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Introduction

There is now considerable evidence to support the belief that susceptibility to dental caries can be influenced by nutritional factors during the period of enamel mineralization. For the rat, the greater part of this period is from birth to twenty-one days of age, that is, up to the time of weaning. Sognnaes (1948, 1949) has shown that the "offspring of rats fed a high sucrose semi-synthetic ration during this period of lactation were much more caries susceptible than the offspring of rats fed a laboratory stock diet of natural foods." A greater degree of susceptibility was produced when this purified diet was fed not only during lactation, but also prior to mating and during pregnancy.

The purified diet used by Sognnaes was Shaw's ration 100 (Shaw (1947))—also called the Harvard ration. The vitamins were added in purified form and the known essential minerals were added as analytical reagent grade mineral salts.

This work of Sognnaes has been confirmed by other investigators. However, Haldi and Wynn (1952) found that the incidence and extent of dental caries produced by feeding a modification of Shaw's Ratio 100 (the Emory diet) was much less than that reported by Sognnaes (1948). The Emory and Harvard diets were similar in the contents of the major constituents, but they did show slight quantitative differences in fat, protein, minerals and vitamins. It was realised that this difference in caries production might have been the result of such variables as genetic factors (different strains of rats) different laboratory conditions, and different drinking water. These variables were controlled by Wynn, Haldi, Shaw and Sognnaes (1953) in an experiment to compare the effect of these two diets. The average number of carious lesions produced in rats on the Harvard diet was about three times that produced by the Emory diet. It was suggested that investigations should be made to determine what the constituent differences were and how these differences altered the caries susceptibility of the teeth.

Since the Emory diet contained a very small trace of aluminium and the Harvard diet contained none, Wynn and Haldi (1954) studied the possible effect of aluminium on caries production. However, no significant alteration in caries susceptibility was produced by the addition of aluminium at the levels used.

Dalderup and Jansen (1955) noted that the Emory ration contained "4% yeast and liver extract" instead of the "4% liver concentrate" of the Harvard ration. They found that a five per cent. brewer's yeast supplement to Shaw's ration 100 caused a marked reduction in the extent, and a less marked reduction in the number of lesions in the rat. However, they could not show the same influence of yeast on caries incidence with three other caries producing diets and they concluded that the effect of the yeast could be supplied or substituted by other foods.

*This series is extracted from a thesis submitted to the University of Queensland in support of candidature for the degree of Doctor of Dental Science. Partial financial support for the study came from Project No. 208, University of Queensland.

By ashing a stock diet and using this ash to replace the analytical reagent grade mineral salt mixture in the Harvard diet, Sognnaes and Shaw (1953, 1954) were able to show a significant reduction in caries production. They suggested that the caries protection afforded might be due "to the presence of certain trace elements in the ash mixture of the natural stock diet."

Nizel and Harris (1950, 1951) have shown that foods grown in different areas have a different caries producing effect. Using a modified Hoppert-Webber-Canniff diet containing 60% corn and 30% whole milk powder, they found that corn and milk from New England states was far more caries producing than products produced in Texas. The difference could not be explained by a difference in fluoride levels but according to Nizel and Harris (1955), New England corn contained at least twice as much magnesium, copper, manganese, iron, aluminium and molybdenum, and significantly less nickel than Texas corn.

Nizel (1955) has also confirmed the observation made by Sognnaes and Shaw (1948, 1949) that caries incidence can be significantly reduced by feeding a ration containing a supplement of the mineral ash of a diet of natural foodstuffs.

More recently, Hewat and Eastcott (1956) have shown an association between certain soils and caries incidence in human beings. As a possible explanation, they have suggested that a different trace element content of the soils may be carried through plants and animals to man, and evidence itself in the difference in caries susceptibility of the teeth.

There is consequently some circumstantial evidence to indicate a role of minerals in building a tooth more resistant to dental caries.

The effect of mineral salts on experimental caries production has been extensively studied and this has been reviewed by Hein (1955). However, the majority of these investigations have administered the compound being studied after the major period of enamel mineralization. They consequently do not provide any information about the contribution of any particular element to the building of a tooth, which will be more resistant to carious attack after it erupts.

The purpose of the present investigation was to see if a number of trace elements had any effect on dental caries in the rat molar. Suitable salts of these elements were administered as daily supplements during the period of enamel mineralization, in an attempt to incorporate them into the enamel structure, and to impart to that enamel a resistance to dental caries attack. This resistance was measured by evaluating the caries produced in the molars after a twenty-week period on a caries producing dietary regime.

Experimental Procedure

Albino rats were used for this investigation, and the caries evaluation was confined to the first and second molars. Breeding stock was obtained from other local laboratories but none of these sources was able to specify any particular strain.

The dietary programme used for the production of dental caries in the rat molars followed that used by Carr (1954, 1954a). In his experiments, Carr made use of a finely divided high sucrose ration during pregnancy and lactation to sensitise the offspring to greater caries susceptibility. He followed this with a coarse cereal particle ration to initiate lesions. Carious progress was then increased by a third diet containing 60% sucrose.

The programme used in the present investigations only differed from Carr's in the breeding diet. The prenatal diet used throughout these experiments was based on Shaw's ration 100 (1947) and was designated D1. It consisted of:

Sucrose	68.0 per cent.
Casein	24.0 per cent.

Salt mix	4.0 per cent.
Peanut oil	4.0 per cent.
Synthetic vitamins ..	see below
Liver meal	2 G to every 100 G of mixed ration.

The salt mix contained analytical reagent grade salts in the following proportions:

Sodium chloride	167.5 G
Potassium dihydrogen phosphate ..	422.5 G
Calcium phosphate (mono acid) ..	95.0 G
Magnesium sulphate	102.0 G
Calcium carbonate	300.0 G
Iron citrate*	27.5 G
Potassium iodide	0.8 G

To every 400 G of peanut oil was added 25 mls. of cod liver oil and 500 mgms. of Vitamin E concentrate (Parke Davis).

The other "vitamin" mixture was added so that every 100 G of mixed ration contained:

Thiamine	350 μg.
Riboflavin	350 μg.
Pyridoxine	350 μg.
Nicotinic acid	2.5 mgm.
Pantothenic acid	2.0 mgm.
Choline chloride	100.0 mgm.
Inositol	100.0 mgm.
p-amino-benzoic acid	30.0 mgm.

Because of the heat labile and light labile nature of some of these components, only small batches were mixed at a time and these were stored in a refrigerator.

After weaning, the coarse cereal particle was fed for 8 weeks. This was the post-weaning diet of Carr (1954a), but for convenience in our laboratory was designated D2. It contained:

Corn meal	67.0 per cent.
Wheat germ	18.5 per cent.
Whole milk powder	4.0 per cent.
Sodium chloride	1.0 per cent.
Calcium carbonate	0.5 per cent.
Casein	9.0 per cent.

