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ELECTROPHORESIS OF PROTEINS FROM HUMAN PAROTID SALIVA

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Summary—Polyacrylamide gel electrophoretic patterns of parotid salivary proteins from healthy young adults were obtained with a standard procedure. A uniform pattern showing fourteen anodal bands is found in most of the samples. Repeated studies on the same subjects over a period of more than a year demonstrated a striking constancy of patterns throughout that period.

Amylase, the most prominent fraction, studied from eluates of the original gel, revealed a complex of 6 isoenzymes.

The patterns described are correlated with those described by other authors using polyacrylamide gel electrophoresis.

The importance of the technique as a tool for clinical research and studies of large groups is emphasized.

INTRODUCTION

ZONE electrophoresis, one of the simplest and most direct of analytical methods with a high resolution for the study of complex protein mixtures, has found broad application in biological research. Most of the biological fluids have been investigated by this method and defined patterns have been described for many of them.

However, its application to the study of salivary proteins has not been so successful. The low protein content of saliva, its great heterogeneity and the alteration by inorganic ions of migration rates have been, among others, important sources of difficulty. The concentration and dialysis of saliva samples before electrophoresis have been generally unavoidable steps and it is known that these manipulations may result in alterations of some protein fractions. Thus, a method avoiding such treatments would improve greatly the practicability and constancy of results of electrophoretic studies.

Paper electrophoresis, a technique of limited possibilities, did not provide a clear picture of the complex composition of salivary proteins (KINERSLY and LEITE, 1957; FERGUSON, KRAHN and HILDES, 1958; WEINSTEIN, HESKINS and JACKSON, 1960; HOLEN, HABER and TUCK-WEISS, 1961; MANDEL and ELLISON, 1961; D'SILVA and FERGUSON, 1962; FISCHER, WYSHAK and WEISBERGER, 1962; WEISS and HABER, 1964; MANDEL, 1966; BELLAVÍA, 1967). Similar results were obtained with agar and cellulose acetate (D'SILVA and FERGUSON, 1962).

More recently, the introduction of certain gels as supporting media has notably improved the sensitivity and resolution of zone electrophoresis. The use of starch gel, nevertheless, is not adequate for saliva because of the extreme conditions of pH or

ionic composition of buffers needed to inactivate amylase (HOERMAN, 1959; D'SILVA and FERGUSON, 1962; SHAW, BOZEK and CONNELL, 1962). Polyacrylamide gel, on the other hand, seems particularly suitable for the study of this material. Several reports (CALDWELL and PIGMAN, 1965; GIFFORD and YUKNIS, 1965; MEYER and LAMBERTS, 1965a; STEINER and KELLER, 1968) have demonstrated its value. However, differences in the previous treatments of the sample and in the buffer systems used, as well as confusion in nomenclature, make it difficult to ascertain the relationship between the multiple fractions described, precluding a general application of these results.

Considering the biological and clinical interest of this type of study, it is important to develop a standard technique, simple enough to be performed in general laboratories, which would provide a reproducible pattern for proteins in saliva.

MATERIALS AND METHODS

Collection of saliva

Parotid saliva was collected by means of a Lashley cup, as modified by CURBY (1953), 5 min after starting stimulation with lemon-flavoured candies. Saliva from 40 healthy persons of both sexes, aged between 18 and 25 yr, was obtained 2–3 hr after breakfast. A volume of 3·5 ml was enough for the study. Although the presence of insoluble material was evident in only a few cases, all the samples were centrifuged immediately for 10 min at 3000 rpm and the supernatant used for the analysis. An aliquot was taken for total protein determination.

Total protein determination

This was performed by the method of LOWRY *et al.* (1951) using crystallized bovine serum albumin as standard.

Electrophoresis

The technique of DAVIS (1964) was modified to allow the study of up to 2·5 ml of sample. The glass columns for gels were selected to be exactly 115 mm long and 9.5 mm i.d. Two marks were engraved on the columns, one at 4 mm from the lower end and another at 45 mm from the upper end.

