fate groups per hexose in the chondroitin sulfates, or one per repeating unit, as opposed to 1.25 sulfate per hexose in heparin or 2.5 per repeating unit). It is of interest that amylopectin sulfates with as little as 0.57 and 0.17 sulfate per hexose, have distinct inhibitory potency, whereas the chondroitin sulfates with about 0.5 sulfate per hexose are without effect. This difference might be due to interference by either N-acetylhexosamine or carboxyl groups with the inhibitory potency of the polymer. An analogous situation has been encountered in the interaction of various polyanions with human serum beta-lipoproteins(3).

The infrequency with which viral infections of joints, cartilage, bone and other connective tissues have been reported, may be related to a protective effect afforded by the high content of acid mucopolysaccharides present in these tissues. This effect may be due not only to the negative charge which these compounds possess, but also to their capacity to serve as filtering barriers to virus particles. The latter concept is supported by the observations, reviewed by Duran Reynals (10), that subcutaneous injections of hyaluronidase can induce extension of local dermal viral infections to deeper tissue layers. In this regard, the *in vitro* finding(11) that larger sized particles (30 mμ) are delayed in their passage through hyaluronic acid may also be pertinent.

Summary. Several synthetic and biological sulfated polymers were investigated for their inhibitory effect on herpes simplex virus in tissue culture. Of the biological preparations tested, heparin was the only one which inhibited this virus. Nineteen other sulfated polymers were assayed and found to inhibit herpes simplex to varying degrees. The inhibitory potency of the compound depended within limits on the degree of sulfation and on the size of the molecule. The viral inhibitory effect was independent of the polysaccharide portion or branching of the molecule, and of the type of glycosidic or sulfur bond.

1. Nahmias, A., Kibrick, S., *Bact. Proc.*, 1963, 101.
2. Milovanovic, M. V., Enders, J. F., Mitus, A., PROC. SOC. EXP. BIOL. AND MED., 1957, v95, 120.
3. Bernfeld, P., Nisselbaum, J. S., Berkeley, B. J., Hanson, R. W., *J. Biol. Chem.*, 1960, v235, 2852.
4. Schoch, T. S., *Cereal Chem.*, 1941, v18, 121.
5. Meyer, K. H., Brentano, W., Bernfeld, P., *Helv. chim. acta*, 1940, v23, 845.
6. Mandel, B., *Virology*, 1957, v3, 444.
7. Takemoto, K., Liebhaber, H., *ibid.*, 1962, v17, 499.
8. Feltz, E. T., Regelson, W., *Nature*, 1962, v196, 642.
9. Liebhaber, H., Takemoto, K. K., *Virology*, 1963, v20, 559.
10. Duran Reynals, F., *Bact. Rev.*, 1942, v6, 197.
11. Laurent, T. C., Pietruszkiewicz, A., *Biochim. Biophys. Acta*, 1961, v49, 258.

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Characterization of Serum Amylase Activity in Normal and Pancreatectomized Dogs. (29099)

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Isoenzymes of amylase have been reported in sera of various experimental animals as well as man(1-7). These variant forms of amylase presumably are elaborated more or less specifically by certain tissues, but their exact sites of origin are poorly delineated. In the hope of extending available information on these points, certain properties and enzyme kinetics of electrophoretic and chromatographic fractions of serum amylase were investigated in normal and pancreatectomized dogs.

Materials and methods. Blood was obtained from 9 normal dogs and from 6 dogs

pancreatectomized 9 or more months previously.* Aliquots of 50 μl of serum were placed on duplicate filter paper strips and separated electrophoretically(3). Following electrophoresis, one strip of each pair was stained for protein with bromphenol blue. The duplicate strip was placed on a starch-agar gel block at 37°C for 24 hours. The strip was then removed and the gel surface was flooded with iodine solution(8). Areas of starch hydrolysis were identified as colorless zones against a blue background.