After 11 weeks of age, the animals were transferred to Carr's third ration and maintained on this for 9 more weeks. This ration, designated D3 here, contained:

Sucrose	60.0 per cent.
Wheat germ	23.5 per cent.
Casein	8.0 per cent.
Oat meal	7.0 per cent.
Sodium chloride	0.5 per cent.
Calcium carbonate	1.0 per cent.

A sieve analysis of these three diets is shown in Table 1.

Pregnant rats were transferred to individual wooden breeding boxes lined with paper waste, and each litter was kept there until weaned at 21 days of age. Distilled water was supplied to the mother and the D1 ration was contained in a tapering pot of glazed pottery as recommended by Shaw (1954). By using these and changing

*A.R. grade not available.

TABLE I

Sieve analysis of the three diets used in the production of dental caries.

B.S.S. Sieve Number	D1	D2	D3
Retained on a 16 mesh sieve	4.0%	10.0%	10.0%
Retained on a 22 mesh sieve	18.6%	53.5%	15.0%
Retained on a 44 mesh sieve	44.4%	3.5%	20.0%
Retained on a 60 mesh sieve	30.5%	18.0%	40.0%
Passed through a 60 mesh sieve	2.5%	15.0%	15.0%

D1 = prenatal semi-synthetic high sucrose ration.

D2 = coarse cereal particle ration to initiate carious lesions.

D3 = high sugar ration to increase the progress of lesions initiated by D2.

the pots daily, it was hoped to reduce the tendency to contamination by faeces and urine. The purpose of this was to minimise coprophagy during the period when test solutions were being administered.

The soluble salts of the elements to be tested were to be administered during the major period of enamel mineralization of the first and second rat molars and according to the table of Schour and Massler (1949) this period occupies the first fifteen days of life. The general accuracy of these figures was confirmed by a histologic series done on the rat strain used for these experiments.

It was not found practicable to begin administration before the fifth day, partly because of the smallness of the animals but mostly because of the danger of upsetting the mother. When this occurred, she usually became savage and ate her young. The administration period adopted was from the fifth day and daily thereafter to the seventeenth day.

All solutions were given by intraperitoneal injection. Hein (1955) has described the disadvantages of administration by injection. As well as this, other studies have shown that certain minerals, *e.g.*, cobalt, show a particular response when administered by mouth but do not give this same result when injected. However, despite these disadvantages, intraperitoneal injections were used because it was felt that a more constant daily dose could be given to very small animals.

To study any effect the injections might have on general growth, all animals were weighed daily from age five days to the time of weaning (twenty-one days) and after weaning were weighed weekly up to the end of the experiment (age twenty weeks).

When the experimental period was completed, the animals were destroyed in a coal gas chamber. The skulls were cleaned by the dermestid beetle method.

For the examination of carious lesions in the rat molars, a dissecting microscope with a magnification of 3OX was used.

For caries evaluation, in the first experiment, the method of Cox, Dodds, Dixon and Matuschak (1939) was used, but it was difficult to maintain accuracy with the 1 to 10 grading scale of Rosebury, Karshan and Foley (1933) employed in this method. In all other experiments, the caries evaluation method of Carr (1954) has been used and has proved reliable yet simple.

Experiment I—The Effect of Manganese.

In this experiment, manganese was administered as a solution of manganese sulphate in normal saline so that the approximate daily dose was 0.15 mgm. of manganese. This was injected intraperitoneally daily from the fifth day to the seventeenth day. Approximately one half of the animals in a litter was treated in this way, and the remaining litter mates acted as controls. They received a daily injection of normal saline. Altogether, four litters were used, giving a final total of fifteen control rats and fourteen experimental rats.

Experiments II, III and IV—The Effect of Boron, Copper, Fluorine, Manganese and Molybdenum.

These three series of experiments were designed to study five elements.

a. Copper as copper nitrate in normal saline.
b. Boron as boric acid in normal saline.
c. Molybdenum as ammonium molybdate in normal saline.
d. Fluorine as sodium fluoride in normal saline.
e. Manganese as manganese chloride in normal saline.

Solutions of the salts of each of these five were mixed so that each was available at three different dosage levels—designated "zero", "low" and "high". It was considered desirable to have the "high" level as high as would be tolerated or at least some several multiples of the low dose. This was mainly to see if there were a gross difference in the effect of the two levels either separately or in their combined effect with some other element. The following dosages were used:

a_0—zero level of copper—normal saline only.
a_1—"low" level of copper—daily amount approximately 0.005 mgms. of copper.
a_2—"high" level of copper—daily amount approximately 0.02 mgms. of copper.
b_0—zero level of boron—normal saline only.
b_1—"low" level of boron—daily amount approximately 0.005 mgms. of boron.
b_2—"high" level of boron—daily amount approximately 0.025 mgms. of boron.
c_0—zero level of molybdenum.
c_1—"low" level of molybdenum—daily amount approximately 0.002 mgms. of molybdenum.
c_2—"high" level of molybdenum—daily amount approximately 0.007 mgms. of molybdenum.
d_0—zero level of fluorine.
d_1—"low" level of fluorine—daily amount approximately 0.054 mgms. of fluorine.
d_2—"high" level of fluorine—daily amount approximately 0.108 mgms. of fluorine.
e_0—zero level of manganese.
e_1—"low" level of manganese—daily amount approximately 0.05 mgms. of manganese.
e_2—"high" level of manganese—daily amount approximately 0.15 mgms. of manganese.

Experiment II followed the same simple experimental plan of Experiment I.

In this experiment, the lower level of each element was mixed to provide a solution of the five—called the "five mineral salt solution." Altogether, fourteen control animals and sixteen experimental animals were used from eleven litters.

TABLE 2

Design of Experiment III.

	Four Mineral Salt Solution_0	Four Mineral Salt Solution_1
Fluoride_0	4 Min_0F_0 (*i.e.* normal saline only)	4 Min_1F_0 (*i.e.* 4 mineral salt mix in normal saline)
Fluoride_1	4 Min_0F_1 (F in normal saline)	4 Min_1F_1 (4 mineral salt mix plus fluoride in normal saline)

Experiment III design—a 2 × 2 factorial set of treatments—is shown in Table 2. In this experiment, the low levels of copper, boron, molybdenum and manganese (called the "four mineral salt solution") were tested for their effect on rat molar caries with and without the addition of the fifth element, fluorine. The design also allowed for the study of the effect of fluoride alone.

The caries production in each of these three could be compared with the fourth litter mate which received the zero level of fluoride and the zero level of the four mineral salt solution. It consequently served as a control.