Small-pore gel solution was poured in the tubes up to the upper mark. Spacer gel was then added to a height of exactly 10 mm. Both solutions were prepared according to DAVIS (1964). The sample was applied directly without gel. The volume of saliva inserted was that necessary to provide 2·5 mg of total protein. As protein concentrations ranged between 1·0 and 4·5 mg per ml, the volumes needed to obtain 2·5 mg ranged between 0·55 and 2·5 ml. When the sample was less than 1·5 ml it was diluted with distilled water up to that volume. In most cases that was the "sample size"; only four of the salivas had less than 1·6 mg of protein per ml and required higher volumes.

To prevent mixing with the buffer, two drops of saturated sucrose solution were added.

Tris-glycine buffer, pH 8·4, ionic strength 0·1, was used. A constant current of 5 mA per tube was applied at room temperature until the marking dye (Bromphenol blue) reached the mark at 4 mm from the bottom of the tube, which took about 3 hr.

Staining

Gels were submerged for 1 hr in a 1 per cent Amido Black solution in 7 per cent acetic acid. Decoloration was carried out by rinsing repeatedly in 15 per cent acetic acid for 3 days. Although this method takes considerably longer than electrophoretic destaining, it is preferable because it results in a better quality of patterns.

Localization of amylolytic activity was carried out by the method of WOLF and TAYLOR (1967).

Elution

Once the region of the gel corresponding to amylase was recognized, it was cut out and suspended in an equal volume of distilled water by using a Potter–Elvejhem homogenizer. The suspension was left overnight in a refrigerator at 4°C and cleared afterwards by centrifugation at 25,000 *g* for 1 hr. About 80 per cent of the original activity inserted was recovered.

Amylase activity was determined by the method of BERNFELD (1951).

RESULTS

Electrophoretic patterns

The volume of saliva containing 2·5 mg of total protein was selected because it showed the best results in terms of total number of separated bands. In most cases at least fourteen bands were visible. Figure 1 shows electrophoretic patterns of parotid salivary proteins from eleven different persons and from the pooled saliva of six other subjects. The uniformity of patterns is evident.

Bands have been numbered beginning with the fraction having the highest mobility. There is a group of three closely spaced bands which migrate rapidly (Fig. 2). The first band appears as a faint and narrow area, the second stains grey and is overlapped by a third band with a bright blue colour. About 5 mm behind the fast complex, there is a pair of faint bands, 4 and 5. As Fig. 1 demonstrates, there are individual variations in the relative concentration of these fractions and, in some cases, slight modifications on the mobility of the fifth band.

Band 6 is a prominent fraction; it is a broad and intensely stained area. Immediately behind this dark zone is another fraction, clearly visible after the first rinses, but being washed out progressively by repeated rinsing. Once the destaining procedure was completed, band 7 was barely detectable.

The area behind bands 6 and 7 usually showed two diffuse fractions (8 and 9) but, frequently, a complex of three or more bands could be demonstrated in this region (Fig. 1).

Band 10 is the heaviest and most intensely stained fraction of the pattern. This is a complex of several fractions, as can be demonstrated by subjecting an eluate of this region, or that amount of saliva which contains 1·0 mg of total protein, to electrophoresis for twice as long as usual (6 hr), when it becomes clear that this area is composed of at least five fractions (more usually six) as Fig. 3 shows.

Applying the method of WOLF and TAYLOR (1967) or eluting the gel and assaying for enzyme activity, it was demonstrated that this zone corresponded to the amylase. All the multiple bands showed amylolytic activity and represent multiple molecular forms of the enzyme (isoenzymes) already recognized by other authors (MUUS and VNENCHAK, 1964; WOLF and TAYLOR, 1967; STEINER and KELLER, 1968). We have designated the isoenzymes by Roman numerals, I to VI, beginning with the most rapidly migrating.

