CHRONIC TOXICITY OF BREAD ADDITIVES TO RATS. PART II

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In an earlier investigation[1] of the chronic toxicity of certain bread additives the ingredients tested (chlorine dioxide, polyoxyethylene (8) monostearate, sodium propionate and a mixture of propyl gallate and butylated hydroxyanisole) were incorporated in bread at 50 times the normal concentration and used for 52 weeks at 75 per cent. of the diet of rats. Under the conditions of the experiment no adverse effects were noted and these results were at variance with some of the experimental evidence obtained by other investigators, particularly in regard to the polyoxyethylene (8) monostearate[2,3,4,5]. Possibly this difference was due to experimental technique. It was of interest, therefore, to retest the bread ingredients previously studied, with the difference that they were not mixed with water, baked into bread, and the bread dried and ground but were added directly to the basal ration.

Diet I contained the ingredients of an essentially normal bread (75 per cent.) added to a basal medium supplying minerals, vitamins, protein, fat, and bulk (25 per cent.). In diet II, flour treated with 50 times the normal amount of chlorine dioxide, lard containing 50 times the usual level of antioxidants, and sodium propionate and polyoxyethylene (8) monostearate each at 50 times the usual concentration were incorporated in the bread ingredient portion of the diet. Diets III, IV, V and VI were similar to diet II, except that one ingredient in each, respectively emulsifier, mould inhibitor, antioxidants, or flour improver, was reduced to the normal level. Diet VII was a commercial cubed ration ground to a consistency similar to that of the other diets. Diets were mixed at intervals of no greater than 2 weeks and were kept in closed cans in the refrigerator. The details of diet composition for convenience are reproduced from the previous publication[1] in Table I.

A group of 210 Wistar strain male albino rats ranging in age from 23 to 36 days and in weight from 35 to 79 g. was divided into 14 sub-groups by arranging them in descending order of weight and randomly assigning the rats in successive lots of 14 to the 14 sub-groups. These sub-groups, each consisting of 15 animals, were assigned to the 7 diets yielding two series, called for convenience replicates A and B, and numbered according to the number of the diet given. The oldest rats had been maintained on diet VII from weaning at 22 days of age until placed on test. The 14 groups of rats were housed in cages with wire screen floors. Each cage held 1 group (15 rats). Cages were moved in a regular rotation on the racks so that all spent approximately equal intervals at the 4 different levels above the floor. Food and water were permitted *ad libitum*. Records of weekly weight, food consumption and mortality

TABLE I

DIET COMPOSITIONS

Part A.—75 per cent. of the diet. Ingredient	Diet I	II	III	IV	V	VI	VII
Commercial bread flour[1]	70·5	—	—	—	—	70·5	G
Bread flour 50 × ClO_2[1]	—	70·5	70·5	70·5	70·5	—	R
Commercial lard[2]	1·8	—	—	—	1·8	—	O
Lard 50 × Antioxidants[2]	—	1·8	1·8	1·8	—	1·8	U
Sodium propionate[3]	0·1	5·0	5·0	0·1	5·0	5·0	N
Polyoxyethylene (8) monostearate[4]	0·3	15·0	0·3	15·0	15·0	15·0	D
Cerelose (glucose)	20·4	0·8	15·5	5·7	0·8	0·8	
Salt (NaCl)	1·8	as I	as I	as I	as I	as I	M
Skim milk powder	2·2	"	"	"	"	"	A
Malt flour	0·5	"	"	"	"	"	S
Yeast food[5]	0·2	"	"	"	"	"	T
Wytase[6]	0·9	"	"	"	"	"	E
Yeast	1·3	"	"	"	"	"	R
	100·0	100·0	100·0	100·0	100·0	100·0	

B.—25 per cent. of the diet. Ingredient	Diet I	II	III	IV	V	VI	F O X C U B E S
Casein	60·0	as I	as I	as I	as I	as I	
Alphacel[7]	9·4	"	"	"	"	"	
Corn oil	12·8	"	"	"	"	"	
Mineral mix[a]	12·0	"	"	"	"	"	
Vitamins and excipient[b]	5·8	"	"	"	"	"	
	100·0	100·0	100·0	100·0	100·0	100·0	

[a] Minerals—the 12·0 g. of mineral mix present in 100 g. of basal diet were made up as follows:—