TABLE 3

Design of Experiment IV.

a_0 b_0	a_1 b_0	a_2 b_0	b_0 c_0	b_1 c_0	b_2 c_0	c_0 d_0	c_1 d_0	c_2 d_0	d_0 e_0	d_1 e_0	d_2 e_0
a_0 b_1	a_1 b_1	a_2 b_1	b_0 c_1	b_1 c_1	b_2 c_1	c_0 d_1	c_1 d_1	c_2 d_1	d_0 e_1	d_1 e_1	d_2 e_1
a_0 c_0	a_1 c_0	a_2 c_0	b_0 d_0	b_1 d_0	b_2 d_0	c_0 e_0	c_1 e_0	c_2 e_0			
a_0 c_1	a_1 c_1	a_2 c_1	b_0 d_1	b_1 d_1	b_2 d_1	c_0 e_1	c_1 e_1	c_2 e_1			
a_0 d_0	a_1 d_0	a_2 d_0	b_0 e_0	b_1 e_0	b_2 e_0						
a_0 d_1	a_1 d_1	a_2 d_1	b_0 e_1	b_1 e_1	b_2 e_1						
a_0 e_0	a_1 e_0	a_2 e_0									
a_0 e_1	a_1 e_1	a_2 e_1									

a = copper

b = boron

c = molybdenum

d = fluorine

e = manganese

0 = zero level

1 = "low" level

2 = "high" level

Each block in the design represents one animal and each of the other three blocks a litter mate. The experiment requires replications of groups of four animals. Altogether eleven litters were used representing forty-four animals.

Experiment IV is a more complicated design and is an attempt to examine the effect of each element separately and combined with another. Table 3 shows the outline of the design. Each of the squares represents one animal and six of these squares, as litter mates, make up a litter or block. In the table, the blocks or litters of six are separated by double lines. Altogether, ten litters are used representing sixty animals. Each animal received a different treatment combination, and the design allows for the study of some elements at three levels and others at two.

Results

Experiment I:

In this experiment, the caries evaluation method of Cox *et al.* (1939) provided three sets of figures called Incidence, Scorings and Total Score. These data are recorded in Table 4.

In the method of Cox, a 1 to 10 grading system is used. The scores obtained are consequently much higher than those obtained by Carr's 1 to 3 grading. Because of this, caries scores for Experiment I are much higher than those for Experiments II, III and IV.

TABLE 4

Results of Experiment I.

The Effect of a Five Mineral Salt Solution on Rat Molar Caries.

	Incidence Before Grinding	Scorings Incidence After Grinding	Caries Score
Mean Value of Control Rats ..	21.1	87.1	250
Mean Value of Experimental Rats	21.7	91.1	304
t (to difference of means) ..	0.92 N.S.	0.59 N.S.	1.8 N.S.

Evaluation by the method of Cox *et al.* (1939).
N.S. = Not Significant.

From the results of Experiment I it is concluded that manganese at the level used (0.15 mgms. daily from fifth to seventeenth day) did not reduce the caries susceptibility of the rat molars.

A study of the caries evaluating method itself was made by comparing the coefficients of variation for the three sets of figures, and from comparison, it would appear that the most satisfactory method is also the simplest one to use—*i.e.*, the "incidence".

The pre-weaning weight records of the rats in this Experiment I showed that at fifteen days of age, the experimental animals were significantly smaller than the control rats (t value = 6 for 25 degrees of freedom).

However, in the four days from the completion of injection (seventeenth day) to the time of weaning (twenty-first day) there was no significant difference between the gain in weight of control rats and experimental rats.

After weaning, too, the progress of experiment rats on D2 and D3 diets was similar to that of the control rats. The weekly weight records have been plotted according to the method of Zucker, Hall, Young and Zucker (1941). Semi-log graph paper is used and the logarithm of the weight is plotted against the reciprocal of time. Reasonably parallel lines for control and experimental animals have been obtained from the post-weaning records in this experiment and the graphs are shown in Figure 1. Growth is almost complete between thirteen and fifteen weeks of age and the curves consequently flatten out at this period.

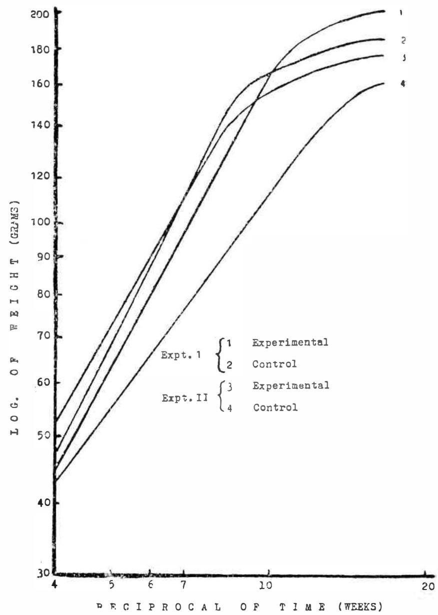

FIGURE I—Post-Weaning Weight Records from Experiments I and II. Control Rat received normal saline; experimental rat (Experiment I) received a solution of manganese sulphate; experimental rat (Experiment II) received a mixture of the salts of all five elements studied. All solutions were injected daily from 5th to 17th day.

Experiment II:

In this experiment, a mixture of the "low" level of each of boron, copper, fluorine, manganese and molybdenum was studied to see if it reduced the incidence of dental caries. The results are shown in Table 5. In the evaluations "caries score" and "incidence after grinding", the effect of the five mineral salt solution

TABLE 5

Results of Experiment II.

Effect of a Five Mineral Salt Solution on Rat Molar Caries.

	Incidence Before Grinding	Incidence After Grinding	Caries Score
Mean Value of Control Rats ..	18.57	14.4	42.7
Mean Value of Experimental Rats..	11.31	10.56	32.0
t (to difference of means)	2.7*	2.2	1.7 N.S.

*Significant at 5% level.
N.S. = Not Significant.

was not significant, but the effect was just significant at the 5% level, in figures obtained with the "incidence before grinding" technique. Additional information is supplied in the results of the next experiment, Experiment III.

Pre-weaning weight records of animals in Experiment II showed no effect on growth by the injection of the "five mineral salt solution." It is consequently not surprising that no difference appears in the post-weaning records. The post-weaning growth curve for experimental rats from Experiment II also appears in Figure 1, and is similar to those of Experiment I.

Experiment III:

A summary of the results obtained with this experiment is contained in Table 6. It can be seen that all treatments, regarded as a whole, were significantly effective at the 1% level in reducing dental caries.

In the breakdown of this treatment effect into its component main effects (also shown in Table 6), fluoride is shown to have exerted a highly significant effect on caries reduction ($P < 0.001$). The "four mineral salt solution" alone (*i.e.* copper, boron, manganese and molybdenum) did not produce a significant reduction, but

TABLE 6

Effect of a Four Mineral Salt Solution, Fluorine, and a Four Mineral Salt Solution plus Fluoride on Rat Molar Caries.

