in males or in rats fed diet 41B. Tumours were mainly chromophobe adenomas of the pituitary, common in rats. Insulomas were seen in two males in each of the groups fed on cake made from chlorinated flour, but an earlier form of this tumour was found in all cake groups and its incidence is thus regarded as unrelated to the flour treatment. The incidence of tumours of the reticuloendothelial system was not related to flour treatment. Covalent chlorine concentrations in the perirenal fat of the cake-fed rats were correlated with treatment levels, with values of 50–912 ppm in males and 59–1174 ppm in females. Since concentrations in the lipid of the diet fed to the animals were much higher than these, accumulation of the additive was absent or negligible. The chlorine concentrations in the perirenal fat of male and female rats fed diet 41B were 62 and 72 ppm respectively.

INTRODUCTION

Certain types of cakes are made from recipes containing a high ratio of sugar and liquor to flour (Gough, Whitehouse & Greenwood, 1978). The flour used in making these cakes needs treatment, in order to prevent collapse on removal of the cake from the oven, to impart desirable eating characteristics to the cake, and, in the case of fruit cake, to prevent sinking of the fruit.

Approximately 0.7% of the total flour production in the UK is chlorinated, the average level of chlorination being about 1250 ppm. As a general rule, the higher the protein content of the parent flour, the higher the level of chlorination used in its treatment. Thus fruit cake is generally made from flours of 11–12% protein content, and may require the use of up to 2500 ppm of chlorine to ensure the correct fruit-holding capacity and to obtain the desired eating qualities.

The chemistry, technology and toxicology of flour chlorination have been reviewed (Fisher, Hutchinson, Berry *et al.* 1979a; Gough *et al.* 1978). The report of the Food Standards Committee (Ministry of Agricul-

*Full experimental details and an extended discussion of the results (Fisher, Hutchinson, Berry, Hardy & Ginocchio, 1979a) are available on loan from FMBRA.

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ture, Fisheries and Food, 1974) noted that adequate short-term toxicological studies were available (WHO, 1967) but called for long-term studies in two species using cake baked from chlorinated flour. Such studies are described in this and the companion paper (Ginocchio, Fisher, Hutchinson *et al.* 1983).

EXPERIMENTAL

Diets. Flour (unbleached, containing statutory vitamins and minerals including chalk) was milled by Spillers Ltd at 3-monthly intervals and aliquots were chlorinated at their Research & Technology Centre in Cambridge. Chlorine of 99.99% purity was supplied by ICI Ltd, the only impurity being water. Flour not used immediately was stored at *c.* 4°C. Stringent quality control was maintained over the production, composition and treatment of the flours. Composition data obtained during the course of the trial were (% w/w flour, mean ± 1 SD); protein 11.5 ± 0.26; moisture 13.3 ± 0.62; colour grade figure 2.9 ± 0.39; chlorine (ppm) control 581 ± 40; additional chlorine in treated flour (ppm) '1250' 1257 ± 74; '2500' 2506 ± 81. Cake batters were mixed in a planetary mixer using the 'all in' method, according to the following recipe (parts by weight): flour 100, egg 70, sugar 100, water 43, skim milk powder 8, oil (corn; groundnut, 3:1) 2, salt 1, sodium bicarbonate/sodium dihydrogen pyrophosphate 5, glyceryl monostearate 2.6. Batters were deposited in trays and baked for 25 min at 177°C.

Cake was crumbed and dried at 49°C to a moisture content averaging 12.6%, then incorporated into the following diet (% w/w): cake 79.1, gluten (dried, commercial, baker's grade) 12.6, minerals (Jones & Foster, 1942) 4.0; wheat bran (autoclaved) 3.0, vitamin mixture (Fisher, Hutchinson, Berry *et al.* 1979b) 1.0, L-lysine hydrochloride 0.3. The cake diets provided protein 17.2%, fat 5.5%, carbohydrate 58.7% and digestible energy 14.8 MJ/kg. The three cake diets differed only in respect of the treatment or non-treatment of the flour used in their manufacture with chlorine, or in the concentration of chlorine used. Analysis of diet 41B gave (% as received): protein 16.0, moisture 10.7, fat 4.3, carbohydrate 64.3 (dietary fibre approx. 20), ash 4.7, digestible energy 12.6 MJ/kg.

Animals. Weanling rats (specific pathogen free, MRC category 3) of the Wistar-derived Porton strain were supplied by Olac Southern Ltd, Bicester, Oxon. (now Olac 1976 Ltd). Each main diet group (test and control), comprised 60 males and 60 females and an additional 39 males and 39 females per group were maintained for biochemical and haematological investigations (a total of 792 rats in four treatment groups). Animals free from obvious disease and comparable in weight were acclimatized for 3 days and randomly assigned to their diet groups. They were caged in groups of three in polypropylene cages (50 cm × 32 cm × 19 cm) with stainless-steel mesh tops and floors. Cages were arranged in the racks so that each treatment group was represented as evenly as possible at each level. Room temperature was maintained at 22 ± 2°C and relative humidity at 40–50%, with positive air pressure and twelve air changes/hr.

Procedure. Diets and water were available *ad lib.* Diet spillage was recovered and food intakes were corrected accordingly. All animals were inspected daily for abnormal appearance or behaviour and signs of ill-health. Sick animals were isolated, to be returned to their cages on recovery or killed if their condition deteriorated. A gross autopsy was carried out on all animals unless precluded by cannibalism or advanced autolysis. Body weights of individual animals and food intakes of cage groups were recorded weekly for the first 13 wk and monthly thereafter. Water intakes of cage groups were determined monthly.

Animals surviving at 104 wk were killed by ether overdosage, any macroscopic abnormalities were noted, and brain, heart, liver, kidneys, spleen, adrenals, gonads, uterus, thyroid and pituitary were removed and weighed. Samples of these organs, and of trachea, lungs, prostate, seminal vesicles, pancreas, salivary gland, eyes, skin, skeletal muscle, cervical lymph node, thoracic aorta, oesophagus, stomach, duodenum, jejunum, ileum, colon, urinary bladder, tongue, optic nerve, sciatic nerve and spinal cord were removed and preserved in 10% buffered formalin. Stained smears of bone marrow were prepared.

Haematology, blood chemistry and urine analyses. Blood for haematological and biochemical examination was obtained by cardiac puncture under light ether anaesthesia at 1, 3, 12, 18 and 24 months. Haematological determinations (at 3, 12 and 24 months; ten males and ten females from each diet group) comprised haemoglobin, packed cell volume, erythrocyte and leucocyte counts (Coulter Electronic counter and haemoglobinometer) and differential leucocyte counts.

Blood urea (Fawcett & Scott, 1960), glucose (glucose oxidase method of Werner, Rey & Wielinger, 1970) aspartate aminotransferase and alanine aminotransferase (Bergmeyer & Bernt, 1970), alkaline phosphatase (Bessey, Lowry & Brock, 1946) and total protein (Weichselbaum, 1946) were determined in the blood of five rats of each sex/group at 1, 12, 18 and 24 months.