Electrophoretic separation of 850 μl of serum was accomplished using a paper strip, 28.5 cm in width. Following electrophoresis, a section measuring 1.5 cm in width was cut lengthwise from the strip and stained with bromphenol blue. The remainder of the strip was cut into 0.5 cm segments, each of which was eluted with 6.7 ml of 0.9% NaCl solution. Amylase activity of the eluates was measured saccharogenically using dinitrosalicylic acid(9).

For chromatographic fractionation, a column (40 × 4 cm) of Sephadex G-75† (10) was equilibrated with 0.01 M NaCl and Tris buffer (pH 8.6, 0.005 M). A 3-ml aliquot of serum was placed on the column and eluted with NaCl-Tris buffer solution at a flow rate of approximately 40 ml/hour. The effluent was collected in 10-ml aliquots which were analyzed individually for amylase activity and protein concentration(11).

Pooled eluates from electrophoretic strips and pooled effluent collections were also dialyzed for 24 hours against distilled water. The dialysates were then lyophilized and reconstituted in appropriate amounts of distilled water. The optimum pH for starch-hydrolysis of the concentrates was determined saccharogenically(9) over a pH range of 4.0 to 8.6, using Lintner soluble starch dissolved in 0.05 M phosphate buffer and 0.9% NaCl. Michaelis-Menten constants (Km) were also measured at 37°C using the same substrate buffered at pH 6.9.

Results. Electrophoretic studies. The average amylase activity of the unfractionated serum of normal dogs was 119.3 mg maltose/ml/hour (range 74.4-213.0), whereas a mean of 69.8 mg maltose/ml/hour (range 46.4-97.3) obtained in pancreatectomized animals. For both types of animals, the total recoveries of amylase activity in electrophoretic fractions were less than 70% of that in whole dog serum. Protein recoveries from the same fractions averaged slightly more than 100% of that in unfractionated serum.

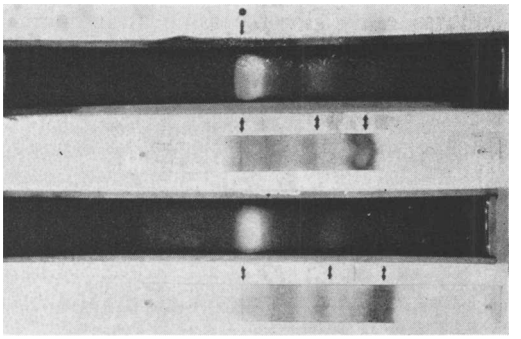

FIG. 1. Electrophoretic patterns of amylolytic activity on starch-agar gel from normal (top) and pancreatectomized (bottom) dog serum.

Sera from normal dogs showed a major hydrolytic area on starch-agar gel which corresponded to the electrophoretic position of gamma-globulin (Fig. 1). Additional zones exhibiting lesser degrees of hydrolysis were observed at positions corresponding to the beta-globulin and albumin areas. Sera from the pancreatectomized dogs yielded patterns that were similar to those of normal dog serum, except for a visible reduction in hydrolytic activity in the gamma-globulin electrophoretic position.

The electrophoretic distribution of amylase activity was determined quantitatively using sera from 5 normal and 5 pancreatectomized dogs. In normal dog sera, the highest saccharogenic activity was routinely extracted from segments containing the gamma-globulin fraction (Fig. 2). Smaller amounts of amylase activity were found in eluates from the beta-globulin area. Much less saccharogenic activity was observed in material isolated from the albumin zone.

The electrophoretic distribution of saccharogenic activity in the beta-globulin and albumin zones of pancreatectomized dog se-

* Generously donated by Dr. Piero P. Foà, Sinai Hospital of Detroit.

† Pharmacia Fine Chemicals.

rum was essentially the same as in normal dog serum. However, pancreatectomized dog serum showed a distinct decrease in amylolytic activity at the electrophoretic gamma-globulin position (Fig. 2). In normal dog sera, the average saccharogenic response associated with the gamma-globulin zone equaled 37.7 mg maltose/ml/hour (range 23.4-79.9) as compared with a mean of only 18.4 mg maltose/ml/hour (range 11.1-26.5) in sera from pancreatectomized animals.