Effect	Sum of Squares	Degrees of Freedom	Mean Square	F	P
Litter	613.00	10	61.3	1.96	N.S.
Treatment	1113.00	3	371.03	11.9	< 0.01
Residual Error	936.5	30	31.22		
Total	2662.5	43			
Fluoride	837.82	1	837.82	27.32	< 0.001
Error	306.68	10	30.67		
4 Min. Salt	29.45	1	29.45	—	N.S.
Error	478.05	10	47.81		
4 Mins. Salt + F	245.82	1	245.82	16.20	0.01 >< 0.001
Error	151.68	10	15.17		
Litters	613.00	10	61.3		
Total	2662.5	43			

N.S. = Not Significant.

the "four mineral salt solution" plus the fluoride solution did have a significant effect. This effect, however, was not as highly significant as fluoride alone— $0.01 > P < 0.001$ compared with $P < 0.001$.

In other words, the addition of the "four mineral salt mixture" of boron, copper, manganese and molybdenum to the fluoride solution reduced the effectiveness of the fluoride.

The pre-weaning weight records of animals in this experiment did not show any significant effect on growth rate by the solutions injected, and the post-weaning weight records (shown in Figure II) exhibit parallel growth curves.

Experiment IV:

A summary of the results obtained with Experiment IV is contained in Table 7.

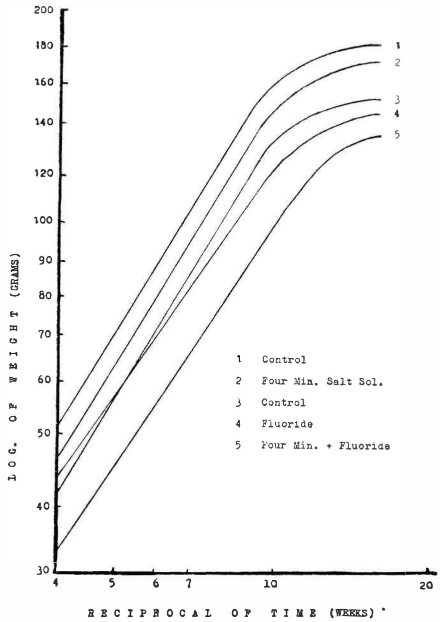

FIGURE II—Post-Weaning Weight Records from Experiment III. Solutions given from 5th to 17th days of life.

In studying the effect of element a, *i.e.* copper, the figures obtained for the incidence of carious areas before grinding and the caries score do not show a significant difference. (Their t values were 2.4 and 2.1 for 4 degrees of freedom.) However, the incidence of lesions after grinding did give a significant effect at the 5% level. Because of this significant result and the size of the other two t values (approaching significance at 5%), copper would seem worthy of further investigation.

TABLE 7

Results of Experiment IV.

The Effect of Five Elements on Rat Molar Caries.

Doses: 0 = zero level; 1 = "low" level; 2 = "high" level.

Element	Differences between Dosages and their Significance			
	Dosages Compared	Incidence Before Grinding	Incidence After Grinding	Caries Score
a copper ..	a_0—a_1	1 N.S.	1 N.S.	8 N.S.
	a_0—a_2	8 N.S.	5 *	11 N.S.
b boron ..	b_0—b_1	10 N.S.	8 *	31 *
	b_0—b_2	11 *	9 *	35 *
c molybdenum	c_0—c_1	4.5 N.S.	8 N.S.	33 N.S.
	c_0—c_2	14 †	13 *	60 *
d fluorine ..	d_0—d_1	9.5 N.S.	5.2 N.S.	17.2 *
e manganese	e_0—e_1	− 0.2 N.S.	− 1.7 N.S.	− 8.7 N.S.

† = significant at 1% level. * = significant at 5% level. N.S. = not significant.

When the results for element b, boron, were analysed, it was found that in all three evaluation techniques, the "high" level dose of boron significantly reduced caries production (at the 5% level). With the "low" dose, a significant effect (at 5% level) was produced in the analysis of figures of the incidence after grinding and the caries score. However, a value which was not significant was obtained when the *t* test was applied to the difference of the means of b_0 and b_1 figures for the incidence before grinding. With this exception, the experiment shows that boron was effective in reducing rat molar caries production.

Interesting results were also obtained for element c, molybdenum. In all three caries evaluations, no significant reduction in caries was produced by the "low" dose of molybdenum. However, the effect of the "high" dose of molybdenum was significant. The differences between c_0 and c_2 were significant at the 5% level for the incidence after grinding and the caries score, and even more significant (at the 1% level) for the incidence before grinding.

The experimental design does not allow for studying elements d and e each at three different dosage levels. For d, fluorine, the high dose, d_2, can only be computed from results combined with e and for this reason, d_2 was not considered. In this experiment, the low level of fluoride only showed a significant effect in caries score figures and an approach to significance in the incidence before grinding. The result obtained with the incidence of lesions after grinding was not significant.

For element e, manganese, only the low level (e_1) can be compared and all three evaluations showed no significant effect. If anything, the general trend would be toward a slightly higher incidence in those animals which received manganese (e_1) than in the controls (e_0).

Inspection of the pre-weaning weight records of Experiment IV animals did not show any gross effects of the solutions injected. The post-weaning weight records of some randomly selected rats in this experiment were plotted and parallel growth curves were obtained (see Figure III).

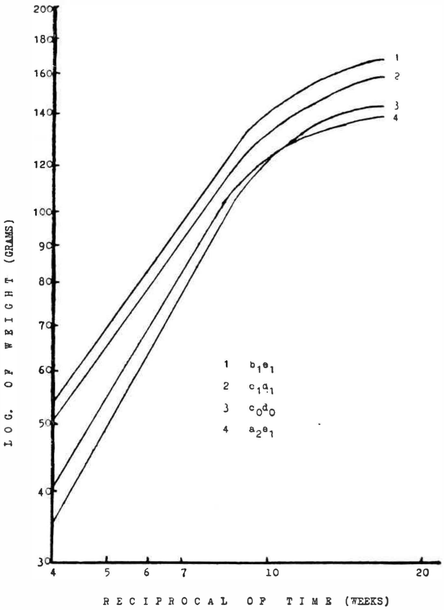

FIGURE III—Post-Weaning Weight Records from Experiment IV.
a = copper; b = boron; c = molybdenum; d = fluorine; e = manganese.
0, 1, 2 = zero, low and high doses, respectively.

Discussion

Stephan and Harris (1953) had previously shown that manganese significantly reduced caries incidence in the rat molar, but the salt was not administered directly to the young. Instead, pregnant rats were fed diets of varying manganese concentrations and the offspring after weaning were maintained on the same rations. Litter mate controls were consequently not used and any difference in caries production was measured by comparing the total number of carious teeth between whole litters.