KCl	1·580	$FePO_4$	0·195
KH_2PO_4	4·100	$KAl(SO_4)_2.12H_2O$	0·001
$Ca_3(PO_4)_2$	1·970	$CuSO_4.5H_2O$	0·080
$MgSO_4$	1·190	NaF	0·076
$MnSO_4.H_2O$	0·027	KI	0·001

[b] Vitamins—the 5·8 g. of vitamins and excipient in 100 g. of basal diet were made up as follows:—

Thiamine hydrochloride	0·0020 g.	Folic acid	0·0008 g.
Riboflavine	0·0040 g.	*p*-Aminobenzoic acid	0·0040 g.
Calcium pantothenate	0·0080 g.	Liver fraction‡	0·8000 g.
Pyridoxine	0·0020 g.	Menadione	0·0004 g.
Nicotinamide	0·0080 g.	E concentrate*	0·0560 g.
i-Inositol	0·0800 g.	A and D concentrate†	0·1600 g.
Choline chloride	0·4000 g.	Corn oil	4·2830 g.

* Vitamin E concentrate, 350 mg. of *d*, α-tocopheryl acetate equivalent per g.
† Navitol, 65000 A, 13000 D per g.
‡ Wilson's Liver fraction L.

NOTES

[1] Chlorine dioxide.—Canadian No. 1 patent flour was treated with 0·3 g. of chlorine dioxide per barrel (196 lb.) to yield commercial bread flour and with 15·0 g. of chlorine dioxide per barrel to yield high ClO_2 flour.

[2] Antioxidants.—Tenox II, a commercial preparation containing 20 per cent. of butylated hydroxyanisole, 6 per cent. of *n*-propyl gallate, 4 per cent. of citric acid and 70 per cent. of propylene glycol was used at the rates of 0·05 per cent. in commercial lard and 2·5 per cent. in high antioxidant lard.

[3] Propionate.—The commercial propionate sold by Du Pont de Nemours and Co., under the trade name Mycoban was used.

[4] Polyoxyethylene monostearate.—The material sold by Atlas Powder Co. as polyoxyethylene (8) monostearate under the trade name Myrj 45 was used.

[5] Yeast food.—The commercial material sold under the name RKD by Standard Brands was used.

[6] Wytase.—This material is sold by J. R. Short Canadian Mills, Ltd., and is used commercially to improve whiteness.

[7] Alphacel is a commercial cellulose supplying bulk without nutritive value.

were kept. Where the condition of the carcass warranted it, detailed autopsies were performed on animals that died on test; in other instances advanced autolytic changes precluded accurate decision as to the cause of death.

After 16 weeks on the diets 2 rats selected from each group by random choice were killed and a number of tissues were preserved for histological

study. Blood samples from each of these rats were analysed for hæmoglobin content by a slight modification of the pyridine hæmochromogen method of Rimington[6]. After 32 weeks on the diet only 3 rats survived in group II A and the experiment was terminated. All surviving rats were killed and the liver, left kidney, heart and spleen were weighed. Tissue samples were reserved for histological examination and subsequently sections from cerebellum, cerebrum, myocardium, trachea, œsophagus, thyroid, salivary gland, thymus, peri-bronchiolar lymph gland, lung, stomach, small intestine, liver, spleen, adrenal, pancreas, kidney, bladder and testis were studied.

Results

The mean body weights at intervals during the 32-week feeding trial of the rats in the 14 groups are presented graphically in Figure 1. The mean body weights of the survivors on each diet and the standard errors of the means are shown for replicates A and B and for the replicates combined in Table II.

TABLE II

Final weight of survivors at 32 weeks
Weight in g. ± standard error

Diet	Replicates combined		Replicate A		Replicate B	
	Mean weight	Number	Mean weight	Number	Mean weight	Number
I	296 ± 7·4	16	297 ± 12·4	7	295 ± 9·6	9
II	258 ± 7·4	13	257 ± 21·8	3	259 ± 13·2	10
III	268 ± 10·3	15	277 ± 13·5	7	260 ± 15·4	8
IV	281 ± 9·4	21	273 ± 14·2	11	290 ± 10·9	10
V	252 ± 7·0	21	247 ± 10·0	11	258 ± 9·9	10
VI	263 ± 6·5	18	255 ± 10·0	10	274 ± 7·0	8
VII	292 ± 8·1	19	305 ± 16·2	7	283 ± 8·4	12

TABLE III

Analysis of variance of final weight data

Main effect	DF	Mean square	F
Replicates	1	335	1
Diet	5	4,345	4·4*
Replicates X diet	5	792	1
Error	111	984	—

* Significant at P = 0·01.