Chromatographic studies. When sera from 5 normal and 5 pancreatectomized dogs were chromatographed on Sephadex G-75, an average of 97.1% (range 80.3-108%) of the enzymatic activity present in whole serum was recovered in the fractions. Protein recoveries in these chromatographic fractions averaged 95.8% of that of whole serum.

Normal serum exhibited 2 well-defined peaks of amylase activity (Fig. 3). The first peak (Peak 1) contained a sizable amount of protein; the second peak (Peak 2) contained only trace amounts of protein. Sam-

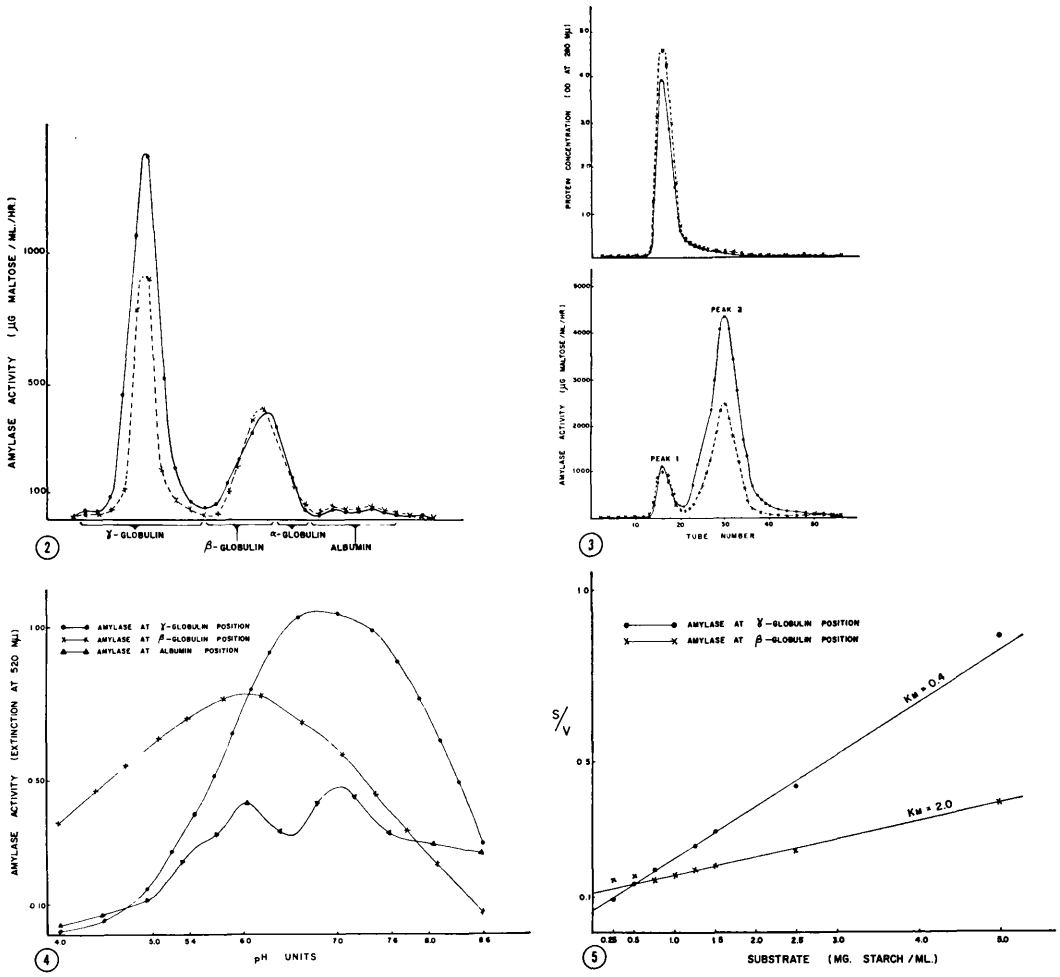

FIG. 2. Electrophoretic distribution of saccharogenic activity of normal (●———●) and pancreatectomized (×– – –×) dog serum.

FIG. 3. Chromatographic distribution of protein (top) and saccharogenic activity (bottom) of normal (●———●) and pancreatectomized (×– – –×) dog serum.