Urine analyses were carried out at 12 and 18 months, using ten animals of each sex/group. Renal concentration and dilution tests were carried out at 6, 12, 18 and 24 months with five rats of each sex/group.

Determination of covalently bound chlorine in adipose tissue. Fat extracted from the dried, ground adipose tissue with water-saturated *n*-butanol was purified, dried and burnt in oxygen (Dobbs, 1966). The chloride-containing combustion products were dissolved in water, and the solution, or a suitable aliquot, titrated with phenyl mercury (II) nitrate at pH 1.5. The resulting phenyl mercury chloride was extracted into chloroform, in which its concentration and hence the concentration of chlorine in the fat was determined by gas–liquid chromatography, using solutions of known chloride content, similarly treated, as standards (Belcher, Majer, Rodriguez-Vaquez *et al.* 1971).

Histopathology. Sections (5 μm) of the organs and tissues listed above were stained with haematoxylin and eosin for histopathological examination.

Table 1. Cumulative mortality of rats fed cake diets or diet 41B

Wk no.	Diet group†	Cumulative mortality (%) 0	1250	2500	41B
		Males			
19		—	—	—	2
24		—	—	2	3
30		2	—	2	5
36		2	—	2	8
48		2	—	3	17
64		5	2	5	17
80		17	12	12	18
92		30	25	27	30
96		45	42	45	32
104		63	51	53	40
		Females			
23		—	—	—	2
27		—	—	3	2
28		3	3	3	5
35		3	5	3	5
52		12	15	10	5
56		13	23	13	5
68		38	40	43	10
85		78	83	77	43
92		87	93	88	62
104		98	98	97	85

†Diets designated '0', '1250' and '2500' contained cake made from untreated flour, flour treated with 1257 ppm chlorine and flour treated with 2506 ppm chlorine, respectively.

Statistical analysis. Analysis of variance and covariance (Texas Instruments 980B computer) and paired t tests were carried out. Rejection of data was controlled by statistical tests of the validity of this procedure. Dose-response relationships in cake-fed groups were determined by linear regression analysis.

RESULTS

Appearance and behaviour

No differences attributable to the flour treatment were observed during the trial.

Mortality, weights and food intakes

Cumulative mortality data are given in Table 1. Death of one animal represents a mortality of 1.67%. No differences in mortality due to flour treatment were observed, but females in cake-fed groups experienced significantly higher mortalities than those fed diet 41B.

Mean body weights and food intakes at various stages of the trial are shown in Tables 2 and 3.

Normalized food intakes were within the range 37.6–38.2 g/kg body weight/day (SD = 19.4) for cake-fed males, and 50.9–52.3 (SD 16.6) for the corresponding females and were not dose-related. Normalized intakes of 41B-fed rats (mean ± 1SD: 46.6 ± 16.2 for males, 66.2 ± 12.0 for females) were higher than those of the controls by 24% for males and 27% for females ($P < 0.01$ for both sexes). The intakes of chlorine (in the form of its reaction products) were calculated from the normalized food intakes to be 12.8 and 25.3 mg/kg body weight/day for males given the cake treated with low and high levels of chlorine, respectively, the corresponding values for females being 17.0 and 35.0 mg/kg body weight/day.

Water intakes were determined for 10–15 animals/diet group at 21 stages of the trial. Neither sex showed significant diet-related differences in water intake, against a background of high scatter. Females drank significantly more than males ($P < 0.01$).

Haematology

Haematological data for male rats at 3, 18 and 24 months are given in Table 4 (there were no dose-related differences at 12 months). Dose-related effects in the males were seen at 3 months (increase in lymphocytes and platelets, $P < 0.05$; lowering of eosinophils, $P < 0.01$); at 18 months (increased erythrocyte (RBC) count, packed cell volumes (PCV) and haemoglobin (Hb) levels, all $P < 0.05$); at 24 months (slight lowering of PCV ($P < 0.05$) but not RBC or Hb levels); other statistically significant differences were sporadic. Dose-related differences in the females were observed at 12 months (lowered reticulocyte counts, $P < 0.05$) and at 18 months (decrease in mean cell volume $P < 0.01$); few other significant differences were observed.

Blood chemistry

Dose-related increases in aspartate ($P < 0.05$) and alanine ($P < 0.01$) aminotransferase were seen at 12

Table 2. Body weights of rats fed cake diets or diet 41B

Diet group†	Mean body weight (g) at wk no. 0	2	12	36	60	92	104	Overall mean body weight/day of trial (± 1SD)
					Males			
0	57	121	408	521	578	556	530	508 ± 106
1250	55	116	418	539	589	588	545	523** ± 111
2500	58	123	419	531	590	571	547	521** ± 109
41B	59	139	386	481	530	541	506	474** ± 92
					Females			
0	54	108	251	302	320	323	233	290 ± 51
1250	54	109	256	303	337	325	348	296** ± 52
2500	52	107	245	292	322	289	279	282** ± 49
41B	53	117	224	277	309	303	302	273** ± 49

†See footnote to Table 1 for definitions.
Values marked with asterisks differ significantly (Student's t test) from those for the corresponding cake-fed control group (**$P < 0.01$).

Table 3. Food intakes of rats fed cake diet or diet 41B

Diet group†	Mean food consumption (g/day) at wk no. 1	8	52	96	Mean food intake (g/day)
Males (1SD = 1.8)					
0	8.7	21.5	17.2	15.1	17.2
1250	8.5	22.3	17.4	14.9	17.9*
2500	9.5	21.8	17.9	16.4	17.7*
41B	10.0	22.4	21.0	21.2	20.7**‡
Females (1SD = 1.3–1.9)					
0	8.8	16.0	15.0	12.2	14.3
1250	8.3	16.1	14.9	14.4	14.2
2500	7.9	15.3	13.7	14.7	14.0
41B	9.2	15.2	18.9	18.0	17.6**

†See footnote to Table 1 for definitions.
‡1SD = 1.5 not 1.8 for this value.
Values marked with asterisks differ significantly (Student's t test) from those for the corresponding cake-fed control group (*$P < 0.05$; **$P < 0.01$).

months in the males but not in the females, for whom all the values were elevated, however. The females showed a dose-related diminution in blood sugar at this stage ($P < 0.05$), not shown by the males. At 24 months, males showed a dose-related decrease in plasma protein concentrations ($P < 0.05$), not shown by the females, nor by the males at any other stage of the trial: but even at 24 months, the lowest mean protein value in the males fed on cake was not significantly different from that of males fed on diet 41B. Other significant differences were sporadic and unrelated to flour treatment levels.