Growth was depressed, particularly during the first few weeks on diets II, III, V and VI, i.e. on those diets containing the high level of sodium propionate. A detailed examination of the data revealed that the total weight gain subsequent to the first 4 weeks on the diet was approximately the same for each diet and the apparent depression of the final weights on these 4 diets high in propionate was the result of the curtailed growth during the first few weeks. Growth rates based on weight changes between the 5th and 32nd weeks were not significantly influenced by diet. Growth on diet I was very similar to that on diet VII throughout the test.

Statistical analysis of the final weight data for diets I to VI using Snedecor's methods for disproportionate groups[7] revealed a significant

($P = 0{\cdot}01$) influence of diet on final weight (Table III). The effect was mainly that of the high level of sodium propionate, for growth on diet IV was not significantly depressed.

Cumulative feed consumption per rat per day over the 32-week period is shown in Figure 2. As in the previous study[1] the rats ate more of the stock diet (VII) than of any of the test diets. In this experiment, feed consumption figures for all diets were higher than the corresponding

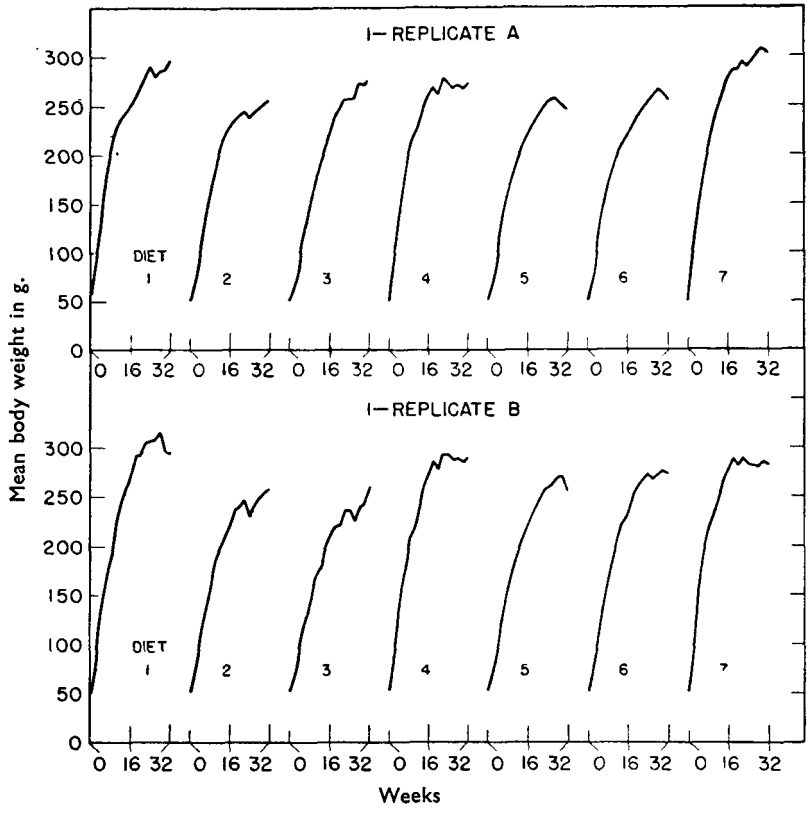

FIG. 1. Mean body weight of the rats in the 14 groups.

figures from the earlier data by an average of some 8·5 per cent. Mean body weights overall were only 3 per cent. higher. These differences are reflected in the generally poorer feed efficiency also depicted graphically in Figure 2. The striking effect of diet which may be observed here is the very poor feed efficiency during the first weeks on diets II, III, V and VI, particularly on diet III.