Bands 11 and 12 are well defined but band 11 was absent in three cases. Band 13 and 14 appear as clear lines very close to the origin.

Patterns from different subjects were very uniform provided the conditions of stimuli, time of collection, etc., were kept constant. No differences between sexes were evident.

Figure 4 shows electrophoretic patterns of saliva from the same person taken on 6 occasions at weekly intervals. The patterns demonstrated a striking uniformity throughout that period. The same constancy was shown in patterns repeated after 6 and 12 months.

The individual differences usually observed were in the relative intensity of bands and/or absence of any of the minor fractions 4 or 11.

The most striking variation, recorded in two cases, was complete absence of band 6, near absence of bands 2, 3, and 4, and intense staining of band 5 (Fig. 5). The only known departure from the usual procedure in the collection of those salivas had been a stronger stimulation (peppermint tablets). Studies repeated on the same patients with the usual conditions gave a pattern similar to that described as normal.

The same more powerful stimulation did not elicit modifications of protein fractions in 18 other cases.

DISCUSSION

Several years have elapsed since the introduction of polyacrylamide gel electrophoresis. However, clinical research in the field of salivary proteins has not taken full advantage of the possibilities of the method.

Electrophoretic analyses of organic fiuids, particularly if they are to be used in clinical investigations, must avoid procedures which are complicated and liable to lead to variable results. Most of the differences in patterns from parotid salivary proteins described by different authors can be accounted for by the diverse conditions in which saliva has been obtained and by the various manipulations to which the sample has been subjected.

Parotid saliva is the most suitable salivary secretion for analysis because it may be easily obtained in a pure state. Mixed saliva is always contaminated with foreign material.

The method described needs a relatively small volume of saliva, thus reducing the time of collection and the discomfort to the patient. It uses the sample without further manipulations, reducing to a minimum the risk of alterations and artifacts.

For our purposes, only anodal migrating fractions have been studied. Other authors (STEINER and KELLER, 1968) found a number of bands migrating toward the cathode. However, some of the fractions demonstrated in cathodal gels are the same as the slowly migrating bands found in anodal gels and the exact identity of the additional bands is not clear.

The quality and resolution of the patterns obtained are comparable to the best reported by other authors using more complicated techniques. The advantage of staining with Coomassie brilliant blue has been emphasized by some authors (MEYER and LAMBERTS, 1965b; STEINER and KELLER, 1968). We have not used that dye, however, our results with Amido Black have detected as many bands as those mentioned by these authors.

The procedure is a convenient tool for clinical research or systematic studies of large groups. With this objective in mind, it is necessary to define a normal pattern and to stablish a common nomenclature. In an attempt to unify comparable observations, we have studied the correlation between our patterns and those described by GIFFORD and YUKNIS (1965) and by STEINER and KELLER (1968). Table 1 summarizes the correspondence between fractions.

An interesting problem is that of individual variations in electrophoretic patterns. It remains to be determined whether genetic factors are involved. A previous report

TABLE 1. CORRELATION OF ELECTROPHORETIC FRACTIONS FROM NORMAL HUMAN PAROTID SALIVARY PROTEINS DESCRIBED BY DIFFERENT AUTHORS

GIFFORD and YUKNIS (1965)	STEINER and KELLER (1968)	BELLAVÍA (1970)
13	C.1	1
12	C.2	2
11	*	3
10	C.3	4
—	—	5
9	C.4	6
—	New components	7
—	revealed with	8
—	Coomassie B. B.	9
7	B.1	10 I
6	B.2	10 II
5	B.3	10 III
4	B.4	10 IV
—	—	10 V
—	Occasional	11
3	A.1	12
2	A.2	13
1	A.3	14

* The authors make reference to one bright blue band.

by BURGESS *et al.* (1966) on identical twins suggested that possibility. However, great care must be taken to avoid false interpretations. Two "variant" patterns were observed which reverted to normal. The present results have not distinguished between different types of stimulation or other unrecognized factors which might cause differences. If the difference in stimuli was the cause, it must be a response characteristic of the individual because it was not observed in other subjects. In any case, these observations emphasize the need for following scrupulously the standard conditions of the study.