Renal function tests and urine analyses

Apart from a dose-related decrease at 12 months in urine volume of male but not female rats deprived of water, no other dose-related effects were observed in the concentration/dilution tests. With normal access to water, no treatment-related differences in urinary pH, protein, glucose, blood, bilirubin, urobilinogen or ketones were observed. At 12 months, there was a dose-related increase in urinary aspartate aminotransferase in the males, but not in the females. No statistically significant differences were seen in the N-acetylglucosaminidase activity per mg creatinine in the urine of the rats in different diet groups, and the values obtained were (surprisingly) not indicative of kidney damage. Urinary cellular material and deposits showed no significant differences.

Post-mortem *findings*

Organ weight data are shown in Tables 5 and 6. A dose-related reduction in spleen weight in the females was observed (Table 6) which did not occur in the males. No other dose-related effects were found.

Histopathology

The histopathological findings are shown in Table 7. Glomerulonephrosis of varying severity was a frequent finding in cake-fed animals, as well as in a high proportion of 41B-fed rats, the latter however being less severely affected. Irrespective of flour treatment, all of the cake diets promoted the deposition of calcareous material in the kidney, especially prevalent in the females. Increased haematopoiesis was noted in the spleens of about half the females in cake diet groups but less frequently in 41B-fed animals.

Tumours were mainly chromophobe adenomas of the pituitary common in rats. More tumours of other types were found in males than in females (Table 8). Insulomas were seen in two males in each of the groups fed on cake made from chlorinated flour, but an earlier form of this tumour was found in all cake groups and its incidence is thus regarded as unrelated to the flour treatment. No evidence was found of treatment-related production of tumours, including tumours of the reticuloendothelial system (on the basis of pooled data for insulomas and giant islets).

Covalently bound chlorine in flours, diets, and rat perirenal fat

Chlorine contents of the lipids of the flours and

Table 4. Mean haematological data for male rats fed on cake diets or diet 41B

Month	Diet group†	Hb (g/100 ml)	RBC (10^6/mm^3)	PCV (%)	Platelets (10^3/mm^3)	Leucocytes: Total (10^3/mm^3)	Differential (%): N	L	M	E
3	0	15.0	7.5	41.5	*|4105	5.9	19 *|79	0	**|2.1
	1250	15.1	7.6	40.6	|4280	5.6	17 |82	0	|0.9*
	2500	15.1	7.5	40.5	|5605*	5.5	13 |87	0	|0.7*
	41B	15.5	7.3	39.4	3360	5.4	17	82	0	0.9*
18	0	*|14.0	*|6.8	*|38.8	—	5.9	17	80	1	2.2
	1250	|15.4*	|7.4*	|42.3	—	7.3	20	78	0	0.8
	2500	|15.7	|7.6	|43.4*	—	6.4	17	78	2	3.4
	41B	13.9	7.5	39.0	—	6.3	18	81	0	0-6
24	0	15.1	7.4	43.6*	—	6.8	22	74	2	2.7
	1250	15.1	7.4	42.3*	—	5.5	17	78	1	2.2
	2500	14.1	6.9	39.5**	—	6.3	17	80	0	2.8
	41B	14.6	7.2	41.9	—	6.0	27	72	2	1.9

Hb = Haemoglobin RBC = Red blood cell count PCV = Packed cell volume
N = Neutrophils L = Lymphocytes M = Monocytes E = Eosinophils

†See footnote to Table 1 for definitions.
Values marked with asterisks differ significantly (ANOVA and Student's t test) from those for the corresponding cake-fed control group (*$P < 0.05$). Vertical lines joining values indicate a statistically significant dose relationship (*$P < 0.05$; **$P < 0.01$).

Table 5. Absolute organ weights (g) of rats fed cake diets or diet 41B

Diet group†	Brain	Heart	Liver	Kidneys	Spleen	Adrenals	Gonads	Pituitary	Thyroid	Uterus	Terminal body weight
Males											
0	2.17	1.63	17.0	4.20	1.37	0.064	3.30	0.015	0.035	—	534
1250	2.16	1.70	17.4	4.25	1.31	0.065	3.18	0.023	0.040	—	535
2500	2.27	1.63	17.5	4.14	1.28	0.062	3.34	0.016	0.046	—	544
41B	2.21	1.63	16.7	4.08	1.12**	0.056	3.35	0.013	0.031	—	502
Females											
0	2.12	1.42	12.2	3.38	1.00	0.074	0.082	0.047	0.022	0.760	249
1250	2.23	1.23	13.9	2.86	0.96	0.048	0.034	0.117	0.016	0.685	315
2500	2.13	1.32	13.4	3.30	1.01	0.068	0.050	0.079	0.024	0.916	285
41B	2.13	1.10	10.3	2.09	0.77	0.044	0.066	0.040	0.019	0.830	294

†See footnote to Table 1 for definitions.

Values are means for all males including those which died before the end of the study and groups of five females determined at 72 wk. Thyroid weight was correlated with brain weight, and when this effect was taken into account the value for the low-dose group was significantly lower ($P < 0.05$) than that of the control but the value for the high-dose group did not differ significantly from that of the control.

diets fed to the rats, and in the perirenal fat of the rats, are presented in Table 9. The raw data for the chlorine contents of the perirenal fat showed a log normal distribution, the variance increasing with treatment level. The analysis of variance was therefore carried out on the logarithms of this data, and the means given are geometric means.

DISCUSSION

Wheat as harvested contains chlorine in both inorganic and organic, covalently bound forms. Thus all grain-based commercial 'control' diets contain chlorine. The only valid control diet in trials of chlorine treatment of flour is that based on cake made from the untreated flour.

Data from the National Food Survey show that the cake (about 20% moisture content) formed 1.7% of the national diet in the UK in 1976. Chlorinated flour formed one third of total cake flour, so that cake made from chlorinated flour formed about 0.57% of the national diet. On this basis, the rats in the present trial received about 150 times the average dietary concentration of cake consumed by the population of the UK, and animals in the high-dose groups received diets containing cake made from flour treated with about twice the average concentration of chlorine used in the UK cakemaking industry, increasing by a factor of two the relative exposure of animals compared with that of man to the products formed by the additive.

The relative exposure may also be calculated in terms of the additional chlorine intake, in mg/kg body weight/day, resulting from eating cake made from chlorinated flour. For a man of 65 kg, a woman of 55 kg and a child of 30 kg mean values of 0.038, 0.045 and 0.082 respectively are obtained. The corresponding values for rats of the high-dose group were 25.3 and 35.3 for males and females respectively. The relative exposures thus become 666 (males), 784 (females) and 370 (children). The intake values for rats and humans are not entirely comparable since the former are derived from "weighted mean" body weight and food intake data which are not available for humans, but the interpretation should not be seriously affected.