Curtailed growth, slightly lower feed consumption, and markedly lowered feed efficiency on diets II, III, V and VI during the early weeks indicate that sodium propionate fed at high levels to the young rat had a deleterious effect. That this effect was not solely due to lack of palatability of the diet is obvious from the fact that feed efficiency was much

more seriously affected than was feed consumption. Although the data are not amenable to statistical assessment, they suggest that polyoxyethylene (8) monostearate at high levels in conjunction with high sodium

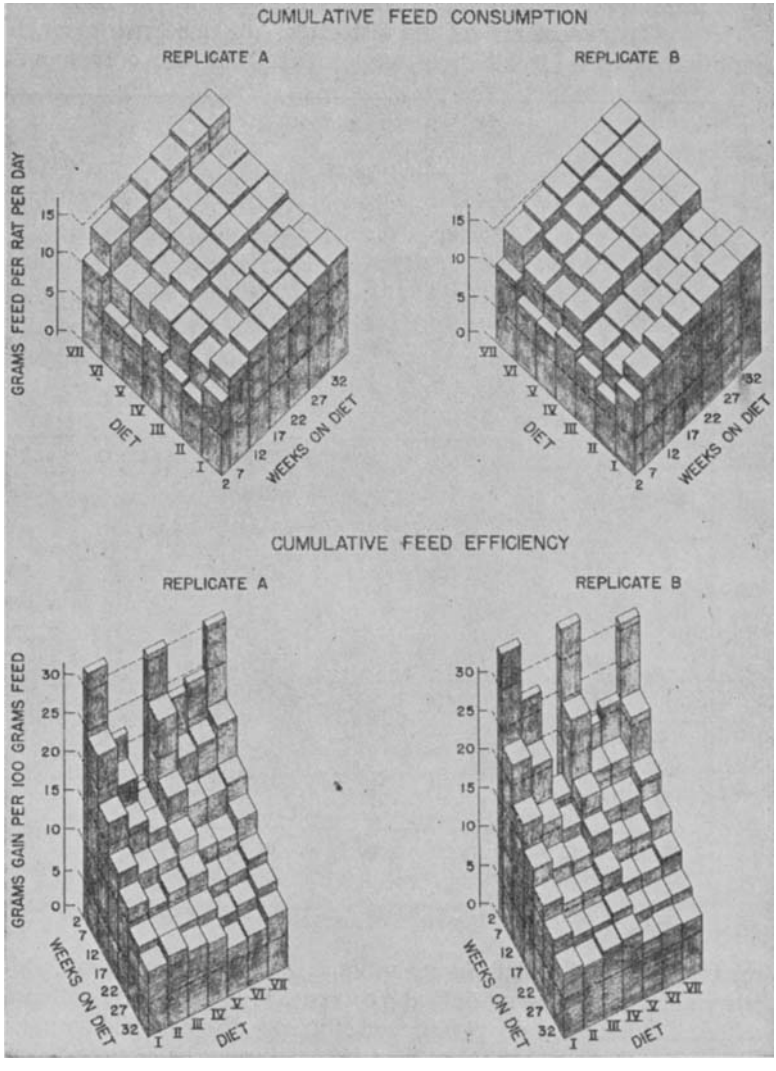

FIG. 2. Cumulative feed consumption per rat per day over the 32-week period.

propionate (diets II, V and VI) increased the efficiency with which the high propionate diet was utilised by young rats (compare diet III). Apparently the rats became acclimatised to the high propionate diet, for growth rate, feed consumption and feed efficiency approached the control levels within a few weeks.

The hæmoglobin levels in the blood of the 28 rats killed at the end of 16 weeks were all within the normal range for rats in this colony.

Sections from the tissues of 151 rats were stained with hæmotoxylin and eosin and examined microscopically. As in the previous investigation[1] the groups to which the individual rats belonged were unknown