In the experiments reported here, manganese was not shown to have a significant reducing effect on dental caries. In fact, there seemed to be a tendency of manganese to predispose the rat molars to an increased carious attack. However, a different method of administration was used here, in that the manganese salt was given directly to the young by intraperitoneal injection. It is not possible to assess the average amounts of manganese actually received by the rat pups in the study by Stephan and Harris and so their doses cannot be compared with the dose used in the present experiments. The different experimental procedures used in these studies does not really allow for adequate comparison of the results.

The dose of manganese used in the Experiment I was high enough to significantly affect the rate of growth of the young while receiving manganese salt injections. From this experiment, it would seem that the level of manganese, approaching the maximum tolerated dose did not significantly reduce caries. In Experiment IV, a lower dose (one-third of the dose in Experiment I) did not affect growth, and it too did not have a significant effect on dental caries. Over this range, manganese is ineffective.

The results with sodium fluoride are interesting in that a significant effect has been shown by the daily administration of fluoride for almost the whole period of enamel mineralization. With few exceptions, most other investigations of fluoride administration to rats have been on the caries controlling effect of fluoride given in drinking water *after weaning*. In such reports, high fluoride levels have been used, and any fluoride uptake by the rat molar enamel must have been mainly by surface adsorption, as the molars had erupted.

According to Hein (1955), an increased production of dental caries has been shown to follow the intraperitoneal injection of sodium fluoride and other fluoride compounds. Carr (1954), however, has shown a caries controlling effect of fluoride as sodium fluoride when injected parenterally into rats on the fifteenth day (15th) in one group, and on each of the fifteenth (15th), sixteenth (16th) and seventeenth (17th) days of life in another group.

In the experiments reported here, the injections were carried over a wider period—from the fifth day of life to the seventeenth day of life, as this more adequately covered the total period of amelogenesis and initial enamel maturation.

A less significant effect was shown with fluorine in Experiment IV than in Experiment III, even though the same dose was used in each experiment. The difference in result might depend on the very small number of fluoride treatments available for analysis in the design of Experiment IV. In Experiment III, eleven litters were used (a total of forty-four rats) and the results with sodium fluoride in III are therefore possibly more reliable.

It is interesting to note from the results of Experiment III that the addition of a solution of four other mineral salts to fluoride reduced the effectiveness of the fluoride solution.

All elements in Experiment III were used at the "low" level and one of these, manganese, had been shown not only to reduce caries but to possibly predispose to an increased attack. Of the "low" doses of the other three (boron, copper and molybdenum) only boron was shown by Experiment IV to have a significant effect on caries. Apparently in Experiment III, the significant effect of the "low" level of boron did not counteract the effect of copper and molybdenum, together with that possibly predisposing effect of manganese.

In Experiment IV both boron and molybdenum were shown to exert a significant effect on rat molar caries and are worthy of much more investigation. Copper, too, has shown sufficient effect to merit further study.

From the experiments, it would seem that the effectiveness of any of the elements studied in reducing rat molar caries must largely be due to some contribution during enamel mineralization, because after tooth eruption, all animals were placed on the same dietary regime.

Summary (Part I)

— Five elements as suitable salts in normal saline were administered to albino rats during the period of amelogenesis of the first and second molars. Their possible contribution during enamel mineralization has been determined by the effect on caries production in the rat molars after eruption. The five studied were boron, copper, fluorine, manganese and molybdenum.

— Of these five, boron, molybdenum and fluoride have been shown to significantly reduce rat molar caries. In one caries evaluation, copper was significantly effective, and approached significance in another. Manganese was ineffective in reducing rat molar caries, and, in fact, tended to increase the carious attack.

— A mixture of the salts of boron, copper, manganese and molybdenum (fluorine omitted) did not reduce dental caries.

— A mixture of the salts of the five elements significantly reduced dental caries but was not as significantly effective as sodium fluoride alone.

Part II — A Study of Aluminium, Boron, Fluorine, Iodine and Vanadium.

In Part I of this series some experiments were reported which showed the significant effect that a number of trace elements had on the caries susceptibility of the first and second rat molars. Elements which were shown to be effective were boron, fluorine and molybdenum. Copper was significantly effective in one evaluation procedure and its response in another evaluation indicated that it would be worthy of further study. Manganese did not have a significant effect on rat molar caries, and, in fact, tended to predispose to an increased attack of caries.

Experimental Procedure

The general principle of the experiments reported here in Part II was to study further the possible effect of trace elements on rat molar caries when injected parenterally as suitable salts into young albino rats. The administration period was from the fifth day of life to the seventeenth, as used in Part I experiments. Amelogenesis of the first and second rat molars occurs during this period.

Other general experimental considerations in Part II were also the same as in Part I—diets used, length of experiment, caries evaluation, etc.

In the experiments reported here, a factorial design has been used, and two replications have been studied. These two replications are outlined in Tables 8 and 9, and have been taken from Yates (1937).

Each of the elements was tested at two dosage levels—a "low" level and a "high" level. No zero level was used for any element. It was attempted to have the "high" level approximate the maximum dose which would be tolerated, and to have it several multiples of the "low" dose.

It was hoped that the plan of having two widely separated dosages might show any possible divergent effects. If there were divergencies and if these were sufficiently large, then further experiments could be designed to study particular elements over a greater range of doses.

TABLE 8

First Replication.

Confounding AB, CD, ACE.

Presence of letter = high level.

Absence of letter = low level.

Litter Number	Rat	Rat	Rat	Rat
1	—	abe	cde	abcd
2	e	ab	cd	abcde
3	ce	abc	abde	d
4	c	abce	abd	de
5	ae	acd	b	bcde
6	a	acde	be	bcd
7	ac	ade	bce	bd
8	ace	ad	bc	bde

In the design (Tables 8 and 9) every row in each replication represents one litter, and in all, sixteen litters are required. Each row contains four animals—litter mates.

The order of animal injections is randomised both in columns and rows. The daily injection which every rat receives contains a mixture of one of the levels of each of the five elements (as suitable salts in normal saline).

TABLE 9

Second Replication.

Confounding AC, DE, ABC.
Presence of letter = high level.
Absence of letter = low level.

Litter Number	Rat	Rat	Rat	Rat
9	—	abc	bde	acde
10	b	ac	de	abcde
11	be	ace	abcd	d
12	e	abce	acd	bd
13	ab	ade	c	bcde
14	a	abde	bc	cde
15	ae	abd	bce	cd
16	abe	ad	ce	bcd

The identity of each of the elements studied was as follows:

a = fluorine as sodium fluoride in normal saline.
b = boron as boric acid in normal saline.
c = iodine in normal saline.
d = aluminium as aluminium acetate in normal saline.
e = vanadium as vanadium chloride in normal saline.