No significant treatment-related differences in mortality were observed in this trial. Comparing rats fed cake diets with those fed diet 41B, mortalities were similiar in the males but there was an unexplained

Table 6. Organ weights, adjusted for covariance with body weight, of rats fed cake diets or diet 41B

Diet group†	Adjusted organ weight (g)									
	Brain	Heart	Liver	Kidneys	Spleen	Adrenals	Gonads	Pituitary	Thyroid	Uterus
Males										
0	2.25	1.81	16.5	4.87	1.28	0.072	2.83	0.024	0.036	—
1250	2.24	1.79	16.4	4.88	1.26	0.070	2.83	0.028	0.040	—
2500	2.27	1.79	16.4	4.76	1.15	0.072	2.75	0.026	0.037	—
41B	2.20	1.53	15.3	3.66**	1.13	0.059	3.26	0.015	0.030	—
Females										
0	2.09	1.59	12.0	3.49 **	1.31	0.075	0.050	0.055	0.022	0.632
1250	2.09	1.61	12.6	3.77	1.14	0.070	0.046	0.111	0.017	0.579
2500	2.09	1.52	12.6	3.53	1.02**	0.070	0.048	0.079	0.024	0.615
41B	2.04	1.28**	11.4	2.76**	0.94	0.071	0.050	0.039	0.019	0.680

†See footnote to Table 1 for definitions.

Animals that died before the end of the study are included. Values marked with asterisks differ significantly (ANOVA and Student's t test) from the corresponding value for the cake-fed control group (**$P < 0.01$). The vertical line joining values indicates a statistically significant dose relationship (**$P < 0.01$).

Table 7. Incidence of histopathological findings in rats fed cake diets or diet 41B

	Males				Females			
Diet group* ...	0	1250	2500	41B	0	1250	2500	41B
No. of animals examined ...	70	68	69	63	68	67	69	65
Affected organ/type of lesion								
Kidneys								
Glomerulonephrosis	63	66	61	55	77	76	65	64
Calculi or calcium deposits	5	11	10	0	60	57	54	6
Other lesions	20	16	16	23	17	13	9	6
Liver								
Cell vacuolation	20	25	26	7	15	12	11	14
Foci or areas of necrosis	3	0	1	3	6	3	1	1
Leucocytic foci & leucocytic infiltration	2	3	2	1	2	3	2	4
Other lesions	4	8	9	17	7	10	7	16
Heart								
Interstitial fibrosis	31	32	16	20	21	24	19	8
Other lesions	4	6	9	3	3	6	5	4
Lungs								
Pneumonitis or pneumonia	38	40	37	42	29	38	45	44
Other lesions	47	49	47	28	31	49	50	26
Spleen								
Increased haematopoiesis	11	9	5	5	35	41	44	19
Thyroid								
Various minor lesions	20	21	18	29	8	6	4	13
Pancreas								
Pancreatitis	1	0	1	3	1	1	2	1
Periarteritis	8	9	5	2	8	11	18	7
Giant islets	4	1	1	0	1	1	3	0
Pituitary								
Cyst	5	5	3	1	0	1	1	1
Hyperplasia	3	5	1	3	1	3	1	1
Testes								
Periarteritis	12	12	9	5				
Atrophy	14	7	11	9				
Ovaries								
Cystic					8	7	5	8
Atrophy or partial					18	27	27	19
Uterus								
Cystic dilatation					10	10	6	11
Adrenals								
Various ageing pathology	23	18	20	26	35	38	53	44
Stomach								
Various minor lesions	17	21	18	10	4	12	18	11
Lymph nodes								
Hyperplasia	2	2	0	3	10	7	10	2
Tumours								
Benign	31	31	30	13	48	41	44	45
Malignant	5	2	6	6	1	2	1	9
Total	36	33	36	19	49	43	47	54

*See footnote to Table 1 for definitions.

excess of mortality in the cake-fed females ($P < 0.001$). Many of the early deaths could have been due to the earlier onset in cake-fed females of conditions (pituitary adenomas and glomerulonephrosis) which would normally have been expected to appear at a later stage of the trial.

All of the cake diets promoted the deposition of calcareous material in the kidneys although the calculated calcium contents of the 41B and cake diets (including the contributions from the creta added to the flours) were comparable, and the nephrocalcinosis may have been associated with the lower magnesium content of the latter (Goulding & Malthus, 1969).

We did not find any evidence of treatment-related tumour production. This conclusion assumes the validity of pooling the data for insulomas and giant islets. In addition the evidence concerning any possible carcinogenicity which might result from the chlorination of flour is limited by the high mortalities of the female rats in this trial.

Treatment-related concentrations of chlorine have been found in the adipose tissue of the rats (and mice: see following paper, Ginocchio *et al.* 1983). The concentrations of chlorine in the lipid of the diets fed to the animals varied from about 5000 ppm to 7000 ppm. Since the concentrations of chlorine found in the perirenal fat of the animals were substantially lower than this, it has been demonstrated that, even allowing for the dilution of the depot fat by fat synthesized from carbohydrate, there was little or no accumulation of lipid-bound chlorine in conditions of

Table 8. Classification of tumours found in rats fed cake diets or diet 41B

Affected organ/tumour type	Diet group†	No. of rats in which tumour was found*							
		Males				Females			
		0	1250	2500	41B	0	1250	2500	41B
Pituitary									
Adenoma		20	17	19	9	45	39	40	43
Thyroid									
Cystadenoma		1	1	0	0	0	0	2	0
Solid adenoma		1	5	1	0	1	0	0	0
Adrenal									
Medullary tumour		3	2	2	0	0	0	1	0
Skin									
Squamous cell papilloma		1	0	0	1	0	0	0	0
Squamous cell carcinoma‡		1	0	0	0	0	0	0	0
Thymoma		0	0	0	0	0	0	0	1
Thymus									
Lymphosarcoma‡		0	1	1	1	0	0	1	0
Generalized lymphosarcoma‡		2	1	1	0	0	0	0	4
Lymph node reticulum cell neoplasm‡		0	0	3	3	0	0	0	0
Generalized reticulum cell neoplasm‡		1	0	0	0	0	2	0	2+1
Testes									
Interstitial cell tumour		1	0	0	2	—	—	—	—
Lung									
Adenoma		0	0	1	0	0	0	0	0
Kidney									
Lipoma		1	2	0	0	1	0	0	0
Liver									
Hepatoma		0	0	1	0	0	0	0	0
Brain									
Glioma		0	0	1	1	0	0	0	0
Subcutaneous fibroma		2	0	0	0	0	0	1	0
Subcutaneous angioma		0	0	1	0	0	0	0	0
Subcutaneous lipoma		1	1	1	0	0	0	0	0
Fibroliposarcoma‡		1	0	0	0	0	0	0	0
Myoma		0	1	0	0	0	0	0	0
Rhabdomyoma		0	0	0	0	0	1	0	0
Rhabdomyosarcoma‡		0	0	1	1	0	0	0	0
Stomach									
Squamous cell carcinoma‡		0	0	0	0	0	0	0	1
Mammary gland									
Fibroma		0	0	0	0	1	0	0	0
Adenocarcinoma‡		0	0	0	0	0	0	2	1
Cervix									
Papilloma		—	—	—	—	0	1	0	0
Uterus									
Adenocarcinoma‡		—	—	—	—	1	0	0	0
Fibroma					—	0	0	0	1
Pancreas									
Insuloma		0	2	2	0	0	0	0	0
Adenoma		0	0	1	0	0	0	0	0
Adenocarcinoma‡		0	0	0	1	0	0	0	0