FIG. 4. Effect of pH on saccharogenic activity of 3 electrophoretic fractions isolated from paper strips corresponding to gamma-globulin, beta-globulin and albumin positions.

FIG. 5. Relationship between substrate concentration and enzyme reaction speed used to establish Km values for 2 electrophoretic fractions isolated from paper strips corresponding to gamma- and beta-globulin positions.

ples of concentrated material corresponding to each peak were subjected to paper electrophoresis. Peak 1 concentrate demonstrated all the electrophoretic protein fractions of whole dog serum. The minute quantity of protein in the Peak 2 concentrate also exhibited mobilities corresponding to all fractions. When unstained electrophoretic paper strips prepared from the peak concentrates were incubated on starch-agar gel, Peak 1 concentrates showed zones of starch hydrolysis at the beta-globulin and albumin positions, whereas Peak 2 concentrates hydrolyzed starch solely in the gamma-globulin area.

Peak 1 effluents from normal and pancreatectomized dog sera exhibited similar amounts of amylase activity. However, the saccharogenic activity of Peak 2 effluents was less in the sera of pancreatectomized dogs than in normal dogs (Fig. 3). On the average, Peak 2 effluents from normal dog sera released 99.7 mg maltose/ml/hour (range 56.0-194.3) as compared with a mean of 53.4 mg maltose/ml/hour (range 37.5-68.8) from pancreatectomized dog sera.

Optimum pH and Km. Optimum pH for starch hydrolysis was determined using concentrated eluates pooled from the gamma-globulin, beta-globulin and albumin areas on electrophoretic strips prepared from normal and pancreatectomized dog serum. Material isolated from the gamma-globulin and albumin zones of both types of animals showed optimum pH values for starch hydrolysis which were virtually identical, the values ranging from 6.8 to 7.1 (Fig. 4). The optimum pH for material isolated from the beta-globulin zone ranged from 5.9 to 6.2.

Km values were also established for concentrated electrophoretic eluates. Enzymatic material isolated from the gamma-globulin areas of both types of dog sera yielded an average Km of 0.4 mg/ml starch, whereas a mean Km of 2.0 was obtained for beta-globulin zone extracts (Fig. 5). Protein interference prevented determination of the Km for albumin eluates.

Measurements of optimum pH and Km for starch hydrolysis were also performed on material fractionated chromatographically. Peak 2 concentrates separated from normal and pancreatectomized dog sera exhibited the same pH optimum and Km as material associated with the gamma-globulin electrophoretic zone. These kinetics could not be reliably assessed in Peak 1 concentrates which appeared to contain an enzymatic mixture.

Discussion. The electrophoretic distribution of amylase activity in normal dog serum differs from that observed in normal mouse(1), rat(2), rabbit(3), and human sera(5,6,8). At least 3 distinct zones of activity can be detected, corresponding to the electrophoretic positions of serum gamma-globulin, beta-globulin, and albumin. Of special interest is the fact that the amount of amylolytic activity in the area corresponding to serum albumin was always much lower when measured in paper strip eluates than was visibly indicated by starch-agar gel patterns. Such differences between starch-iodine reactions and saccharogenic responses have also been noted in electrophoretic studies of normal rabbit serum (3).

The total recovery of amylase activity from the electrophoretic strips in no case exceeded that of unfractionated dog sera. Similar results have been obtained following electrophoresis of rat and rabbit sera(2,3). The chromatographic separation of dog serum amylase yielded recoveries which in 3 cases were only slightly greater than the activities measured in the same serum prior to fractionation. In normal human sera, other workers(5,6) report electrophoretic recoveries of amylase activity which totaled much more than that of whole sera. The greater activity in the summed fractions of these samples has been attributed to the presence of an amylase inhibitor which is only active in unfractionated serum(5).

