APP, while the control rats were given equivalent amounts of saline. The total crude fatty acids (fatty acids plus unsaponifiable lipid) were then determined (Table 4). In the group given APP, 379 mg. crude fatty acids per 100 g. initial body weight were gained by liver and kidney, while 901 mg. were lost by the other tissues. The difference of 522 mg. had presumably been utilized.

DISCUSSION

It was demonstrated by Barrett, Best & Ridout (1938) that the administration of APP to fasting mice caused rapid mobilization of lipid from the depots with an accumulation of lipid in the liver. Stetten & Salcedo (1944) demonstrated this effect in rats. The results of the present study in mice and rats support and extend these findings.

The glyceride fraction was principally affected in the mobilization of lipid produced by the anterior pituitary stimulation. Glycerides were removed from the depots, were deposited in liver, and were utilized to a greater extent than any other lipid fraction examined. While no attempt is made to explain the mechanism of lipid mobilization, it

seems probable from the evidence available that the rate of mobilization from a tissue may be related to the glyceride content of that tissue.

The liver and, to a much less extent, the kidney were the only structures of those examined whose lipid contents increased during the period of lipid mobilization.

SUMMARY

The mobilization, accumulation in the liver and utilization of lipid in fasting mice injected with an anterior pituitary preparation involves chiefly the glycerides. The amount of lipid transferred from a tissue under these conditions appears to bear a relation to the glyceride content of the lipid of the tissue. The rat appears to respond similarly to treatment with anterior pituitary preparation.

We wish to express our appreciation and thanks to Prof. C. H. Best for his interest and encouragement. We are glad to acknowledge the aid of Dr M. F. Clarkson, who took an active part in the investigation, and of our colleagues Dr Jessie H. Ridout and Dr Jean M. Patterson, who assisted particularly with the phosphorus and cholesterol determinations. Part of the expenses of the investigation have been defrayed by funds supplied by the Banting Research Foundation.

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The Effect of a High-Sucrose Diet on the Calcium and Phosphorus Content of the Enamel and Dentine of Rat Incisors

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So far as is known no investigation has been carried out to determine the effect of a large excess of a major dietary constituent on the process of mineralization. The work reported here was concerned with investigating the effect of a diet containing 67% sucrose on the calcium and phosphorus content and the calcium/phosphorus ratio of rat incisors. In recent years such a diet has been used for the experimental production of dental caries in the white rat, the hamster and the cotton rat (Sognnaes, 1948*a, b*; Shaw, Schweigert, Elvehjem & Phillips, 1944). It is of interest, therefore, to know if teeth produced on

a high-sugar diet differ chemically from those of animals receiving a typical stock laboratory ration, when both diets contain adequate amounts of the known vitamins and necessary minerals. Work in the past appears to have been concerned, in the main, with deficiencies of vitamins and minerals, several factors having been shown to influence the structure and composition of growing teeth. In general, however, dietary deficiencies need to be much more severe to influence the composition of the teeth than similarly to affect bone. For example, diets which cause severe rickets may produce only slight changes

in the teeth (Templin & Steenbock, 1933; Gaunt & Irving, 1939, 1940; Smith & Light, 1945).

Gies & Perlzweig (1916) and Toverud (1923) showed that, in the rat, diets low in calcium produced weak incisors having a reduced mineral content. Prolonged deficiency of vitamin A results in severe changes in the rat incisor (Smith & Lantz, 1933); lack of vitamin D in the same species gives rise to a decreased total ash and calcium content (Karshan & Rosebury, 1933), and the incisor becomes distorted (Boyle & Wesson, 1943). Recently, Sobel & Hanok (1948) have shown in the white rat that variations of the calcium and phosphorus contents of serum lead to small variations in the calcium and phosphorus of the upper incisors.

The incisor teeth of the rat (unlike the molars) grow continuously throughout life and can therefore be used to study the influence of diet upon mineralization. No evidence can be found to suggest that the actual processes of mineralization in incisor teeth differ fundamentally from those in molar teeth. It appears reasonable to assume, therefore, that conditions which affect the incisors would also affect the growing molars during their period of development.