In the replication designs (Tables 8 and 9), the presence of a letter represents the high level of that particular element, and the absence of a letter represents the low level, *e.g.*, abe would be a mixture of the salts of elements a, b and e at the "high" level and c and d at the "low" level.

The normal saline solutions of the salts of these elements were prepared so that the following levels were available:

a, the "high" dose of a—daily amount approximately 0.108 mgms. F.
–, the "low" dose of a—daily amount approximately 0.054 mgms. F.
b, the "high" dose of b—daily amount approximately 0.025 mgms. boron.
–, the "low" dose of b—daily amount approximately 0.005 mgms. boron.
c, the "high" dose of c—daily amount approximately 0.005 mgms. iodine.
–, the "low" dose of c—daily amount approximately 0.002 mgms. iodine.
d, the "high" dose of d—daily amount approximately 0.025 mgms. aluminium.
–, the "low" dose of d—daily amount approximately 0.008 mgms. aluminium.
e, the "high" dose of e—daily amount approximately 0.025 mgms. vanadium.
–, the "low" dose of e—daily amount approximately 0.005 mgms. vanadium.

TABLE 10

Results of Series D.
The Effect of Five Elements on Dental Caries Incidence in the Rat
(Incidence Before Grinding).

Source		Sum Squares	d.f.	Mean Sq.	F
"Litters"		604.75	15	40.3	
Main Effects and First Order Inter-actions	a	63.9	15	63.9	6.0‡
	b	162.3		162.3	15.3 †
	c	20.22		20.22	N.S.
	d	12.25		12.25	N.S.
	e	33.00		33.00	N.S.
	*ab	45.12		45.12	4.25‡
	*ac	0.5		0.5	N.S.
	ad	30.2		30.2	N.S.
	ae	0.06		0.06	N.S.
	bc	0.06		0.06	N.S.
	bd	1.56		1.56	N.S.
	be	12.25		12.25	N.S.
	*cd	24.5		24.5	N.S.
	ce	18.03		18.03	N.S.
	*de	0.125		0.125	
Error		350.2	33	10.6	
Total			63		

‡Significant at 5% level. †Significant at 0.1% level. *Confounded in one replication.
a = fluorine; b = boron; c = iodine; d = aluminium; e = vanadium.

TABLE 11

Results of Series D.
The Effect of Five Elements on Dental Caries Incidence in the Rat
(Incidence After Grinding).

Source		Sum Squares	d.f.	Mean Sq.	F
"Litters"		2051.06	15	136.7	
Main Effects and First Order Inter-actions	a	523.3	15	523.3	18.8‡
	b	819.4		819.4	29.5‡
	c	9.77		9.77	N.S.
	d	0.14		0.14	N.S.
	e	221.3		221.3	7.93†
	*ab	10.1		10.1	N.S.
	*ac	34.0		34.0	N.S.
	ad	284.8		284.8	10.21†
	ae	21.4		21.4	N.S.
	bc	78.8		78.8	N.S.
	bd	5.6		5.6	N.S.
	be	23.8		23.8	N.S.
	*cd	28.1		28.1	N.S.
	ce	23.77		23.77	N.S.
	de	306.3		306.3	10.98†
Error			33		
Total			63		

‡Significant at 0.1% level. †Significant at 1% level. *Confounded in one replication.
a = fluorine; b = boron; c = iodine; d = aluminium; e = vanadium.

Results

The analysis of the results for this experiment is summarized in Tables 10 and 11. The caries evaluation method of Carr (1954) was used. However, the caries score was not determined, because no additional information had been supplied by it in earlier experiments.

From the analysis of the results, it can be seen that the rats which received boron during enamel mineralization were resistant to molar caries. The effect of boron was highly significant ($P<0.001$). This highly significant effect was obtained in both evaluation techniques.

In the evaluation of caries incidence after grinding, fluorine as sodium fluoride was highly significant ($P<0.001$), even though the F value was nowhere near as large as was obtained with boron. In the incidence of caries before grinding, fluorine was effective but at the 5% level of significance.

Both iodine and aluminium were ineffective in reducing rat molar caries, but some interesting results were obtained with vanadium. In the evaluation of caries incidence before grinding, vanadium was not significantly effective, but in the figures for the incidence of caries after grinding, the effect of vanadium was significant (at the 1% level).

The analysis showed some interesting first order interactions. A combined effect of fluorine and boron (*i.e.*, ab) seemed to be significant at the 5% level in the incidence of caries before grinding. A similar significant effect was shown by the combination of aluminium and vanadium (*i.e.*, de). The interaction ad (*i.e.*,

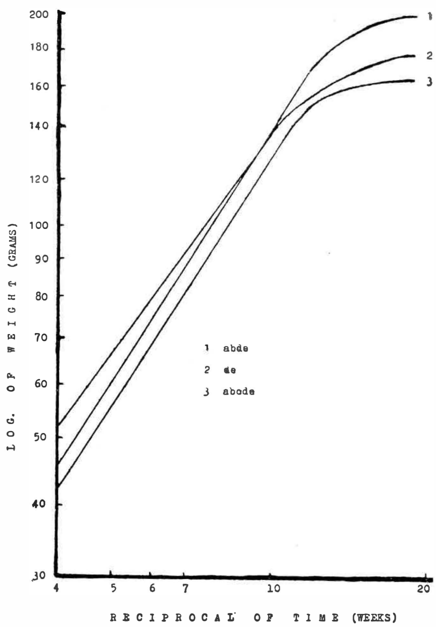

FIGURE IV—Post-Weaning Weight Records. Solutions injected from 5th to 17th day.
a = fluorine; b = boron; c = iodine; d = aluminium; e = vanadium.
abde = a mixture of the high dose of a, b, d and e and the low dose of c.
de = a mixture of the high dose of d and e plus a low dose of a, b and c.
abcde = a mixture of the high dose of a, b, c, d and e.

fluorine and aluminium) also showed a significant effect at the 5% level. No significant effects were determined in this design with interactions of a higher order than a combination of two elements.

Daily weight records of all animals were kept up to twenty-one days of age, at which time the animals were weaned. After weaning, weekly weights were recorded up to the end of the experiment (twenty weeks of age). Inspection of these showed no effect of the solutions injected on the growth of the rats.

The post-weaning weight records of several rats (randomly selected) were plotted according to the method of Zucker, Hall, Young and Zucker (1941), and parallel growth curves were obtained—see Figure IV.

Discussion

Even though boron and fluorine were both highly significant in their effect on reducing rat molar caries, boron tended to be more effective than fluorine in the figures obtained with the caries evaluation used. However, only occlusal caries incidence was considered because smooth surface lesions were not produced by the dietary regime used in this experiment. There is some evidence that a different occlusal crown morphology of the rat molar is produced by boron administration from that produced by fluoride.