The amylolytic activities of whole serum and the fraction isolated from the electrophoretic gamma-globulin zone were reduced by more than 40% in pancreatectomized dogs. A similar diminution in amylase activity of pancreatectomized dog serum was also consistently confined to a specific chromatographic fraction (Peak 2). This fraction contained amylase with an electrophoretic mobility similar to that of serum gamma-globu-

lin. It may be inferred from these data that, in the dog, the amylase activity normally located in the gamma-globulin area originates at least partially from the pancreas. It is noteworthy in this respect that the starch hydrolytic activity of recrystallized hog pancreatic amylase, when subjected to paper electrophoresis, likewise exhibits a mobility similar to that of serum gamma-globulin(3).

The optimum pH for starch hydrolysis of material extracted from the beta-globulin electrophoretic position differed from that isolated from the gamma-globulin zone. Km values for beta-globulin and gamma-globulin zone extracts were also dissimilar. These differences in enzyme kinetics may be construed as suggesting that amylases localized in the beta- and gamma-globulin zones represent different molecular forms of the enzyme. The only enzyme property that could be established for material extracted from the albumin area was that of optimum pH for starch hydrolysis. This value was similar to that obtained from gamma-globulin zone extracts but differed from that obtained with beta-globulin zone materials.

The presence of serum protein, as well as other substances, may modify optimum pH and Km values for starch hydrolysis(12,13). It appears significant, therefore, that optimum pH and Km values of the Peak 2 chromatographic fraction, which contained minute amounts of protein, and that isolated from the gamma-globulin area on electrophoretic strips, were essentially the same. Hence, the gamma-globulin present in electrophoretic eluates does not appear to alter detectably the enzyme kinetics of amylase studied.

The optimum pH and Km values of amylases in corresponding electrophoretic zones were indistinguishable in normal and pancreatectomized dogs. Perhaps, some pancreatic and extra-pancreatic amylases possess similar physiochemical properties even though available data indicate that pancreatic and extra-pancreatic amylase differ immunologically(7). On the other hand, pancreatic and salivary amylase appear to be immunologically similar(7).

Summary. Three amylolytic fractions with different mobilities were distinguished in normal dog serum by paper electrophoresis and 2 distinct peaks of activity were demonstrated by Sephadex chromatography. The amylase activity located in the beta-globulin position yielded optimum pH and Km values for starch-hydrolysis different from those in the gamma-globulin zone. The optimum pH of the beta-globulin zone material also differed from that of material obtained from the albumin zone. These dissimilarities suggest, but do not conclusively prove, that at least the amylolytic activities residing in the beta- and gamma-globulin areas stem from molecularly different amylases. Based on reductions in activity following pancreatectomy, it would appear that the saccharogenic and amylolytic activity in the gamma-globulin electrophoretic zone of dog serum arises in part from the pancreas.

1. Delcourt, A., Delcourt, R., *C. R. Soc. Biol.,* 1953, v147, 1104.

2. McGeachin, R. L., Potter, B. A., *Nature,* 1961, v189, 751.

3. Berk, J. E., Kawaguchi, M., Zeineh, R., Ujihira, I., Searcy, R. L., *Nature,* 1963, v200, 572.

4. ———, *Science,* 1963, v141, 1182.

5. McGeachin, R. L., Lewis, J. P., *J. Biol. Chem.,* 1959, v234, 795.

6. Dreiling, D. A., Janowitz, H. D., Josephberg, L. J., *Ann. Intern. Med.,* 1963, v58, 235.

7. McGeachin, R. L., Reynolds, J. M., *J. Biol. Chem.,* 1959, v234, 1456.

8. Baker, R. W. R., Pellegrino, C., *Scand. J. Clin. and Lab. Invest.,* 1954, v6, 94.

9. Noelting, G., Bernfeld, P., in *Methods in Enzymology,* edit. by Colowick, S. P., and Kaplan, N. O., (Academic Press, New York), 1955, v1, 149.

10. Blandamer, A. H., Beechey, R. B., *Nature,* 1963, v197, 591.

11. Kalckar, H. M., *J. Biol. Chem.,* 1947, v167, 461.

12. Whitaker, J. R., Tappel, A. L., Wormser, E., *Biochim. Biophys. Acta,* 1962, v62, 300.

13. Whitaker, J. R., Tappel, A. L., *ibid.,* 1962, v62, 310.

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