A STUDY OF CARBOHYDRATE LEVELS IN BRITISH AND DUTCH ENAMEL SAMPLES

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I. *Report from the Amsterdam Laboratory (Dr. Egyedi)*

It is about 30 years since Bodecker, reporting to a meeting of the International Association for Dental Research, positively confirmed for the first time, by histological evidence, that enamel contains organic matter. Since then a number of authors have studied the composition of the organic matrix of enamel.

Keratin is known to be the chief protein constituent and attention has been given mainly to this. Our present knowledge of its finer chemical structure (amino acid constituents) is due chiefly to recent studies (Hess, Lee and Neidig, 1953; Stack, 1954; Battistone and Burnett, 1956).

The chief problem in carrying out these investigations is to separate the organic portion from the inorganic portion without altering the structure of the former. Moreover, only a small amount of organic matter is retained after removal of the minerals from the enamel. Another factor is that a choice has to be made as to which part of the enamel is being studied.

Losee and Hess (1949) differentiate between "enamel proper" and "inner enamel", the latter containing the tufts, spindles and dentino-enamel junction. In addition it is essential to separate enamel with great care from the dentine, which contains from 30—40 times as much organic matter as the enamel.

In studying the composition of the proteins of the enamel Stack also attempted to estimate the amounts of carbohydrate associated with them; as a result of analyses with an orcinol reagent, carbohydrate was found to form about 5 % of the total quantity of organic matter. This was reported at the International Dental Congress in London (1952) and subsequently reported in a publication (1954).

At about the same time the presence of glycogen in enamel was being studied in the Biochemistry Laboratory of the Kliniek voor Inwendige Ziekten of the Binnengasthuis, in Amsterdam. This study, in which anthrone was the reagent used, showed that:

(1) Carbohydrate levels were three times as high as those found by S t a c k,
(2) Carbohydrate present in the enamel occurred as the glucose polymer glycogen, which so far had generally been assumed to be absent from the organic matter of the fully developed tooth.

These findings led to correspondence and subsequently to personal contact between Drs. E g y e d i and S t a c k. Later, more extensive studies were undertaken and samples were exchanged. The causes of the differences between results from the two laboratories were thus identified and a more detailed knowledge of the carbohydrate present in the enamel was gained.

The following is an account of these later developments:

1. *Problems of Demineralization*

In starting our investigations in the Biochemistry Laboratory of the Binnengasthuis in Amsterdam we were confronted with the difficulty that so far hardly anything had been published on the subject; all details had therefore to be elaborated. Taking a study of dentine by S t a c k as a basis, we decided to perform the necessary demineralization of the enamel with a ten-fold amount of 3.6 % hydrochloric acid for 36 hours. By this means we obtained sufficient organic matter by centrifugation and washing.

Further studies showed that prolonged demineralization could be avoided. Adequate demineralization was obtained when enamel powder was mixed with 3.6 % HCl for 30 minutes (the mixture being stirred), followed by removal of the supernatant fluid; the sediment was then mixed again with 3.6 % HCl for 10 minutes. The method was simplified further by using the anthrone reaction with enamel powder which had not previously been demineralised. A detailed report on the reagents used and the carbohydrate levels found was published in this Journal (T. v. T., No. 8-9, 1953). We found about 9 % glycogen and about 15% carbohydrate in the organic matter isolated from the enamel.

2. *Distribution of Carbohydrates within the Enamel*

In view of the differences between the carbohydrate levels found by ourselves and those found by S t a c k it was essential to compare enamel samples from Bristol and Amsterdam and to standardize the methods used. Table 3 in the second report shows that the amounts of carbohydrate (hexose) found in the exchanged enamel powder samples, using anthrone reagent, were markedly similar in the two laboratories.

The discrepancies were found to be due to the different distribution of carbohydrate in various parts of the enamel. In his initial studies S t a c k ground off the outermost part of the enamel. This part showed much

higher carbohydrate levels than did the "enamel proper" plus "inner enamel" (L o s e e and H e s s) which he used in his investigations. The enamel powder used in the Amsterdam laboratory consisted of "outer enamel" plus "enamel proper".

This is thought to be the reason for finding carbohydrate levels which were three times as high.

3. *Studies on the Nature of the Carbohydrate Found*

These studies were concerned with the extent to which the alkali-stability of the carbohydrates could be regarded as evidence of the carbohydrate being glycogen.