*Nos of rats examined are given in Table 7.
†See footnote to Table 1 for definitions.
‡Malignant tumour types.
§Uterus principally involved or ovary.

continuous feeding of the additive over the entire lifespan of the animals. In the more normal conditions of consumption of this very minor dietary component (high-ratio cake) by human subjects, there would be time, between successive intakes, for metabolism and excretion of the additive, and much lower levels in adipose tissue would consequently be expected. The findings should also be considered in relation to the relative levels of exposure of the test animals and the human population, discussed earlier.

A number of papers have reported toxic effects of feeding chlorinated flour, chlorinated flour lipids, or chlorinated gluten to rats for 2–10 wk (Cunningham & Lawrence, 1978; Cunningham, Lawrence & Tryphonas, 1977). A 4-wk trial (Fisher, Berry & Hardy, 1983) on the lines of those of Cunningham *et al.* failed to confirm the histological changes in the liver reported by these authors, although the weights of the livers and kidneys increased, as they reported. The present work offers no support for the view that

Table 9. Covalently bound chlorine in flours, diets and rat perirenal fat

Sample	% fat extracted from sample	Chlorine concentration in fat (ppm)	Chlorine concentration in perirenal fat (ppm)	
			Males	Females
Flour				
0	1.32	1450	—	—
1250	1.27	30,800	—	—
2500	1.60	56,490	—	—
Diet				
0†	3.91	1260	*** 50	*** 59
1250†	4.13	5010	468**	871**
2500†	4.29	6820	912**	1174**
41B	2.22	3400	62	72

†See footnote to Table 1 for definitions.
Values marked with asterisks differ significantly (ANOVA) from the corresponding control value (**$P < 0.01$). Vertical lines joining values indicate a statistically significant dose relationship (***$P < 0.001$).

chlorination of flour to levels used industrially in the manufacture of cake is a potential hazard to health.

Indeed on the basis of the evidence submitted (Fisher *et al.* 1979a) the Committee on Toxicity of Chemicals in Foods, Consumer Products and the Environment has recently recommended that chlorine should be reclassified in Class A, additives which the available evidence suggests are acceptable for use in food.

In the United States, chlorine has recently been cleared for continued use as a flour treatment agent by the Food & Drug Administration on the basis of lifetime feeding studies in rats and mice and shorter term studies in dogs (Gumbmann & Gould, 1979).

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LONG-TERM TOXICITY AND CARCINOGENICITY STUDIES OF CAKE MADE FROM CHLORINATED FLOUR

2. STUDIES IN MICE

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Abstract—Male and female Theiller's Original strain mice were fed for 16 and 17 months respectively on diets in which cake, prepared from flours treated with 0, 1250 or 2500 ppm chlorine, formed 79% by weight on a 12.6% moisture basis. Body weights and food intakes were unaffected by flour treatment but all of the animals on cake diets showed significant increases in body weight compared with controls on a standard diet and became obese. Mortalities in the males were not related to treatment, but in the females there was excess mortality in the treated groups compared with the cake control group, after 13 months in the 1250 group and after 15 months in the 2500 group. No consistent treatment-related effects were observed in the haematological, biochemical and renal-function studies. Dose-related increases in heart and kidney weights and a dose-related decrease in ovary weight were seen in females. No evidence of carcinogenicity resulting from flour treatment was obtained but the early ending of the study, necessitated by high mortalities, greatly diminished the value of this finding. Concentrations of covalently bound chlorine in the perirenal fat were positively correlated with treatment level, but were considerably below those present in the lipid content of the diets on which the mice were fed.

INTRODUCTION

Studies in rats of the cake-flour treatment agent chlorine have previously been described (Fisher, Hutchinson, Berry *et al.* 1983). The results of a study of similar design using mice are described in the present paper.

EXPERIMENTAL

Mice of Theiller's Original strain were obtained from A. Tuck & Son, Rayleigh, Essex and housed in groups of five in an animal room kept at 21 ± 2°C and 40–50% relative humidity at Consultox Laboratories, under the supervision of their staff. Experimental protocols, including diet formulation and supply, and overall project control were the responsibility of FMBRA. The cake batter used in the present trial (which started earlier than the rat trial described in the preceding paper) contained eight parts by weight of a 1:1 mixture of corn and groundnut oils, instead of two parts of a 3:1 mixture, but was otherwise identical with that used in the rat trial. The mouse cake diet provided protein, 17.0%, fat, 7.2%, carbohydrate, 57.3%, digestible energy, 15.2 MJ/kg. Other materials and methods were as described in the preceding paper except that the 41B group, included mainly for haematological and biochemical comparisons, consisted of 30 males and 30 females and the duration of the trial was 70–73 wk.

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RESULTS

Appearance and behaviour

No differences attributable to the flour treatment were observed during the trial.

Mortality, body weights and food intakes

Cumulative mortality data are presented in Table 1. The death of one animal represents a mortality of 1.67% in the cake fed groups, and 3.33% in the 41B group. There were no treatment-related differences in mortality among the male mice, but there was increased mortality ($P < 0.001$) in female mice in the treated groups compared with cake-fed controls starting after 13 months in the 1250 group and 15 months in the 2500 group. In the later stages of the trial there was higher mortality in all of the cake-fed

Table 1. Cumulative mortality of mice fed cake diets or diet 41B

Week no.	Diet group*...	Cumulative mortality (%) 0	1250	2500	41B
	Males				
13		0	0	0	17
19		0	0	2	17
49		0	2	2	17
52		2	2	7	17
53		8	5	7	23
60		45	40	23	23
65		62	50	50	30
70		82	70	75	43
	Females				
13		0	0	0	13
51		0	2	0	13
57		0	12	3	13
59		2	20	7	13
65		20	35	27	13
70		43	70	65	30
73		60	78	82	33

*Diets designated '0', '1250' and '2500' contained cake made from untreated flour, flour treated with 1257 ppm chlorine and flour treated with 2506 ppm chlorine respectively.

groups of both sexes than in the group fed diet 41B. Because of the high mortalities, treatment was ended sooner than planned, after 70 wk (males) or 73 wk (females).

Mean body weights at various stages of the trial are shown in Table 2. Body weights of animals fed diet 41B were consistently lower than those of cake-fed mice, which became obese, but among whom differences in weight were not treatment-related.