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Résumé—Les motifs électrophorétiques par gel polyacrylamide des protéines salivaires parotides de jeunes adultes sains ont été obtenus par un protocole type. Un motif uniforme montrant quatorze bandes anodales est retrouvé dans la plupart des échantillons. Des études répétées sur les mêmes sujets sur une période de plus d'une année ont démontré une constance frappante dans les motifs pendant toute cette période.

L'amylase, la fraction la plus frappante, étudiée à partir d'éluats du gel original a révélé un complexe de 6 isoenzymes.

Les motifs décrits sont en corrélation avec ceux décrits par d'autres auteurs faisant usage de l'électrophorèse en gel polyacrylamide.

L'importance de la technique comme moyen de recherche clinique et d'études sur de grands groupes est soulignée.

Zusammenfassung—Polyacrylamid-Kolloid elektrophoretische Proben von Ohrspeichelprotein junger, gesunder Erwachsener sind in einem Normalverfahren erhalten worden. Ein gleichförmiges Muster, das vierzehn anodische Bänder zeight, ist in den meisten Proben gefunden worden. Wiederholte Untersuchungen derselben Gegenstände im Laufe von mehr als einem Jahre wiesen eine auffallende Beständigkeit der Muster während dieser Zeitdauer auf.

Der hervorstechendste Bruch, die aus Eluaten des ursprünglichen Kolloids untersuchte Amylase, ergab einen Komplex von 6 Isoenzymen.

Die beschriebenen Muster sind auf die bezogen worden, welche von anderen Verfassern unter Anwendung von Polyacrylamid-Kolloid Elektrophorese beschrieben wurden.

Es wird die Bedeutung der Methode als Mittel für klinische Forschung und für Untersuchungun grosser Gruppen betont.

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PLATES 1 AND 2 OVERLEAF

PLATE 1

FIG. 1. Polyacrylamide gel electrophoretic patterns of parotid saliva proteins from eleven different persons (2–12). One is from a pool of six other salivas mixed in such proportion that each contributed one sixth of the total of 2.5 mg of proteins inserted.

FIG. 2. Schematic representation of a normal gel electrophoretic pattern from human parotid saliva. Numbering begins with the most rapidly migrating band.

FIG. 3. Polyacrylamide gel electrophoretic patterns of proteins from human parotid saliva. (A) Eluate from band 10 was subjected to electrophoresis for 6 hr. (B) A volume of saliva giving 1 mg of total protein was inserted and subjected to electrophoresis for 6 hr. Bands demonstrating amylolytic activity have been numbered from I to VI.

