(1968).
6. Gregory, R. A. and Tracy, H. J., Gut 5, 103 (1964).
7. Wormsley, K. G., Mahoney, M. P., and Ng, M., Lancet 1, 993 (1966).
8. Johnson, L. P., Brown, J. C., and Magee, D. F., Gut 7, 52 (1966).
9. Dinoso, V., Chey, W. Y., and Lorber, S. H., Clin. Res. 14, 295 (1966).
10. Stunkard, A. J., Van Itallie, T. B., and Reis, B. B., Proc. Soc. Exptl. Biol. Med. 89, 258 (1955).
11. Dotevall, G. and Kock, N. G., Gastroenterology 45, 364 (1963).
12. Fishlock, D. J. and Parks, A. G., Brit. Med. J. 2, 666 (1963).
13. Gaddum, J. H., Brit. J. Pharmacol. 8, 321 (1953).
14. Wilcoxon, F. and Wilcos, R. A., "Some Rapid Approximate Statistical Procedures," Lederle Laboratories, New York, (1964).
15. Bodanszky, M., Ondetti, M. A., Levine, S. D., Narayanan, V. L., Von Saltza, M., Sheehan, J. T., Williams, N. J., and Sabo, E. F., Chem. Ind. 42, 1757 (1966).
16. Beacham, J., Bentley, P. H., Gregory, R. A., Kenner, G. W., MacLeod, J. K., and Sheppard, R. C., Nature 209, 585 (1966).
17. Gleason, P. G., N. Y. State J. Med. 65, 252 (1965).
18. Danhof, I. E., Physiologist 9, 164 (1966).
19. Pallin, B. and Skoglund, S., Acta Physiol. Scand. 60, 358 (1964).
20. Ågren, G., Skand. Arch. Physiol. 81, 234 (1939).
21. Vagne, M. and Grossman, M. I., Am. J. Physiol. 215, 881 (1968).

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Macroamylasemia: Observations on the Nature of the Macroamylase* (33827)

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Evidence has been advanced indicating that prolonged elevation of serum amylase activity may result from the existence in the serum of a macromolecule of amylase that is too large to be excreted by the kidneys (1–4). Certain observations made on the nature of this large amylase structure constitute the basis of the present report.

Materials and Methods. Serum samples were obtained from 12 patients with so-called "macroamylasemia." The existence of a macromolecular serum amylase component was established in each case by dextran gel (Sephadex G 200[1]) filtration as previously described (2). This was confirmed in one case (Case 5) by filtration through a column of polyacrilamide gel (Bio-Gel P-300[2]). Additional verification was obtained in 2 cases (Cases 1 and 8) by sucrose density-gradient ultracentrifugation using essentially the same method as that employed by Levitt and Cooperband (3) except that the buffer was 0.1 *M* Tris at pH 7.4 instead of 0.01 *M* phosphate at pH 7.0. The sedimentation coefficient of the large amylase component in each case was roughly estimated from its Sephadex G200 elution position as compared with the elution positions of the major protein fractions (5).

To test the effect of dissociation of possible amylase–protein complexes on the amylase elution pattern (6), urea was added to serum specimens from each of the 12 patients to a final concentration of 6 *M*. The urea–serum mixtures were allowed to stand at room temperature for 30 min and were then applied to a dextran gel column prepared by exposure to 6 *M* urea in 0.85% sodium chloride. The same urea–saline solution was used as the eluant.

The Michaelis constant for starch using

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[1] Sephadex G 200, Pharmacia Fine Chemicals, Inc., Piscataway, N. J.

[2] Bio-Gel P-300, Bio-Rad Laboratories, Richmond, Calif.

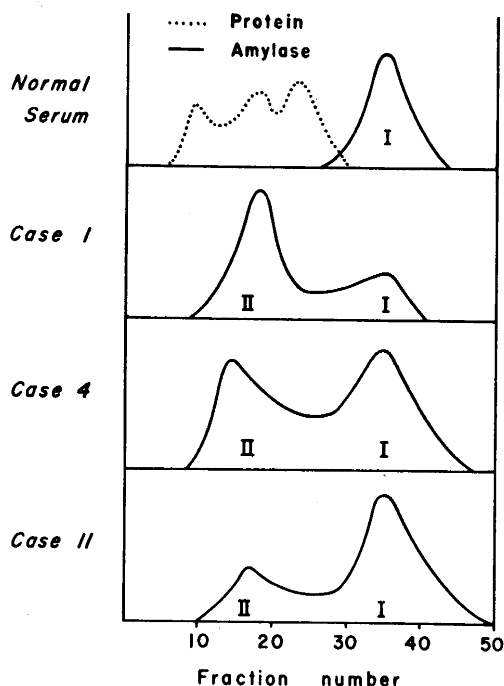

FIG. 1. Dextran gel (Sephadex G 200) elution patterns of normal and representative macroamylasemic sera.