Food intakes of the mice are shown in Table 3. Normalized food intakes (g/kg body wt/day) ranged from 107 to 114 for cake-fed males, and from 140 to 147 for cake-fed females; values for 41B-fed mice were 197 and 210 for males and females respectively.

Haematology

Haematological findings for male mice are summarized in Table 4. Dose-related decreases in red blood cell count were seen among cake-fed mice after 3 and 12 months but not after 16 months. There was also a dose-related increase in total leucocyte count after 3 months but not after 12 or 16 months. Results for females showed differences between groups fed cake and those fed diet 41B but not among the cake-fed groups.

Table 2. Mean body weights of mice fed cake diets or diet 41B

Diet group*	Mean body weight (g) at week no. 0	13	52	65
	Males			
0	18	46	53	45
1250	18	46	54	50
2500	18	45	52	44
41B	18	35	36	34
	Females			
0	17	36	42	38
1250	17	37	41	38
2500	17	37	43	39
41B	17	29	31	30

*See footnote to Table 1 for definitions.

Blood chemistry

No treatment-related differences in serum chemistry were observed in male mice. There was a dose-related decrease in blood urea nitrogen in females after 1 month, but this was not seen in subsequent tests. A dose-related increase in blood glucose was seen after 12 months but not after 1 or 17 months. Dose-related reductions in alanine aminotransferase (but not in aspartate aminotransferase), and alkaline phosphatase were observed at 17 months but not at 1 or 12 months. The differences were not considered to be of clinical significance.

Renal function tests and urine analyses

While a slight but statistically significant increase in urine specific gravity with increasing treatment levels was seen in males but not females at 16 months, neither this nor other sporadic significantly different values noted occasionally during the trial appeared to be of any clinical significance. The concentration-dilution tests showed the kidneys of the mice to be functioning normally on each occasion of testing.

Post-mortem *findings*

Autopsy revealed no evidence of treatment-related effects among the cake-fed mice apart from the

Table 3. Food intakes of mice fed cake diets or diet 41B

Diet group*	Mean food intake (g/day) at week no. 1	13	52	65
	Males			
0	23	32	43	33
1250	23	31	42	38
2500	29	26	43	41
41B	45	44	40	44
	Females			
0	23	43	39	38
1250	24	23	43	38
2500	33	26	48	31
41B	38	48	37	38

*See footnote to Table 1 for definitions.

Table 4. Mean haematological data for male mice fed on cake diets or diet 41B

Month	Diet group‡	Hb (g/100 ml)	RBC (10^6/mm^3)	PCV (%)	Leucocytes: Total (10^3/mm^3)	Leucocytes: Differential (%) N	L	M	E
3	0	13.7	*|7.9	40	*| 9.2	21	77	2	1
	1250	13.7	|7.4	40	|10.7	26	75	0	0
	2500	14.4	|7.2*†	41	|12.4	11	89	0	0
	41B	14.2	8.2	41	10.3	11	88	1	0
12	0	9.8	*|7.3	28	31.0	18	82	0	0
	1250	11.4	|7.1	31	29.7	34*†	66*†	0	0
	2500	10.0†	|5.9*†	27	39.7†	30	69	0	0
	41B	13.3*	7.2	31	22.5	11	88	0	0
16	0	12.8	8.6	38	21.3	30	69	0	0
	1250	12.2	7.8	34	24.7	26	73	0	0
	2500	12.7	8.1	35	18.6	31	68	0	0
	41B	12.1	8.4	35	29.0	31	67	0	1

Hb = Haemoglobin RBC = Red blood cell count PCV = Packed cell volume
N = Neutrophils L = Lymphocytes M = Monocytes E = Eosinophils

‡See footnote to Table 1 for definitions.

Values marked with asterisks differ significantly (ANOVA and Student's t test) from those for the corresponding cake-fed control group ($P < 0.05$). Those marked with daggers differ significantly from the corresponding group fed diet 41B ($P < 0.05$). Vertical lines joining values indicate a statistically significant dose relationship (*$P < 0.05$).

organ-weight differences mentioned below. Excess adipose tissue was seen in both male and female mice in groups fed cake when compared with mice fed the stock diet 41B, consistent with the higher energy density of the cake diets. Absolute organ weights are given in Table 5; the adjustment for covariance with body weight made negligible difference to the values recorded. Dose-related increases in heart and kidney weights and a dose-related decrease in ovary weight were seen in females.

Histopathology

The findings are summarized in Table 6. The predominant lesion in both males and females was amyloidosis, involving principally spleen, liver, heart, ovaries and uterus. There was a higher incidence of this condition in cake-fed mice than in those fed diet 41B, but its incidence was not correlated with treatment levels in the cake-fed groups.

Covalently bound chlorine in adipose tissue

Analysis of perirenal fat for covalently bound chlorine gave the results shown in Table 7. The means are geometric means (see Fisher *et al.* 1983). Treatment-related concentrations of chlorine were found in the adipose tissue of both males and females.

DISCUSSION

The intakes of chlorine by the mice consuming cake made from chlorinated flour were increased thereby by a maximum of 286 and 350 mg/kg body weight/day for males and females respectively. The relative exposure factors compared with average human intakes of chlorine in cake are approximately 7500 compared with adult males, 7800 compared with adult females and 3900 compared with children.

Despite this heavy loading, few dose-related effects were seen during the trial and these were sporadic and mostly quite small. The additional stress to the animals caused by the obesity induced by the cake diets must also be remembered in assessing the findings. No satisfactory explanation of the obesity of the mice was obtained. Two hypotheses (that the obesity-inducing factor was the high sugar content of the diet, or that it was due to overfeeding to compen-

Table 5. Mean organ weights of mice fed on cake diets or diet 41B

Diet group‡	Organ weight (g): Brain	Heart	Liver	Kidneys	Spleen	Adrenals	Gonads	Pituitary	Thyroid	Uterus
					Males					
0	0.42	0.30	3.93†	0.84†	0.44†	0.015	0.19	0.001	0.014	—
1250	0.38	0.29	3.35†	0.81†	0.40†	0.012	0.20	0.001	0.012	—
2500	0.44	0.33†	3.21†	0.86†	0.32†	0.015	0.21	0.001	0.015	—
41B	0.42	0.22**	1.84**	0.56**	0.20**	0.012	0.21	0.001	0.011	—
					Females					
0	0.42	*|0.25†	2.60†	*|0.63	0.48†	0.017	*|0.07	0.001	0.013	0.49
1250	0.44	|0.27†	2.53†	|0.68†	0.35*†	0.019	|0.04*	0.001	0.013	0.33
2500	0.42	|0.29†**	2.45†	|0.72*†	0.46†	0.016	|0.04*	0.001	0.014	0.40
41B	0.42	0.18*	1.82**	0.47**	0.26**	0.015	0.06	0.001	0.011	0.31

‡See footnote to Table 1 for definitions.

