Technical Papers

Demonstration of Hemoglobin-Reactive Substance in Human Serum

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During a previous study (*1*) I found that fetal and adult hemoglobins exhibit electrophoretic mobilities similar to that of the β-globulin fraction of serum when they are analyzed by filter-paper electrophoresis using veronal buffer at *p*H 8.6. The addition of serum of new-born infants to the hemoglobin solutions before electrophoresis did not appear to alter the electrophoretic characteristics of either of the two types of hemoglobin. It was later observed that hemoglobin present in specimens of serums obtained from older children exhibited a mobility more nearly that of α_2-globulin. This observation prompted the studies described here.

Filter-paper electrophoresis was carried out as is previously described (*1*), using Whatman 3-mm filter-paper strips 1 in. wide. After the completed strips were dry, each was cut longitudinally into two ½-in. strips, one of which was stained with bromphenol blue to show the location of the major serum proteins; the other was stained with benzidine to localize hemoglobin or other hematin compounds. Substances giving a positive benzidine reaction were therefore accurately localized with respect to the serum proteins.

Hemoglobin present in the serum of newborn infants as a result of hemolysis during withdrawal of blood and separation of serum migrated at a rate similar to that of β-globulin. The addition of either fetal or adult hemoglobin (identified by the method of Singer *et al., 2*) to serums obtained from newborn infants resulted in an increase in the intensity of the benzidine stain in the β-globulin area on the completed electrophoretic record. In contrast, the electrophoretic mobility of hemoglobin in serum of older children and adults was such that the hemoglobin migrated to a point corresponding to that of the α_2-globulin. Addition of serial dilutions of either adult or fetal hemoglobin to adult serum followed by electrophoresis resulted in a progressive increase in intensity of the benzidine strain in the α_2-globulin area corresponding to the amount of hemoglobin added to the specimen of serum. As the hemoglobin concentration was progressively increased, a point was eventually reached at which no further increase in intensity of the benzidine stain in the α_2-globulin area occurred. At this point a benzidine-positive compound, presumably hemoglobin, began to appear at the β-globulin position; further increase of the concentration of hemoglobin in the specimen resulted in a progressive increase in intensity of the benzidine reaction in this area. Specimens of serum from a limited number of individual children have been "titrated" in this manner and considerable variation was observed in the extent to which the serum was capable of altering the mobility of hemoglobin. Dialysis of serum and hemoglobin solution against the buffer solution prior to electrophoresis did not modify the results.

Serum was obtained from a well child aged 10 yr. The major serum proteins were separated from 0.1 ml of the specimen using a strip of Munktell No. 20 filter paper 4 in. wide. After electrophoresis, while the paper was still wet, one 1-in. strip was cut longitudinally from each side of the paper. These smaller sections were quickly dried and stained with bromphenol blue and then were used as guides for sectioning the remaining 2-in. center strip of paper, so that each section contained one of the major serum proteins. Each of these sections was then placed in a separate tapered centrifuge tube into which had been placed seven or eight small clean glass beads. The tubes were then centrifuged and the liquid in the bottom of each tube, which represented a relatively pure diluted sample of each of the major serum proteins, was removed. Each fraction obtained in this manner was then mixed with a small amount of dilute hemoglobin, after which each protein-hemoglobin mixture was subjected to electrophoresis and stained as is described in the second paragraph. Hemoglobin not mixed with other protein was run simultaneously as a control. The mobility of hemoglobin was not increased by albumin, α_1-, β-, or γ-globulin. Two hemoglobin bands were present in the α_2-globulin-hemoglobin mixture, however; a faint but definite band was observed in the area corresponding to free hemoglobin, but the major part of the hemoglobin migrated at the rate of the α-$_2$-globulin.

These data indicate that the serum of children and adults ordinarily contains a substance (or substances) capable of altering the electrophoretic mobility of hemoglobin, presumably by molecular interaction resulting in complex formation. This substance is absent in the serum of newborn infants, and, according to results of our preliminary observations, it begins to appear during the first few weeks of life.

The activity of this substance does not seem to be influenced by the type of hemoglobin present in the serum, for reactions with both fetal and adult hemoglobins have been demonstrated. The nondialyzable nature of the substance suggests that it is of relatively large molecular size. Electrophoretically, under the experimental conditions employed, it has been identified with α_2-globulin(s).

Qualitative experiments indicate that the concentration of this hemoglobin-reactive substance may undergo alterations in certain diseases; however, the number of determinations carried out to date is not adequate for final interpretation.

It is perhaps significant that the interaction of a

compound that is primarily protein in nature—that is, hemoglobin—with another substance found in the serum results in a profound change in the electrophoretic characteristics of the hemoglobin. This emphasizes the possibility that characteristic alterations in the electrophoretic pattern of serum proteins seen in certain diseases may be the result, at least in part, of complex formation occurring between normal serum proteins and other substances not ordinarily present in the serum.