The results reported here show statistically significant differences in the calcium and phosphorus content of the incisors of two groups of animals given a stock diet and a high-sugar diet. A parallel study is being carried out on bone and will be reported later.

EXPERIMENTAL

Animals. Albino rats from our own inbred colony were used. All animals were born to parents receiving a stock diet of Lever rat cubes.

Group 1. Twenty-two animals were weaned at 21 days, maintained on the rat cube diet and killed when 91–95 days old.

Group 2. Twenty-eight animals were weaned at 21 days on to the diet containing 67% sucrose and killed when 91–95 days old.

Diets. The stock diet of Lever cubes is, in the experience of this and other laboratories, nutritionally sound; rats grow and breed well when it is the sole source of food. The diet is made up as follows:

	(%)
Wheat germ	2.8
Skim milk powder	3.27
Dried yeast	3.74
Fine bran	29.26
Broad bran	7.47
Molasses	11.21
Coconut cake meal	16.81
Groundnut cake meal	5.60
Maize	10.28
Fish meal	5.60
Dried blood meal	1.40
Limestone	0.93
Common salt	0.70
Bone flour	0.93

The diet contains 1.41% Ca and 0.80% P. The high-sucrose diet was essentially the same as that used by Sognnaes (1948a) and Shaw *et al.* (1944), except that groundnut oil was used in place of corn oil, and 'Hepamino' (Evans Medical Supplies), a whole-liver preparation, replaced the liver concentrate. Each kg. of diet contained:

	(g.)
Sucrose (granulated)	670
Casein (Glaxo; unextracted)	200
'Hepamino'	40
Groundnut oil	50
Salt mixture (Osborne & Mendel, omitting NaF)	40

The following supplements were added to each kg. of diet:

	(mg.)
Aneurin	5
Riboflavin	5
Pyridoxine	5
Nicotinamide	25
Calcium pantothenate	25
p-Aminobenzoic acid	500
Choline chloride	1000
Calciferol	40 µg.
Carotene	20 mg.
α-Tocopheryl acetate	20
2-Methyl-1:4-naphthoquinone	20

The diet contains 0.87% Ca and 0.50% P.

The diets and water were fed to the animals *ad lib.* The experimental period (10 weeks) was long enough to allow for the complete replacement of the continuously growing incisors; any differences should therefore be noticeable at the end of this period. The animals were weighed twice a week and at the end of the experiment were killed with ether, the heads removed and placed in 96% ethanol.

Treatment of teeth. The upper and lower incisors of each animal were removed from the jaws, stripped of adhering tissues, split into fragments and placed in absolute ethanol overnight. The teeth were then extracted with ether in a Soxhlet apparatus for 6 hr. and dried at 110° for 3 hr. The dry fat-free incisors of each animal were pulverized in a percussion mortar so as to pass a 60-mesh sieve. The powdered teeth were fractionated into their several components by the flotation method of Manley & Hodge (1939). First, pulpal material, tissue debris and cementum were floated off in a bromoform-acetone mixture (sp.gr. 2.06) and the remaining enamel and dentine separated by flotation in a mixture of sp.gr. 2.60. Manley & Hodge (1939) recommend the use of a liquid of sp.gr. 2.70 for separating human enamel and dentine, but the corresponding rat tissues appear to be slightly less dense and in a liquid of sp.gr. 2.70 practically no enamel separates. At sp.gr. 2.65 small particles of pigmented enamel were retained in the dentine fraction; at sp.gr. 2.60 they were absent.

Use of fluorescence excited by ultraviolet light as an indication of the purity of the enamel and dentine samples. When teeth are irradiated with ultraviolet light they fluoresce. The dentine fluoresces intense blue-violet, the enamel a much paler blue and the pigmented labial surface of the enamel not at all. On examining the two fractions any brightly fluorescing granules in the enamel sample indicate the presence of dentine; conversely any dull granules in the dentine show the presence of enamel. When flotation is carried out at sp.gr. 2.60 the separation of the two tissues appears to be complete.