The results with vanadium are interesting. They confirm findings by Geyer (1953), even though the designs of the two experiments were different. Geyer (1953) had shown that vanadium as vanadium pentoxide effectively reduced caries in the molars of golden hamsters. His investigations were started after weaning, and consequently, after the major period of enamel mineralization. In all of his experiments, the hamsters were taken over at about thirty days of age, and fed a caries producing diet for twenty-one to twenty-four days. At this age (seven to eight weeks) Geyer could observe the first symptoms of fissure caries in the hamster molars. Three series of controlled experiments were then performed (and repeated several times). In two, vanadium pentoxide was added as a daily supplement to the diet and in the third, the vanadium pentoxide in bidistilled water was subcutaneously injected once a week for six weeks. Geyer observed that the vanadium pentoxide had "an inhibitory effect on the dentinal caries" and that "no new caries of the enamel arose as long as the vanadium salt was given."

In the experiments reported in this article, vanadium chloride in normal saline was used and injected parenterally during the period of amelogenesis of the rat molar. It was not possible to have quantitative spectrographic analyses done on these teeth, and any contribution of the vanadium to the enamel during mineralization was evaluated by the effect on caries production.

Both Geyer's and this present study probably demonstrate a systemic influence and an uptake of vanadium by the tooth. Since the hamsters used by Geyer were seven to eight weeks old, it is probable that the inhibition of enamel caries which he observed is at least partly due to the influence of the environment on fully erupted enamel—vanadium content of the diets and secretion of vanadium in the saliva. Results of work quoted and performed by Fanning, Shaw and Sognnaes (1954) led them to suggest that final enamel maturation, possibly by means of the saliva, occurs in rat molars soon after they erupt.

Significant first order interaction effects were found for fluorine and boron (ab), for fluorine and aluminium (ad), and aluminium and vanadium (de). Of these, the interaction ab was confounded in the first replication and de was confounded in the second replication.

However, in these two cases, the analysis concerned itself with figures taken from that replication in which each particular interaction was not confounded. Consequently the significant effect produced can be assigned to the interaction.

Aluminium, alone, did not show a significant effect, but the interactions aluminium and fluorine (ad) and aluminium and vanadium (de) were effective.

Summary (Part II)

Five elements, as suitable salts in normal saline were administered to albino rats during the period of amelogenesis of the first and second molars. The elements studied were aluminium, boron, fluorine, iodine and vanadium. Their contribution during the period of enamel mineralization was evaluated by the effect on caries production in the rat molars after eruption.

Of the five elements studied, boron, fluorine and vanadium were significantly effective in reducing rat molar caries, and iodine and aluminium were not significantly effective. The most significant effect was shown by boron.

Significant effects were produced by the interactions: boron and fluorine, aluminium and fluorine, and aluminium and vanadium.

Part III — A Study of Boron and Copper.

Parts I and II of this series have shown that a number of trace elements significantly affect caries production in the rat molar, when administered as suitable salts during the period of enamel mineralization. The trace elements studied were aluminium, boron, copper, fluorine, iodine, manganese, molybdenum and vanadium. Of these, boron, copper, fluorine, molybdenum and vanadium were shown to have a significant reducing effect on rat molar caries.

Experimental Procedure

It was decided to study the effect of two of these elements over a wider dosage range. The two studied were boron as boric acid in normal saline and copper as copper nitrate in normal saline. A 4×4 design was used so that four levels of dose of each element could be tested. The design of this factorial experiment was taken from Yates (1937).

In this design, the two elements are designated "a" and "b", and the four levels as a_0, a_1, a_2, a_3 and b_0, b_1, b_2, b_3. These levels were chosen so that a_0 to a_3 and b_0 to b_3 were each in arithmetic progression.

Three replications were to be used and were taken from Yates as:

A′B′	A′B″	A′B‴
A″B″	A″B‴	A″B′
A‴B‴	A‴B′	A‴B″

where

$$A' = a_3 + a_2 - a_2 - a_0$$
$$A'' = a_3 - a_2 - a_1 + a_0$$
$$A''' = a_3 - a_2 + a_1 - a_0$$

$$B' = b_3 + b_2 - b_1 - b_0$$
$$B'' = b_3 - b_2 - b_1 + b_0$$
$$B''' = b_3 - b_2 + b_1 - b_0$$

When set out in the levels to be used of ab combinations, the three replications become:

First Replication

a_3b_3	a_1b_1	a_2b_2	a_0b_0
a_3b_2	a_2b_3	a_1b_0	a_0b_1
a_3b_1	a_2b_0	a_1b_3	a_0b_2
a_3b_0	a_2b_1	a_1b_2	a_0b_3

Second Replication

a_3b_3	a_2b_0	a_1b_2	a_0b_1
a_2b_3	a_3b_0	a_1b_1	a_0b_2
a_1b_3	a_0b_0	a_3b_2	a_2b_1
a_1b_0	a_0b_3	a_3b_1	a_2b_2

Third Replication

a_3b_3	a_2b_1	a_1b_0	a_0b_2
a_3b_1	a_2b_3	a_1b_2	a_0b_0
a_1b_3	a_0b_1	a_3b_0	a_2b_2
a_1b_1	a_0b_3	a_3b_2	a_2b_0

Each row in each replication represents four litter mates from the one litter.

General experimental considerations (method of administering salt solutions, caries producing dietary regime, caries evaluation) were the same as in previous reports.

The dosage levels of boron (a) and copper (b) used in this experiment were as follows:

a_0 — zero level, normal saline only.
a_1 — daily amount approximately 6.5 μg. of boron.
a_2 — daily amount approximately 13 μg. of boron.
a_3 — daily amount approximately 19.5 μg. of boron.

b_0 — zero level, normal saline only.
b_1 — daily amount approximately 20 μg. of copper.
b_2 — daily amount approximately 40 μg. of copper.
b_3 — daily amount approximately 60 μg. of copper.

These levels were all well received from day five, with the exception of b_3. This level of copper nitrate in some rat pups seemed close to the lethal dose, and in some animals the dosage administered had to be reduced during the period from day five to about day nine. By the ninth day, the animals were larger and seemed better able to tolerate this high level. It was decided to continue with the b_0 — b_3 levels listed above, as it was considered desirable to have the b_3 level as close as possible to the lethal dose for age five days. No allowance is made for any possible influence which may occur through the slight alteration in level for the first four days of injection in those few animals which could not tolerate the higher dose.

Results

The results of this experiment are contained in Tables 12 and 13.

From Table 12, it can be seen that neither boron (a) treatments nor copper (b) treatments produced a significant effect.

TABLE 12

Effect of Copper and Boron on Caries in the Rat.

	Sum of Squares	Degrees of Freedom	Mean Square	F
Litters	522.95	11	47.5	
a	133.49	3	44.5	N.S.
b	42.15	3	14.0	N.S.
ab ..	510.45	9	56.72	N.S.
Error ..	576.31	15	34.4	
Total ..	1725.35	41*		

*6 animals missing. N.S. = Not Significant.