References and Note

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Hydrolysis of Amylotriose by Crystalline Salivary Amylase

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In an earlier publication (*1*) it was shown that partially purified preparations of salivary amylase were capable of hydrolyzing the glucosidic bonds of amylotriose to yield glucose and maltose as the hydrolytic products. Whelan and Roberts (*2*) did not observe any hydrolytic products in digests of amylotriose (maltotriose) with highly purified salivary amylase, and they concluded that the enzyme was without action on this compound. In our laboratory, crystalline salivary amylase (*3*) has now been shown to effect a hydrolysis of amylotriose and of 1-C^{14}-amylotriose. The results of our studies are presented in this paper (*4*).

Four milligrams of amylotriose were dissolved in 0.05 ml of water and treated with 0.05 ml of a solution of crystalline salivary amylase. The enzyme solution was buffered to *p*H 6.8 with 0.1*M* phosphate and assayed 12 units (*5*) of amylase activity per milliliter. An aliquot of 0.01 ml of the digest was placed on a paper strip immediately after the addition of the enzyme and was heated at 100°C for 5 min to inactivate the enzyme. Subsequent samples were obtained at 6- and 24-hr reaction periods. The reducing sugars in the aliquots were separated in *n*-butyl alcohol-pyridine-water (6:4:3 by volume) solvent and located on the paper with copper sulfate reagent (*6*). A photograph of the chromatogram is reproduced in Fig. 1. Filtered saliva containing 12 units of amylase activity per milliliter was also tested on amylotriose under conditions identical to those of the preceding experiment. The finished chromatogram of this digest was the same as that reproduced in Fig. 1.

In order to determine which bond in the trisaccharide was susceptible to enzyme hydrolysis, 1-C^{14}-amylotriose was subjected to the action of the crystalline amylase. Four milligrams of 1-C^{14}-amylotriose (*7*) dissolved in 0.05 ml of water was treated with 0.05 ml of the solution of crystalline enzyme. Aliquots of the digest were analyzed for reducing sugars by paper chromatography at 0-, 6-, and 24-hr reaction periods. The areas at which reducing sugars appeared on the chromatogram were cut from the paper and counted for radioactivity in a conventional counting apparatus. The radioactivities of the various compounds are recorded in Table 1.

An examination of Fig. 1 shows that, under the conditions of our experiments, amylotriose was rapidly hydrolyzed to glucose and maltose by crystalline salivary amylase. In a 6-hr reaction period, considerable hydrolysis of the trisaccharide had already occurred, while in a 24-hr reaction period more than 75 percent of the trisaccharide was hydrolyzed to glucose and maltose. Comparison of the chromatogram in Fig. 1 with that obtained for the samples of amylotriose treated with crude saliva revealed that, at equivalent concentrations of amylase activity, the crystalline and crude enzyme preparations hydrolyzed amylotriose at the same rate. In addition, neither the crude preparation of saliva nor the crystalline amylase preparation exhibited any maltase activity. On the basis of these findings, it would appear that the hydrolysis of amylotriose is the result of amylase activity and not of the activity of maltase or some yet unidentified enzyme in saliva (*8*). The discrepancy in the results obtained by Whelan and Roberts (*2*) and by us may be due to differences in enzyme concentrations or in some other reaction condition.

The values in Table 1 for the radioactivities of the products from 1-C^{14}-amylotriose show that salivary amylase is capable of hydrolyzing the glucosidic bonds at the reducing and nonreducing end of the amylotriose molecule. Hydrolysis of the bond nearest the reducing end leads to the production of radioactive

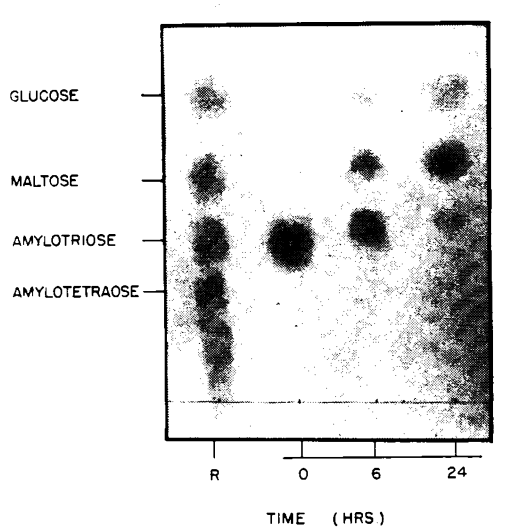

Fig. 1. A triple-ascent paper chromatogram of the digest of amylotriose and crystalline salivary amylase at several stages of enzymolysis: R, reference amylooligosaccharides.