Analytical procedure. Samples of the enamel (10–20 mg.) were dissolved in 5 ml. of 5 N-HCl, the solution made alkaline with NH_3 , re-acidified with acetic acid and made up to 25 ml. The dentine samples (50–100 mg.) were dissolved in 10 ml. 5 N-HCl, treated as for the enamel and made up to 50 ml. There is a residue after treatment with HCl, since enamel contains about 1% and dentine some 20% protein. Any Ca or P which is bound to this protein is not analysed. The analytical figures refer to the acid-soluble Ca and P in enamel and dentine.

Calcium determinations. Suitable samples of the filtered enamel and dentine solutions were analysed by precipitation of the Ca as oxalate in 15 ml. centrifuge tubes. Further operations of centrifuging, washing and titration with 0.01 N-KMnO₄ were all carried out in the centrifuge tube.

Phosphorus determinations. Suitably diluted portions of the filtered enamel and dentine solutions were analysed for phosphorus by the method of Berenblum & Chain (1938) using a Spekker photoelectric absorptiometer.

Both methods gave reproducible results with an accuracy within 2%.

RESULTS

The average growth rates for the two groups of animals were almost identical.

		Females (g./day)	Males (g./day)
Group 1	Stock diet	1.70	2.27
Group 2	Sugar diet	1.75	2.31

The general health of both groups was good, and the diets were readily eaten.

The results for the calcium and phosphorus analyses and the calcium/phosphorus ratios are given in Table 1. The ratios are expressed stoichiometrically.

The percentage of calcium and phosphorus in the dry fat-free enamel and dentine of rat incisors was greater when the animals were maintained on the high-sugar diet. The ratio of calcium/phosphorus was, however, lowered. All these differences are statistically significant, the probability of their

being due to chance is in all cases less than one in twenty and in four of the six cases less than one in a hundred.

DISCUSSION

The ingestion of large amounts of refined carbohydrates and especially of fermentable sugars is considered to be an important factor in the aetiology of dental caries (Jay, 1940). There are three possible ways in which a high-sugar diet may influence this condition. First, it has been shown that an increased intake of sugar increases the acid-producing oral flora (Jay, 1940; Becks, Jensen & Milarr, 1944). Secondly, the composition of the salivary secretions may be changed, although no work seems to have been done on this aspect of the problem. Thirdly, the tooth itself may undergo changes in composition if a high-sugar diet is provided during the period of tooth formation. Recently, Sognnaes (1948*a, b*) has reported that rats and hamsters show increased susceptibility to dental caries when maintained on a diet containing 67% sucrose. They grew and reproduced well on the diet, indicating that it contained adequate amounts of fats, proteins, vitamins, etc. He made the important observation that this susceptibility was enhanced if the high-sugar diet was fed during gestation and lactation, so that the animals were exposed to the effects of the diet during the whole of their pre- and post-natal life. When the diet was continued through three generations there was a further progressive increase in caries susceptibility. It appears, therefore, that a high intake of sucrose exerts an influence other than by its affecting the oral flora; Sognnaes (1948*a*) is of the opinion that the effect may be a systemic one.

The results of our experiments show that the enamel and dentine of the incisor teeth of animals maintained on a purified diet containing 67% sucrose had significantly higher amounts of calcium and phosphorus than the same tissues of animals

Table 1. *The calcium and phosphorus content as percentage of fat-free dry weight of rat incisor teeth on a stock diet and on a high (67%) sucrose diet**