C l a u d e B e r n a r d was the first to provide conclusive evidence of the presence, chemical structure and physiological significance of glycogen (about 1860). P f l ü g e r contributed materially to a more detailed knowledge of glycogen in the early part of this century. His findings and those of his associates have been recorded in an impressive monograph of 500 pages (P f l ü g e r, 1905).

Since then the principles formulated by this author have withstood criticism. The gist of his argument is as follows: The particularly high resistance of glycogen to boiling with concentrated alkali may be utilized in isolating glycogen from organs. Other carbohydrates, proteins and glycoproteins present in the organs are broken down by boiling with alkali. In the fields of medicine and biochemistry it has since been regarded as an established fact that carbohydrates are glycogens if they are resistant to boiling with alkali and are precipitable by alcohol from the alkaline solution.

Table 1 shows data on the proportions of certain carbohydrates remaining after heating with alkali in a boiling water bath. Sucrose was found to resist destruction with alkali in the same way as glycogen. This is probably due to the particular chemical structure of sucrose (no free COH group and no reduction of Fehling's solution). However, carbohydrates having a structure identical with that of sucrose are not found in human tissues.

It should be pointed out that there are difficulties in colorimetric estimations owing to the high chloride content of the solution neutralized after heating with 40 % Na OH. This detracts from the accuracy of the results.

Comment: The above is important evidence supporting E g y e d i's caries theory, not only in view of the absolute carbohydrate levels found, but to an even greater extent owing to the standardization of the methods employed. This considerably increases the value of evidence on variations in the carbohydrate levels of enamel which may be observed in individuals having different diets. It will also be of vital importance in studies on the carbohydrate levels in the enamel of monkeys having diets containing various amounts of carbohydrates. It is hoped that these studies will be undertaken shortly.

TABLE 1

Percentage Resistance of Carbohydrates Heated with Potassium Hydroxide Solutions

Carbohydrate	Heating Time (hours)	Potassium Hydroxide Concentration 3 %	6 %	20 %	40 %
Glycogen	½	100	100	100	100
	1½	100	100	100	100
Dextrin	½	100	100	100	82*
	1½	100	100	100	82*
Sucrose	½			93	94
	1½			95	85
Maltose	½	2			
Galactose	½	0			
Glucose	½	1			10*
	1½				3*

* Samples heated with sodium hydroxide.

4. *Report from the Bristol Laboratory (Dr. Stack)*

Only a milligram of carbohydrate is likely to be present in the greater part of the enamel from the whole of the human dentition, according to the present studies. This is a very small amount, but significant when compared with the protein content, which is twenty times greater. Although so low, such carbohydrate levels can be determined with sensitive chromogenic reagents in strong sulphuric acid solutions. Two such reagents, *anthrone* and *orcinol*, have been used by many who believe that they are specific for hexoses, whether these are free or combined in polysaccharides or proteins. The two substances do not react with the amino sugars likely to be present in most biological tissues.

Similar results were obtained by both reagents with some enamel samples treated with concentrated potassium hydroxide. Table 2 shows hexose levels in the alkali-stable matter present.

Identity of the Carbohydrate

Glycogen has not been found by histochemical methods in the enamel, dentine and pulp of adult teeth (Wislocki, Singer and Waldo, 1948). It is thought to be present in developing dental tissues (Engel, 1948; Wislocki and Sognnaes, 1950). At least one-eighth of the insoluble part of the organic matrix of enamel was believed by Egyedi (1953) to be glycogen, as shown by chemical analysis. Nikiforuk and Burgess (1956) detected hexoses in hydrolysates of the insoluble matrix. They considered that galactose, mannose, fucose and

other carbohydrate components were present in this protein fraction. The reason for the lack of agreement with the present work is not yet known and it will be of great interest if an alkali-stable polysaccharide is later identified as containing a number of carbohydrate components.

TABLE 2

Hexose Levels in Enamel Shown by Anthrone & Orcinol Reagents

Samples from	Hexose Level (mg.%) Anthrone Method	Orcinol Method
Incisors	16	15
Canines	20	19
Bicuspids . . .	17	19
Molars	11	12

Method: Samples from British teeth (10 of each type, surface removed) heated with 40 % potassium hydroxide for 90 minutes at 100° C and reacted with sulphuric acid solutions of anthrone and orcinol.

The courses of the colour reactions during heating with anthrone or with orcinol were found, during the present study, to be consistent with the presence of glucose. The rates of increase and decrease of colour were different when the anthrone reaction was used after adding mannose or galactose, two other likely carbohydrate components, to an enamel sample having a hexose level much lower than usual. With orcinol tests the ratios of the optical densities when two colour filters were used in tests also showed that glucose was the most likely hexose.