Values marked with asterisks differ significantly (ANOVA and Student's t test) from those for the corresponding cake-fed control group (*$P < 0.05$; **$P < 0.01$). Those marked with daggers differ significantly from the corresponding group fed diet 41B ($P < 0.05$). Vertical lines joining values indicate a statistically significant dose relationship (*$P < 0.05$).

Table 6. Principal histopathological findings in mice fed on cake diets or diet 41B for 70–74 wk

Affected organ and/or type of lesion	No. of animals in which lesion was found							
	Males				Females			
Diet group*...	0	1250	2500	41B	0	1250	2500	41B
No. of animals examined...	42	45	48	22	55	43	46	24
Generalized amyloid (mainly of kidneys, spleen, liver, heart)	34	35	38	5	46	35	35	13
Liver								
Minor lesions other than amyloid	14	36	23	10	28	27	20	13
Kidney								
Glomerulonephrosis	28	26	30	7	23	30	24	12
Calcareous deposits or calculi	3	4	11	0	1	1	12	1
Other lesions	22	24	12	20	7	17	15	18
Lungs								
Pneumonitis or pneumonia	5	10	15	7	1	10	10	3
Other lesions	20	28	14	14	32	16	17	24
Heart								
Focal myocarditis, calcification Pericarditis. Fibrosis	10	12	11	3	1	10	10	1
Spleen								
Increased haematopoiesis	1	6	3	2	2	0	1	0
Lymphoid hyperplasia	1	4	1	0	5	5	4	0
Testes								
Atrophy, partial or complete	4	6	2	9				
Uterus								
Cystic endometrial hyperplasia					25	25	26	11
Ovaries								
Cystic					5	3	12	5
Atrophic					3	5	7	4
Neoplasms								
Peritoneal lipoma	1	1	0	0	1	0	0	0
Reticulum cell neoplasm								
(liver, kidney, spleen)	1	1	1	2	0	0	1	0
(ovaries)					0	0	1	0
Lung adenoma	1	2	2	1	1	0	1	0
Lymphosarcoma (spleen)	0	0	0	0	0	0	1	0
Total benign	2	3	2	1	2	0	1	0
Total malignant	1	1	1	2	0	0	3	0
Overall total	3	4	3	3	2	0	4	0

*See footnote to Table 1 for definitions.

sate for a dietary deficiency of lysine, methionine or iron), were tested in Wistar rats in an attempt to avoid a recurrence of the problem in the corresponding rat trial (Fisher *et al.* 1983). Neither hypothesis was found to be tenable, and a reduction in the fat content of the diet to be fed to the rats was decided on without further trials. The obesity of the mice undoubtedly increased the mortalities of the cake-fed groups and led us to make the final kill earlier than planned. Thus although no evidence of carcinogenicity attributable to the treatment of the flour was obtained during the trial, the value of this finding is diminished by the lower probability of observing tumours at the earlier stage at which the trial was ended.

Table 7. Covalently bound chlorine in perirenal fat of mice fed on cake diets or diet 41B

Diet group†	Chlorine concn (ppm)			
	Males		Females	
0	**	115	***	64
1250		624**		575**
2500		1298**		1239**
41B		347		124**

†See footnote to Table 1 for definitions.
Values are geometric means. Those marked with asterisks differ significantly (ANOVA) from the corresponding cake-treated control value (**$P < 0.01$). Vertical lines joining values indicate a statistically significant dose-relationship (**$P < 0.01$; ***$P < 0.001$).

As in the rat trial (Fisher *et al.* 1983) dose-related concentrations of chlorine were found in the perirenal fat of the mice. These concentrations were again very much lower than those present in the lipids in the diets on which the mice had fed, indicating that little or no accumulation of chlorinated lipid had occurred during the trial despite daily intake of reaction products derived from the chlorination of the flour from which the cake was made.

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BROMIDE-ION RESIDUES IN FOOD AND FEEDSTUFFS

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Abstract—The daily intake of bromide ion by humans was estimated from two total-diet studies conducted in the summer of 1976 and in the winter of 1978 and involving in each case investigation of 100 samples. The average daily intake found was 7.8 mg/person in the first study and 7.6 mg/person in the second study (with ranges of 2.9–15.0 and 1.8–17.2 mg/person/day, respectively). National surveys, covering several thousands of samples, showed that certain leafy vegetables and some herbs could occasionally contain high residues of bromide ion (> 200 mg/kg). An important source of these high residues is the treatment of soils with methyl bromide against nematodes. Action has been taken to minimize contamination of the human diet with bromide ion by this route.

Introduction

This paper deals with three different aspects of the bromide problem, namely analytical methodology, residues in total diets and residues in various classified food items. It will be shown that at the moment a good insight can be gained into the bromide content of most commodities sold in The Netherlands, as well as into bromide intake by the consumer. Analytical methodology has been standardized to such an extent that the results reported can be compared with each other without the risk of systematic discrepancies.

Experimental Procedures and Data

Analytical methodology

The method recommended in the manual issued by the Dutch Ministry of Public Health and Environmental Hygiene (1980) was used to obtain the figures reported in this paper. The method is based on gas chromatography and was described in detail by Greve & Grevenstuk (1976). In a collaborative study of the method reported by Greve & Grevenstuk (1979), using lettuce with incurred bromide residues ranging from 5 to 200 mg/kg, the coefficients of variation for repeatability (r) and reproducibility (R) ranged from 4.0 to 9.6% and from 8.3 to 18.4%, respectively. The ratio r/R ranged from 0.47 to 0.52.

A schematic summary of the method is given later.

Residues in total diets

In order to estimate the daily intake of bromide by the consumer, two total diet studies were conducted in which 100 workers of this Institute took part. The participants were asked to collect in stainless-steel containers duplicates of everything they ingested during 24 hr; the contents of each container were processed according to the scheme given below. The participants were also asked to supply details of the food items collected and of their eating habits. Apart from bromide content, many other parameters were also determined in the samples, but that part of the programme falls outside the scope of this paper.

The first study was carried out in the summer of 1976 and the second in the winter of 1978, to take into account possible differences in diets according to the seasons. The results of both studies are summarized in Table 1, which gives the bromide content of the edible part of the diets, as well as the daily bromide intake by the consumer.