	Ca (%) Mean $\pm$ S.E.M.	P (%) Mean $\pm$ S.E.M.	Ca/P stoichiometric ratio Mean $\pm$ S.E.M.	Ca (%) / P (%) (approx. mean)
Enamel, stock diet	33.17 $\pm$ 0.37 (22)	15.99 $\pm$ 0.15 (22)	1.607 $\pm$ 0.014 (22)	2.07 —
	29.25–35.86	14.42–17.30	1.491–1.740	1.92–2.24
Enamel, sugar diet	35.06 $\pm$ 0.31 (28)	17.27 $\pm$ 0.11 (28)	1.569 $\pm$ 0.012 (28)	2.02 —
	31.53–37.43	15.49–18.12	1.411–1.678	1.82–2.17
Dentine, stock diet	26.00 $\pm$ 0.29 (22)	13.54 $\pm$ 0.16 (22)	1.486 $\pm$ 0.012 (22)	1.92 —
	23.09–28.95	11.96–14.64	1.352–1.590	1.74–2.05
Dentine, sugar diet	26.75 $\pm$ 0.23 (28)	14.57 $\pm$ 0.12 (28)	1.425 $\pm$ 0.014 (28)	1.84 —
	24.80–28.65	13.37–16.65	1.248–1.538	1.61–1.98

* The figures represent the mean $\pm$ standard error of the mean, number of observations (in parentheses) and range

maintained on the stock diet. Whether or not this difference, if it occurred in the molars, would be of sufficient magnitude to have any effect on the incidence of dental caries is not known. If demineralization of tooth substance is a cause of caries (Miller, 1890), increased mineralization would not be any bar to the progress of the disease, in fact the better the calcification of the tooth the more likely would it suffer acid decalcification. Sobel & Hanok (1948) point out that comparatively small changes in the composition may affect tooth solubility, e.g. an increased content of carbonate at the expense of phosphate will result in an increased solubility in acid. It may well be that small changes in the composition of teeth are one of the many factors in the aetiology of dental caries.

The high-sugar diet affected both the enamel and the dentine, and its influence seems to have been twofold; first, the absolute amounts of calcium and phosphorus were raised, and secondly the calcium/phosphorus ratios of both tissues were lowered. This may mean that at the site of mineralization relatively less calcium was precipitated than phosphate, and therefore the composition of the fluid from which precipitation occurred had been modified.

In both groups of incisors the calcium/phosphorus ratios of the enamel were distinctly greater than those for the corresponding dentine. This finding agrees with that of Sobel & Hanok (1948). It has been a widespread belief that the mineral phases of enamel, dentine and bone are identical, but it would appear that for the rat this view must be modified, since the mineral phase of enamel has relatively less phosphate and more calcium than that of dentine. If we assume an apatite formula $3\text{Ca}_5(\text{PO}_4)_3 \cdot \text{CaX}$, in enamel X must consist mainly of radicals other than phosphate, whereas in dentine the proportion as phosphate must be considerable. Alternatively, if the basic apatite structure is the same in both tissues, the dentine must contain more adsorbed phosphate. In the rat the stoichiometric calcium/phosphorus ratio is (except in one or two cases) greater than 1.5 for the enamel and less than 1.5 for the dentine.

Stoichiometric calcium/phosphorus ratios are used throughout this paper, because they give a better understanding of chemical composition than a weight ratio. A stoichiometric ratio of 1.5 is equivalent to a weight ratio of 1.94.

That there is some connexion between carbohydrate metabolism and mineralization is becoming increasingly evident. It has been known for some time that the developing tooth contains glycogen. Creighton (1896) described its occurrence in the rodent incisor, and a recent study by Bevelander & Johnson (1946) described its location in the tooth bud. Glycogen disappears when mineralization begins. Engel (1948) reported the presence of glyco-

protein granules in the cytoplasm of the odontoblasts and ameloblasts in the stages prior to the formation of dentine and enamel. Glock (1940) suggests that the phosphorylation processes involved in the breakdown of glycogen are the first source of phosphate esters which set off the mineralization process; later when the glycogen has disappeared calcification continues by the action of the alkaline phosphatase on the phosphate esters of the blood. Boyle & Wesson (1943) made the interesting observation that the teeth formed on low-calcium diets in the presence of vitamin D were better when the diet was rich in carbohydrate than when it was rich in protein.