Hexose Levels Found in Enamel

One pooled sample of enamel was prepared from temporary teeth. Outer surfaces were first cleaned and the dentine removed from the inner surface by grinding. This material was tested fifteen times as a "control" which was included in other tests. Direct reactions with orcinol showed a hexose level of 40 milligrams per cent (mg. %). A higher value of 47 mg. % was found when this was treated with alkali in a boiling water bath before reaction with orcinol. It was thought that the treatment of the enamel with hot alkali had modified the enamel so as to allow more complete reaction. A similar value of 48 mg.% was found when this material was dissolved in a small amount of 25% phosphoric acid before analysis.

The importance of these tests, which were made with suitable controls, was that they showed all the hexose of this enamel to be present in a form which was stable to hot alkali; this is a well-known property of glycogen. These findings were repeated with a number of samples of enamel, and with the fractions prepared by removing the minerals.

However, not more than one-third of the total carbohydrate failed to remain in solution when the alkaline extracts were diluted with ten volumes of alcohol. Some of the "anthrone-positive" material did not dissolve in alkali and some of the material soluble in alkali did not precipitate when alcohol was added. Material thought to be glycogen in other biological tissues behaves in the same way (K i t s v. H e i j n i n g e n and K e m p, 1955).

Methods of Enamel Preparation

Samples were prepared in Amsterdam by grinding enamel from caries-free surfaces which had been thoroughly cleaned. Hexose levels were found to be 50—60 mg.% (E g y e d i). Much lower levels of 10—20 mg.% were found in most enamel samples prepared in Bristol. However, the surface had been ground off before crushing dentine-free pieces of enamel (S t a c k). These lower levels were also noted in samples of enamel separated from dentine by treatment with bromoform of specific gravity 2.75, after removing the surface, and crushing. This flotation process has been shown to remove not more than 3 % of the enamel (S t a c k and W i l l i a m s, 1952). In addition to being freed from dentine the enamel is also separated from particles composed of both enamel and dentine. It was shown that treatment of enamel with bromoform and its removal by washing with acetone did not alter the observed hexose content.

Only a small percentage of the total enamel was thus removed as "surface enamel". The hexose level in this was found to be at least thirty times as high as the level in the main part of the enamel. A somewhat higher level than the average was noted at the inner surface, as would be expected since processes extend into the enamel from the dentine. Dentine itself was found to have a hexose content about five times that of the main part of the enamel.

Separation of Enamel Fractions by Acid and Alkali Treatment

Samples of enamel were prepared as noted above (E g y e d i) and the total hexose levels determined in Bristol. Weighed portions of about 100 mg were freed from minerals by stirring with the minimum volume (15 parts) of 10 % hydrochloric acid (by volume). The "filtrate" was separated from the acid-insoluble material, which was then heated for 90 minutes at 100° with 40 % potassium hydroxide. Two further fractions were thus obtained — an alkaline "extract" and an insoluble "residue". These were analysed at the same time as portions of the "filtrate" fraction. Results are shown in Table 3, where they are compared with some on enamel samples prepared in Bristol and analysed in Amsterdam.

It was concluded that nearly one-third of the carbohydrate of these enamel samples was not soluble in hot alkali. Nearly one-half was soluble in cold acid, an one-quarter would not dissolve in cold acid but was soluble in hot alkali. The hexose level of this fraction was not changed by exposure to acid for several hours. Recovery of hexose in these fractions was similar to the total amount of hexose found in the enamel samples.

TABLE 3

Proportions of Total Enamel Hexose in Fractions Separated by Treatment with Acid and Alkali (Anthrone Reagent)

Sample		Total Hexose (mg %)	% in Acid Filtrate	% in Alkali Extract	% in Alkali Residue	% in Three Fractions
1	A	49	48	20½	25½	94
	B	53½	41½	25½	31	98
	C	50	49	25	30	104
2	A	20	41	26½	23½	91
	B	17½	46	23½	33½	103
	C	16	44	28	37	109
3	A	49	46	22	29	97
	B	46	44	24½	33½	102
	C	19	45	23½	31½	100
	D	17	41	27	28	96

Samples: (1) A, B, C and (3) A, B were prepared in Amsterdam from cleaned enamel. They were analysed in Bristol. Those marked (3) A, B were prepared in Bristol from Dutch teeth.

(2) A, B, C, and (3) C, D were prepared in Bristol from cleaned enamel, excluding the outer layer. Those marked (3) C, D were from Dutch teeth analysed in Bristol. Those in the first group were analysed in Amsterdam.