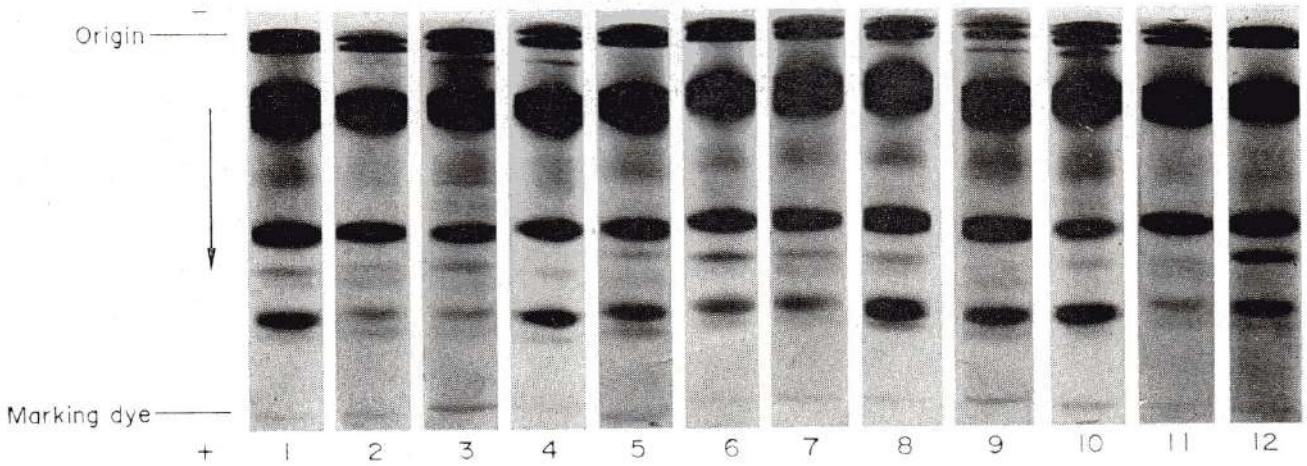

FIG. 1

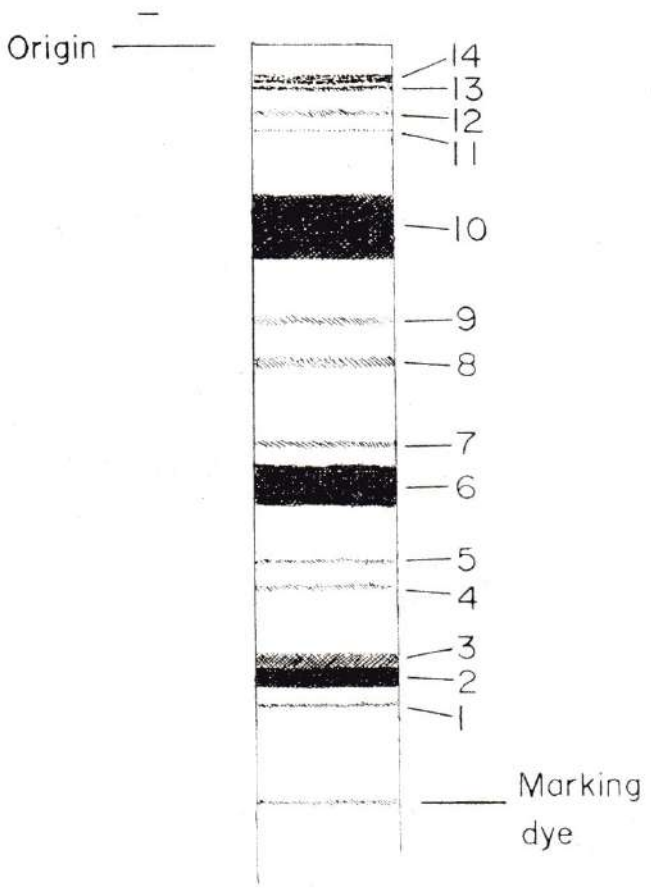

FIG. 2

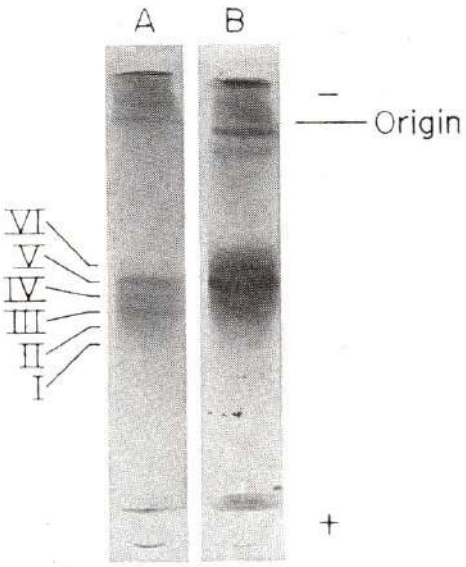

FIG. 3

PLATE 1

A.O.B. f.p. 758