TABLE 13

Mean Total Values of the Incidence of Caries for the Four Levels of Boron (a) and Copper (b) Treatments.

	a_0	a_1	a_2	a_3	Totals
b_0	4.4	1.9	−1.0	−0.3	5.0
b_1	−0.7	−1.9	1.0	2.0	0.4
b_2	−4.7	2.9	−2.5	−1.1	−5.4
b_3	2.9	6.0	−7.2	−1.7	0
Totals	1.9	8.9	−9.7	−1.1	

Table 13 gives the total values (mean of three) of the caries incidence for the four levels of each of the treatments "a" and "b". The mean total caries incidence values for boron (the "a" treatments) in increasing doses from the zero level (a_0), show an apparent increase in caries production by the a_1 level. The level a_2 caused a reduction in caries when compared with the level a_1 with the zero level a_0. The level a_3 was certainly not as effective as a_2 and only slightly reduced the caries incidence when compared with a_0.

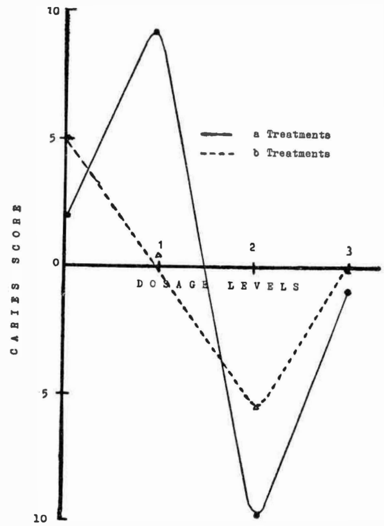

FIGURE V—Effect of the four dosage levels of boron and copper on mean caries incidence in the rat molar.

The mean total caries incidence values for copper (b treatments) in increasing doses from the zero level (b_0) show a reduction in caries with the b_1 level and a still lower incidence with the b_2 level. The highest dose, b_3, reduced caries incidence when compared with b_0 but was no more effective than b_1.

The mean caries incidence values obtained with these two treatments were plotted against the four levels and the curves are seen in Figure V.

Pre-weaning and post-weaning weight records were kept as previously described. Early work showed b_3 to be close to the lethal dose for five day rat pups, but when commenced at nine days, no effect on growth rate was seen. Other levels of copper (b) and all levels of boron (a) did not affect the growth of the rats. The post-weaning weight records parallel one another for all rats in this experiment.

Discussion

In view of the results obtained in Parts I and II, it was expected that boron, at least, would show a significant effect. The results in Table 12 showed no significant effect by boron or copper, but the analysis of variance in Table 12 analyses the effects of each element as a summation of the levels used.

In previous experiments, it had been shown that significant effects had been produced by that level giving a daily dose of approximately 25 μg. of boron. A significant effect and one approaching significance had also been produced with 20 μg. of copper. The dosage levels used were arbitrarily chosen, and in this present experiment they were only selected to range in arithmetic progression. The levels used in the present experiment were approximately 0, 6.5 μg., 13 μg., 19.5 μg. of boron and 0, 20μg., 40μg., and 60μg., of copper.

In studying Table 13 and Figure 5, there is a more distinctive trend in caries reduction for copper (b treatments) than for boron (a treatments). It could be claimed that the levels b_0 and b_3 were both effective in reducing the caries incidence but that b_2, the most effective level was the optimal dose. The figures obtained with b_3, of course, may not be truly representative, because b_3 was close to the minimum lethal dose for five day rat pups. Because of this, the injections of b_3 were commenced at the ninth day instead of the fifth, but the original b_3 level was maintained to complete the experimental design. What effect this change in experimental procedure had is not completely known, but it is possible that the b_3 figures were altered by this change. Probably, a better dosage range would have been 0, 15, 30, 45 μg./day instead of 0, 20, 40, 60 μg./day.

When the results for boron were plotted, a grossly fluctuating curve was obtained, and no optimal level could be ascertained from the data. This fluctuation is suggestive of some experimental error.

The general experimental considerations have been reported in Part I, and in the present experiment only one exception to this procedure occurred. Rats used for the other experiments came from a common stock source, and all breeders had been maintained on a semi-synthetic high sucrose ration before mating. As stated previously, this was to sensitize the offspring to a greater susceptibility to dental caries. For the present experiments, however, insufficient breeders of a suitable age were available from this common stock, and a request was made to obtain breeders from another local laboratory. It was intended to maintain these on a caries sensitizing ration (D1) before mating. However, when the rats were received, all were pregnant and some littered within several days. Nevertheless, the experiment was continued using these offspring.

If this variation in experimental procedure had any effect, it could be expected that the effect on both treatments would be similar. However, a similar effect was not produced, as the results for copper (b treatments) did not exhibit the gross fluctuation that was obtained in the results for boron (a treatments). It may be that the levels of copper (b) used in this experiment were of sufficient magnitude to show an overall effect. The level which had been shown to be effective in previous experiments was the one selected for the b_1 level in this experiment.

On the other hand all the levels in the range used for boron (a) were below that level (25 μg./day) which had been previously used and shown to be effective. None of these may have been sufficiently high to show an effect. Probably it would have been better to have used a different dosage range for boron, *e.g.*, 0, 10, 20, 30 μg./day.

Summary (Part III)

Boron and copper as suitable salts were administered during the period of enamel mineralization of the first and second molars of the albino rat. Four dosage levels of each element were studied.

No significant effect on caries incidence was produced by either of the elements, regarding the dosage range as a whole.

The trends in reduction of caries incidence by the separate levels of each element is discussed, and further investigations suggested.

General Summary

A number of experiments has been conducted to test the hypothesis that some "trace-elements", administered as suitable salts during the period of amelogenesis, may contribute toward a more caries resistant rat molar. This caries resistance was comparatively gauged after a twenty week caries producing dietary regime.

Eight elements were studied as suitable salts, some at four dosage levels, others at three and the remainder at two. These element salts have also been investigated in a variety of interactions between two, three, four and five elements and also at a number of combinations of the various dosage levels of the salts. Those studied were aluminium, boron, copper, fluoride, iodine, manganese, molybdenum and vanadium.

Of these, boron and fluoride have been most effective in reducing the caries attack, and copper, molybdenum, and vanadium have also shown a significant reducing effect. Interesting interactions have been observed between the fluoride solution and a solution of the salts of boron, copper, molybdenum and manganese; and also between the fluoride solution and the boric acid solution, fluoride and aluminium acetate, and aluminium acetate and vanadium chloride.

The effect of a wider dosage range of two of the effective elements, boron and copper, was also studied. However, no significant reducing effect was produced, but the trend in reduction by certain levels, and possible explanations for the total negative effect, are discussed.

Acknowledgments

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