Summary

The authors report methods of analysis which show the amount and nature of the carbohydrate fraction of human enamel. It was noted that: (1) The carbohydrate level was high in enamel near the surface. (2) Carbohydrate formed about 3 % of the organic matrix of the rest of the enamel. (3) This proportion was 6—8 times greater than was found for carbohydrate in the organic matrix of the dentine. (4) Most of the carbohydrate found in dental tissues resembles glycogen in its reactions.

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Samenvatting

Ongeveer 30 jaren geleden heeft Bodecker het reeds voordien bestaande vermoeden, dat het glazuur organische substantie bevat, voor het eerst langs microscopische weg overtuigend aangetoond. Het eiwitbestanddeel ervan (keratine) werd in belangrijke mate bekend door de onderzoekingen van Losee en Hess (1949), Stack (1954) en anderen.

Bij een nadere analyse van de door hem aangetoonde twee eiwitten viel het Stack op, dat deze een aanzienlijke hoeveelheid glucose bevatten. Andere onderzoekers, die sinds 1930 bezig waren naar koolhydraten te zoeken, waren minder gelukkig. De toenmalige bepalingen waren te grof om kleine hoeveelheden aan te tonen. Pas de ontdekking van nieuwe kleurmethoden (anthron- en orcinolmethode) opende de mogelijkheid om tot nauwkeurige bepalingen te komen. Stack deelde zijn bevindingen het eerst mede op het F.D.I.-Congres te Londen in 1952. Hij vond 5 % glucose in de organische substantie van het glazuur.

Omstreeks dezelfde tijd werden er te Amsterdam in het Biochemisch Laboratorium van het Binnengasthuis, op grond van de cariëstheorie van Egyedi

(waarin de aanwezigheid van glycogeen in het glazuur was gepostuleerd) onderzoekingen verricht naar de aanwezigheid van laatstgenoemde stof in het glazuur met behulp van de anthron-methode.

Deze leverden de volgende resultaten op:

1. De gevonden hoeveelheid koolhydraten was driemaal zo groot als de door S t a c k vermelde;
2. De in het glazuur aanwezige koolhydraten werden aangetroffen in de vorm van het glucose-polymeer glycogeen, waarvan tot dusverre algemeen werd aangenomen, dat het in de organische substantie van het volgroeide element niet aanwezig is.

De vondsten, zomede de verschillen hierin, gaven aanleiding tot een uitvoerige briefwisseling en later tot een persoonlijk contact tussen beide laatstgenoemde onderzoekers.

Vervolgens werden de onderzoekingen uitgebreid en glazuurmonsters uitgewisseld. Tengevolge hiervan werd de oorzaak der gevonden verschillen duidelijk en werd de kennis omtrent de aanwezige koolhydraten verdiept.

De reden van de verschillen werd tot in détails opgehelderd, toen bleek, dat de Britse glazuurmonsters van S t a c k, onderzocht in Amsterdam, ongeveer $^1/_3$ van de door E g y e d i gevonden hoeveelheid koolhydraten bevatten, terwijl omgekeerd het Nederlandse tandpoeder, onderzocht in Bristol 3 maal meer koolhydraten opleverde dan de oorspronkelijk door S t a c k gevonden hoeveelheden.

De methode van het winnen van het glazuurpoeder bleek de oorzaak van de verschillen te zijn. Het Amsterdamse laboratorium gebruikte voor de bepalingen de oppervlakte- en middenlaag van van te voren grondig gereinigde tanden, S t a c k daarentegen sleep de oppervlaktelaag af en gebruikte bij zijn bepalingen de middenlaag („enamel proper"). De door S t a c k weggeslepen buitenlaag (voor het ontstaan van cariës vermoedelijk de belangrijkste) bleek nog rijker aan koolhydraten dan de oorspronkelijke getallen van het Biochemisch Laboratorium van het Binnengasthuis te Amsterdam deden vermoeden.

Het derde hoofdstuk van E g y e d i's rapport bevat het verslag van de onderzoekingen, in beide laboratoria verricht, om de structuur van de gevonden koolhydraten te bepalen. De proeven gaven een bevestiging van de oorspronkelijke veronderstelling der auteurs, dat deze substantie glycogeen is, daar zij de typische reacties van het glycogeen vertoonde. S t a c k' s rapport eindigt met een tabel, die enkele resultaten van de koolhydraat-bepalingen van Nederlandse en Engelse glazuurmonsters, uitgevoerd te Bristol en Amsterdam bevat.