TABLE IV

HISTOPATHOLOGICAL FINDINGS

	Replicate													
	A							B						
Diet	I	II	III	IV	V	VI	VII	I	II	III	IV	V	VI	VII
Number of rats examined*	9	5	9	13	13	12	9	11	12	10	12	12	10	14
Normal	4	2	4	2	0	1	1	5	4	1	5	1	1	3
Trachea—inflammation	–	–	–	–	5	2	–	2	–	1	–	4	1	–
Lung—respiratory infection	2	1	–	5	10	7	6	2	1	1	3	8	7	6
Lung—papilloma of bronchus	1	–	–	–	–	–	–	–	–	–	–	–	–	–
Thymus—inflammation	–	–	1	–	–	1	–	–	–	–	–	–	–	–
Stomach—gastritis	–	–	1	–	–	–	–	–	2	–	–	–	–	–
Stomach—erosion	–	–	–	–	–	–	–	–	1	–	–	1	–	–
Adrenal—congestion	–	–	–	1	–	–	–	–	–	1	–	–	–	–
Kidney—tubal necrosis	–	–	–	–	1	–	–	–	–	–	–	–	–	–
Kidney—nephritis	–	1	–	1	1	–	–	–	–	–	–	1	–	–
Kidney—congestion	–	–	–	–	–	–	–	–	–	1	–	1	–	–
Liver—cloudy swelling	–	–	1	2	1	–	–	–	–	–	2	–	–	–
Liver—venous congestion	–	–	–	1	–	–	–	–	1	–	–	–	–	–
Pancreas—fatty degeneration of acinar tissue	–	–	–	–	–	–	–	–	–	–	–	–	1	–
Intestine—enteritis	–	–	1	–	1	–	–	–	1	–	–	–	–	–
Intestine—papilloma	–	–	–	–	–	–	–	–	–	1	–	–	–	–
Testis—reduced spermatogenesis	–	–	–	–	–	–	–	1	–	–	–	–	–	–
Bladder—parasitic cysts	4	3	5	8	5	6	8	5	5	9	6	4	6	8

* 2 rats were killed at 16 weeks, the remainder at 32 weeks.

MORTALITY DATA

Died on test	6	10	6	2	2	3	6	4	3	5	3	3	5	1
Autopsy	3	4	5	2	0	2	3	2	1	4	2	3	4	0
Undiagnosed	3	6	1	0	2	1	3	2	2	1	1	0	1	1
Respiratory infection	1	4	2	–	–	2	1	1	1	4	2	3	3	–
Middle ear disease	2	–	3	1	–	–	–	1	–	–	–	–	2	–
Encephalitis } Probable extension from middle	2	–	4	–	–	–	–	–	–	–	–	–	–	–
Meningitis } ear infection	–	–	1	1	–	–	–	1	–	–	–	–	2	–
Pericarditis	–	–	1	–	–	–	1	–	–	–	–	–	1	–
No visible lesion	–	–	–	1	–	–	1	–	–	–	–	–	–	–

to the pathologist at the time the tissues were studied. The histopathological findings are presented in Table IV and have been summarised by the pathologist as follows:

Findings in the cerebrum, cerebellum, myocardium, œsophagus, thyroid, salivary gland, thymus, small intestine, spleen, adrenal, pancreas, stomach and testis were negative or of no significance. Most of the lungs exhibited some changes due to pneumonia, including emphysema, bronchiectasis and atelectasis, although only acute and sub-acute cases have been listed in Table IV. The predominant bronchopneumonia invariably was accompanied by mild chronic inflammatory changes in the trachea. The two instances in which congestion and polymorphonuclear infiltration of the peribronchiolar lymph nodes were observed probably were the result of infection from the lungs. While cloudy swelling of the liver is the first sign of a degenerative process caused by

bacterial or chemical toxins, the few cases noted here were very mild and of doubtful significance. The incidence of kidney abnormality is similar to that reported previously[1], and again these abnormalities were observed in rats on diets II, III, IV and V, those diets high in chlorine dioxide. The incidence of the kidney changes again is too low to warrant any conclusion. The lung changes, which are of bacterial origin, and the bladder parasites (*Trichosomoides crassicauda*) occur normally in this rat colony. From a histopathological standpoint the tissues were essentially negative as far as consistent changes due to a dietary toxicant are concerned.