Lineweaver–Burk methods (7), the pH for maximum activity, and the sodium chloride molarity for maximum activity were determined for both the Type I amylase eluted from normal human serum by dextran gel filtration, and the Type II amylases eluted in the same manner from the macroamylasemic sera. Identical determinations were also made after treating each sample with urea.

The possible influence of chelation was assessed in Cases 1, 2, 3, and 4 by comparing the dextran gel filtration patterns of the untreated serum with the corresponding patterns obtained after incubating 3.0 ml of the serum with 450 mg of ethylenediaminetetraacetate (EDTA) at 37°C for 24 hr.

The importance of disulfide bonds was evaluated by testing the influence of 2-mercaptoethanol on the sera of Cases 1, 2, and 3 by a modification of the method of Deutsch and Morton (8). Serum samples, measuring 1.5 ml each, were mixed with 10 ml of 0.02 *M* potassium phosphate buffer at pH 7.4 and 0.1 ml of 2-mercaptoethanol. The final concentration of 2-mercaptoethanol in this mixture was 0.13 *M*. After standing at room temperature for 48 hr, each mixture was dialyzed against distilled water for 48 hr and then reconstituted with 3.0 ml of water. This material was then applied to the dextran gel column and eluted with 0.85% sodium chloride.

Attempts were made to locate a protein in macroamylasemic serum which would combine with amylase. Serum samples from Cases 2, 4, 5, and 10 were filtered through dextran gel after exposure to urea. The amylase-free protein fractions recovered from the column were treated variously as follows: in Case 4 normal serum amylase was added; in Cases 5 and 10 these protein fractions were combined with the fractions containing amylase; in Cases 2 and 10 whole normal serum was added. After standing at room temperature for 1 hr, the mixtures were dialyzed against distilled water for 12–16 hr and then applied to the dextran gel column.

In one case (Case 2) the effect of lowering pH was examined by employing 0.2 *M* glycine–hydrochloric acid buffer at pH 3.4 as the chromatographic solvent.

Results. The relative amounts of the two principal serum amylase components (Type I, the form corresponding in elution position to normal serum amylase, and Type II, the larger molecule that eluted with the serum proteins) showed considerable variation (Fig. 1). In one case (Case 5) almost all of the discernible amylase was Type II; in 9 cases (Case 1–3, 6–10, 12) Type II was the dominant component; in one case (Case 4) the two types were about equal in amount; and in one case (Case 11) Type I was by far the major component.

Another variation of note was in the Sephadex G 200 elution position of the Type II amylases as compared with the positions of the major protein peaks. In 2 cases (Cases 1 and 9) the macroamylase was recovered in the same fractions as the 7S globulins; in 7 cases (Case 2[3] 4, 5, 7 8, 10, 11) it was in the

[3] It had previously been reported that the Type II amylases of Cases 2 and 3 were closely related in their recovery positions with the 7S globulins (2). Reassessment of the distribution patterns, how-

TABLE I. Comparative Kinetics of Normal Serum Amylase (Type I) and the Macroamylases (Type II) Found in 12 Patients with Macroamylasemia.

	K_m[a]	pH[b]	Cl[c] (M)
Type I	0.10	7.1	0.2
Type II			
Case 1	0.19	7.0	0.1
2	0.19	6.9	0.1
3	0.18	6.9	0.05–0.2
4	0.16	7.0	0.08–0.2
5	0.15	7.0	0.1
6	0.13	7.0	—
7	0.19	7.0	0.05–0.2
8	0.14	7.0	0.05–0.2
9	0.18	7.0	0.05–0.2
10	0.16	7.0	0.1 –0.2
11	0.14	7.0	—
12	0.18	7.1	0.1

[a] Michaelis constant for starch expressed as g/100 ml.
[b] pH for maximum activity.
[c] Sodium chloride molarity for maximum activity.

fractions whose positions lay between the 7S and 11S globulins; in 2 cases (Cases 3[3] and 12) its elution position corresponded with the one at which most of the 11S globulins would be expected to appear; and in one case (Case 6) the macroamylase was found in the fractions very close to those containing the 19S globulins. Ultracentrifugation confirmed that the macroamylase in Cases 1 and 8 was 7.5 ± 0.5S.

When dextran gel filtration was carried out with serum mixed with 6 M urea, only Type I amylase was recovered in 11 of the 12 cases; in a single case (Case 1) Type II was markedly reduced but some was still present. The Type I amylase was also diminished quantitatively in 9 of the 12 cases after exposure of the serum to urea. In the remaining 3 cases, however, (Cases 3, 5, and 7) the amount of Type I amylase activity rose after urea treatment.

Treatment of the serum with EDTA before dextran gel filtration resulted in a decrease of both amylase components except for Case 2; in this case the Type I amylase showed no appreciable change. Similarly, treatment with 2-mercaptoethanol and dialysis against distilled water resulted in a diminution in both types of serum amylase.