Our results show that a diet containing 67% sucrose leads to the formation of incisor teeth having a higher calcium and phosphorus content than those of animals receiving a stock diet.

The hypothesis is therefore advanced that a diet which is adequate in all known respects, and in addition is extremely rich in carbohydrate, may stimulate the mineralization process by maintaining the glycogen in the developing tooth at a maximum level. This hypothesis implies that local factors are more important in mineralization than humoral ones, which is probably true since, as Sobel & Hanok (1948) have observed, if only humoral factors were involved there should be no difference in the calcium/phosphorus ratios of enamel and dentine in the same animal. The present investigation has been concerned only with calcium and phosphorus since they are the two major inorganic components; variation in them will of course be accompanied by variation in certain other components, notably magnesium and carbonate. This will require further study. The analytical results for the enamel and dentine in both groups show considerable individual variation, and it is evident that the composition of the tooth is not rigidly fixed. Any study of tooth composition must receive statistical treatment.

The next stage in the investigation must be to study the effect of reducing the carbohydrate intake by replacing some of the sucrose in the purified diet with protein or fat and noting any significant change in the calcium and phosphorus content.

SUMMARY

1. The enamel and dentine of the incisors of rats maintained on a diet containing 67% sucrose had significantly higher amounts of calcium and phosphorus than the same tissues of animals maintained on a stock diet of Lever cubes.

2. The calcium/phosphorus ratios on the high-sugar diet were significantly lower than on the stock diet.

3. It is confirmed that in the rat the calcium/phosphorus ratio for the enamel is higher than that for the dentine.

4. The hypothesis is advanced that a diet high in sucrose stimulates the processes of mineralization by maintaining a maximum level of glycogen in the developing tooth.

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Chemistry of Tissues

4. METHYLATION STUDIES ON HYALURONIC ACID

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Despite the many investigations on hyaluronic acid there is no precise knowledge of the structural units of which the complex polysaccharide is constituted. Accordingly, our investigation was undertaken in order to apply chromatographic technique, methylation studies, etc. to gain knowledge of hyaluronic acid structure.

Hyaluronic acid is extracted from human umbilical cords with water. Acidification of the extract yields a precipitate or mucin clot containing the hyaluronic acid in conjunction with protein. Most of the various procedures which have been employed for the isolation of hyaluronic acid have differed in the method of removal of this protein.

In early methods of isolation (Meyer & Palmer, 1934) both the cords and the mucin clot obtained therefrom were subjected to rather drastic acid treatment so that products of low viscosity and with low nitrogen and ash contents were usually obtained. Later (Meyer & Palmer, 1936), it was found that the Sevag chloroform-ethyl alcohol method for protein denaturation (Sevag, 1934) could be applied to the dissolved mucin clot from which a hyaluronic acid product could then be precipitated from aqueous

solution by acetic acid. Further treatment with zinc acetate and alkali effectively removed the protein residue. However, this treatment also reduced the viscosity of the product and it was found difficult to remove the zinc completely. It was necessary also to use dilute solutions owing to the danger of precipitating zinc hyaluronate.

Two general methods have been used to redissolve this clot: (a) 10% calcium chloride at pH 8, and (b) enzymic digestion at a more alkaline pH. Use of method (a) gave a product of slightly higher viscosity but in lower yield (Meyer & Chaffee, 1940a). McClean & Hale (1941) removed the protein residue by tryptic digestion followed by precipitation of the trypsin with trichloroacetic acid. Blix & Snellman (1945) extracted dissected cords with water in an atmosphere of nitrogen six or eight times for 12 hr. each. They found it necessary to apply the Sevag process many times before the last traces of protein could be removed and a product of high viscosity obtained.

Most of the procedures aimed at obtaining, on a small scale, a product of the highest possible viscosity. Since our work was completed (1947),