TABLE V

EFFECT OF DIET ON THE WEIGHT OF VARIOUS ORGANS
Mean tissue weight in mg./100 g. of rat

Diet	Number	Liver	Left kidney	Heart	Spleen
Replicate A—					
I	7	35·4	3·94	3·79	3·26
II	3	38·4	4·07	3·73	3·20
III	7	33·2	3·87	3·46	3·19
IV	11	36·0	3·76	3·54	2·71
V	11	35·7	4·05	3·65	2·85
VI	10	31·7	4·13	3·59	2·84
VII	7	36·3	3·56	3·39	2·69
Overall standard deviation		4·1	0·39	0·33	0·55
Replicate B—					
I	9	27·3	3·66	3·39	3·04
II	10	32·9	3·86	3·62	2·71
III	8	34·3	4·21	3·53	2·94
IV	10	30·0	3·48	3·49	2·68
V	10	32·4	3·83	3·65	2·62
VI	8	32·2	3·91	3·60	2·45
VII	12	30·9	3·56	3·73	2·74
Overall standard deviation		4·5	0·40	0·37	0·45

The mortality data also are presented in Table IV. Those deaths for which no explanation is given occurred at night and autolysis was too far advanced to permit a valid diagnosis. As in the previous study[1], it is confidently believed that most of these deaths may be attributed to respiratory infection. The high mortality in group II A appears to have been due to a particularly severe outbreak of respiratory infection in this cage. It is possible, of course, that the high level of all 4 potential toxicants in diet II predisposed these rats to infection, but the lack of abnormally high mortality in the corresponding group II B argues against such a possibility. In general, it may be concluded that the variations in mortality were of no significance in so far as they might reflect toxicity of the diet.

The mean weights of the livers, left kidneys, hearts and spleens of the rats surviving until killed after 32 weeks on the diets are presented in Table V. For statistical analysis the data summarised in Table V were transformed into percentage of the mean weight for each tissue and analysed by Snedecor's methods for analysis of variance with disproportionate subclass numbers[7]. The results of these analyses are presented in Table VI. Since the diet X replicate interaction was significant in one instance, and was of appreciable magnitude in the other 3 cases, these interactions were used in the calculation of F for diet and replicate. On

this basis spleen weights were significantly different (P = 0·05) between replicates. No explanation of this finding can be offered: it is suggested that this may be one of the 5 out of 100 cases where the difference is due to chance.

TABLE VI

ANALYSIS OF VARIANCE ON TISSUE WEIGHT DATA

		Liver		Left kidney		Heart		Spleen	
Main effect	D.F.	M.S.	F.	M.S.	F.	M.S.	F.	M.S.	F.
Replicates ..	1	2,005	3·6	252	1·1	250	1·1	3,000	6·7*
Diet	5	314	< 1·0	470	2·1	102	< 1·0	742	1·7
Diet X replicates	5	560	4·1*	221	2·1	229	2·5	447	1·8
Error	111	137	—	107	—	90	—	249	—

* Significant at P = 0·05.

DISCUSSION

The transient growth-depressing effect of a high level of sodium propionate in the diet of young rats in this experiment is contrasted with the lack of influence of this compound in the previous study[1]. As was noted at the time, baking the bread and later drying it resulted in a strong odour of propionate and it is probable that the final concentration of propionate in the diets of the previous study was considerably reduced. Since the present diets were unheated, little or no propionate was lost and the material at this higher level in the diet exerted an early growth-depressing effect. Obviously since normal rates of growth were resumed after a few weeks and no pathology attributable to propionate poisoning was detected, this effect of propionate cannot be regarded as too serious. In the human dietary, propionate is used almost entirely in baked goods and it is likely that the baking process would materially reduce any potential hazard.

In agreement with the previous findings, the high levels of the antioxidants butylated hydroxyanisole and propyl gallate, of the emulsifier polyoxyethylene (8) monostearate and of the flour improver chlorine dioxide had no detectable deleterious effect on the growth of rats over a 32-week feeding period. In spite of the change in experimental method, 11·25 per cent. of polyoxyethylene (8) monostearate in the diet failed to exert deleterious effects.

SUMMARY

1. Bread ingredients containing 50 times the normal concentration of chlorine dioxide, propyl gallate and butylated hydroxyanisole, or polyoxyethylene (8) monostearate given as 75 per cent. of the diet for 32 weeks did not harmfully affect growth or mortality of male rats. Sodium propionate at the same high level caused a depression of growth during the first few weeks of feeding, but did not influence mortality.

2. The high concentration of polyoxyethylene (8) monostearate, sodium propionate, antioxidants and chlorine dioxide had no detectable effect on hæmoglobin levels in the blood, on organ weights, or on histopathology of the tissues.

3. No evidence was obtained that the simultaneous presence of a high concentration of more than one potential toxicant in the diet led to any synergistic action.

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