Analytical flow scheme for total diet samples

Sample

(a) ↓

freeze-dried materials $\xrightarrow{(b)}$ ash $\xrightarrow{(c)}$ solution

for GLC $\xrightarrow{(d)}$ bromide content of sample

Table 1. Studies on bromide levels in total diets

	Study I (May–June 1976)		Study II (Jan.–March 1978)	
Calculations*	Bromide content (mg/kg diet)	Bromide intake (mg/person/day)	Bromide content (mg/kg diet)	Bromide intake (mg/person/day)
Mean ± SD	3.6 ± 1.4	7.8 ± 2.8	3.2 ± 1.2	7.6 ± 3.0
Median value	3.4	7.7†	2.9	7.4†
Range	1.2–11.7	2.9–15.0	1.1–8.2	1.8–17.2

*Results are derived from 100 observations in study I and 101 observations in study II.
†From another total diet study (a 'market basket' survey) conducted in The Netherlands (see Table 2), a median bromide intake of 9 mg/person/day was calculated. The range was 7–13 mg/person/day.

Procedures for steps a–d:

(a) Remove inedible parts (bones, stones etc.) and homogenize the remainder. Divide the homogenate into three equal parts. Freeze-dry one part (the rest being used for other studies).

(b) Homogenize the freeze-dried material carefully and weigh accurately *c.* 500 mg into a nickel crucible. Add 1 ml 2N-NaOH and 2 ml water. Allow to stand for 10 min. Place the crucible for 30 min in an oven heated to 100–120°C. Transfer the crucible to a muffle furnace heated to 500°C. Remove the crucible from the furnace after 1 min and break the lumps with a spatula. Place the crucible again in the furnace and leave it there for 30 min. Allow to cool to room temperature.

(c) Add, cautiously, 75 ml 0.6 N-H_2SO_4 to the cool ash. Using 20 ml acetone, transfer the contents of the crucible quantitatively to a stoppered 250-ml Erlenmeyer flask. Add 5 ml of a 4% solution of ethylene oxide in diisopropyl ether. Mix well and allow to stand for 1 hr at room temperature. Transfer *c.* 10 ml of the supernatant to a stoppered 25-ml cylinder. Add 2 g ammonium sulphate, shake vigorously for 2 min and allow to stand for 30 min. Transfer *c.* 5 ml of the supernatant to a reagent tube, add 1 g anhydrous sodium sulphate and allow to stand for 30 min.

(d) Inject 5 μl of the dried solution into a gas chromatograph under the following conditions: Pyrex column 1.80 m × 2 mm ID, packed with 15% polypropylene glycol (UCON LB-550-X; Phase Separations Ltd, Queensferry, Clywd, UK) on a Chromosorb W-AW (80–100 mesh), with temperatures of 120°C for the column and 135°C for the injector, a nitrogen flow rate of *c.* 30 ml/min and a ^{3}H electron-capture detector. Also inject standard solutions of 2-bromoethanol in acetone of comparable strength and calculate the 2-bromoethanol content of the unknown solutions.

Determine the recovery of the procedure by adding known amounts of potassium bromide to a sample and carrying it through the entire procedure. The recovery should be $\geqslant 80\%$. Calculate the bromide content of the sample. Also determine the bromide content of the reagents used by carrying out the whole procedure without the sample. Correct for this blank value if necessary.

Residues in classified food items

For several years, an action plan covering several thousands of samples of food sold in The Netherlands was carried out under the co-ordination of the Veterinary Head Inspectorate and the Head Inspectorate for Food of the Ministry of Public Health and Environmental Hygiene. The Food Inspection Services directed their efforts mainly to products of vegetable origin and to milk, and the National Institute of Public Health to products of animal origin and to animal feedstuffs. The latter also analysed samples of human milk and blood.

The results have been published, together with data from other sources in The Netherlands, in "Het Contaminantenboekje" (List of Contaminants) by the Ministry of Public Health and Environmental Hygiene (1982). A summary of the bromide content of some important food classes is given in Table 2.

Although some food classes still have to be covered, and other classes require further investigation, a good general survey of the bromide situation in the foods consumed by the Dutch population is now possible.

Table 2. Bromide levels in various classified food items sold in The Netherlands

Type of food/feed	Median bromide level (mg/kg)*
Vegetables	
Endive	6
Lettuce	4
Purslane	5
Spinach	3
Turnip tops	5
Lamb's lettuce	3
Brussels sprouts	3
Beans	3
Celery	5
Chicory	3
Tomatoes	4
Beets	5
Radish	7
Carrot	3
Potato	5
Herbs	
Parsley	3
Celery (green)	7
Grains	
Wheat	2
Rice	6
Rye	2
Barley	7
Fruits	
Strawberries	3
Other fruits	0.2
Animal products	
Milk	3
Dairy products	4
Cattle meat	4
Pork meat	4
Pork fat	0.2
Meat and egg products	3
Fish (fresh-water and marine)	7
Eel (fresh-water and marine)	4†
Liquids (excluding milk)	
Drinking water	0.1
Beverages	0.2
Beer	0.3†
Animal feeding-stuffs†	
Products based on rice	0.5
wheat	2.1
soya bean	1.3
maize	1.2
sorghum	1.2
citrus	0.7
alfalfa	1.6
fish meal	12.6

*Data source—Ministry of Public Health and Environmental Hygiene (1982) except where indicated otherwise.

†Unpublished data from author's laboratory.

Occasional high residues ($\geqslant$200 mg/kg) were found in certain leafy vegetables (endive, lettuce, purslane and turnip tops) and herbs (celery). The medium bromide intake from total diet samples composed according to the 'market basket' principle was 9 mg/person/day (range 7–13 mg/person/day).

Discussion

Table 1 shows that the two studies were largely in agreement. In the first study (summer 1976) an average daily intake of 7.8 mg/person was found, while in the second study (winter 1978) this figure was 7.6 mg/person. The median values were all slightly lower than the average, indicating that the distribution is skewed to a moderate extent only. Standard deviations are relatively low, so it is probable that the 201 diets investigated give a good representation of the diets consumed by the participants, and hence of their daily bromide intake. The acceptability of these residue levels, notably the higher ones, is discussed elsewhere in this volume.

The breakdown given in Table 2 for the various food classes shows that one can apparently distinguish three groups of food: (i) a group containing 'low' bromide residues (i.e. generally < 1 mg/kg), consisting of fats (oils), most fruits, water and beverages; (ii) a group containing 'medium' levels of bromide (roughly 3–7 mg/kg), consisting of meat, fish, dairy products, strawberries, most vegetables and grains; (iii) a group occasionally containing "high" bromide residues ($\geqslant 200$ mg/kg) and consisting of certain leafy vegetables (endive, lettuce, purslane and turnip) and herbs (green celery).

Logically, special attention has been given to the latter group and to the main source of the high bromide residues found. This source is the treatment of soils with methyl bromide against nematodes. As a result, it has become obligatory to leach the soils with water after each treatment with methyl bromide. The desired goal (lowering the bromide residues in the crops cultivated in the treated soils) has been achieved in this way, but the problem has at the same time been shifted to the environment, as both unchanged methyl bromide and bromide ion were released into the surface waters around the greenhouses where the crops were cultivated. In the next paper (Wegman, Hamaker & de Heer, 1983) the consequences of these practices are discussed.

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