The results of the comparative kinetic studies made on normal Type I serum amylase and on the Type II amylase fractions isolated from the 12 macroamylasemic sera are given in Table I. Shown in Table II are the corresponding properties of the amylase fractions isolated after urea treatment of normal serum and the macroamylasemic sera. The Type II amylases, regardless of their elution position with respect to the serum proteins, consistently showed less affinity for starch than did normal Type I amylase; after urea treatment, the resultant amylases from macroamylasemic sera closely resembled normal serum in this property. There were no appreciable differences in pH optima between the Type I and Type II amylases, either before or after treatment with urea. The data for chloride maximum exhibited great variation, possibly reflecting continued association of an undetermined amount of chloride with the amylase even after dialysis. In any event, the variation did not permit conclusions re-

TABLE II. Comparative Kinetics of the Amylases Recovered After Treatment of Serum with Urea.

	K_m[a]	pH[b]	Cl[c] (M)
Normal serum	0.12	7.0	0.05–0.25
Macroamylasemic serum			
Case 1	0.12	6.9	0.05–0.3
2	0.10	6.9	0.1 –0.3
3	0.12	7.0	0.05–0.1
4	0.11	6.9	0.05–0.1
5	0.11	6.9	0.1
6	0.12	7.0	—
7	0.11	6.9	0.05–0.2
8	0.13	7.0	0.05–0.1
9	0.10	7.0	0.05
10	0.11	6.9	0.1 –0.3
11	0.11	7.0	0.05
12	0.11	7.0	0.05

[a] Michaelis constant for starch expressed as g/100 ml.
[b] pH for maximum activity.
[c] Sodium chloride molarity for maximum activity.

ever, indicated that the elution positions of these macroamylases would be more accurately located as here indicated.

garding possible differences in this parameter.

Dextran gel filtration of the mixture formed by adding isolated normal serum amylase, or normal whole serum, to protein fractions obtained after urea treatment of macroamylasemic serum failed to recover a macroamylase. Neither was a new macroamylase formed when the amylases derived from urea treatment of the macroamylasemic sera were added to their own protein fractions.

Lowering the pH altered the configuration of the amylase pattern in the single case in which this was attempted; the macroamylase peak largely disappeared leaving only a solitary amylase peak whose molecular size resembled that of Type I serum amylase. Conversion of Type II to Type I amylase by this treatment was suggested by the fact that the total activity in the surviving normal size amylase fraction increased about 3-fold.

Discussion. The relative amounts of the large amylase components in these 12 patients with macroamylasemia varied. The macroamylases were also diversified in the rate at which they filtered through a column of dextran gel. The latter would suggest heterogeneity with respect to molecular size and presumably in molecular weight as well. It is appreciated with respect to the latter that there are distinct limitations inherent in any attempt to assign a sedimentation coefficient to an emzyme based on its chromatographic elution rate. Although filtration of normal human serum through dextran gel typically yields 3 major peaks corresponding to the 4S, 7S, and 19S proteins obtained by ultracentrifugation (9), the position of the 11S globulins can be determined only by interpolation. Uncertainty is further compounded by the fact that Sephadex is a polydextran, not unlike starch in molecular structure. Interaction of this material with amylase (10) could conceivably retard elution and thereby make the enzyme appear to be a smaller molecule than it actually is. Despite these limitations, the fact that sucrose gradient ultracentrifugation performed in 2 of our cases gave sedimentation coefficients reasonably corresponding to those estimated for the same cases from gel chromatography indicates that the latter estimations are not altogether invalid. Furthermore, the findings are in keeping with those of Levitt *et al.* (4) who reported that preparative ultracentrifugation demonstrated that the macroamylase was approximately 11S in size in 2 of their 5 cases and approximately 7S in the other 3. It would seem reasonable to conclude, therefore, that these several data support our earlier observation that the macroamlase complex in cases of macromamylaemia probably is not homogenous (2).

The strong denaturating effect of 6*M* urea on the large amylase component, and the suggestive evidence in at least 3 of the cases that the dissociated Type II amylase was transformed into Type I, may be interpreted as indicating that the macroamylase may be either a polymer of normal serum amylase, or a complex of normal serum amylase and some larger protein molecule. Our efforts to shed some light on which of these situations may obtain gave inconclusive results. Exposure of the macroamylasemic sera to EDTA in an attempt to chelate calcium, zinc, or other cations that might be involved in polymerization of normal amylase chains (11), or in linking amylase to an immunoglobin, resulted in a decrease of both amylase components. Similarly, the addition to these sera of 2-mercaptoethanol, an agent known to reduce immunoglobulin to subunits and to release bound protein by disrupting disulfide bonds (8, 12), diminished both amylase fractions. On the other hand, Levitt *et al.* (3) succeeded in showing that the addition of 2-mercaptoethanol to the serum of a patient in whom the serum amylase appeared to exist principally as an 11S-IgA-linked compound, reduced the sedimentation coefficient of the amylase to approximately 7S.

Our studies dealing with the kinetic properties of the macroamylases isolated from the sera of these 12 patients gave results similar to those of Levitt and Copperband (3). The findings suggest that the active portion of the large component differs only slightly, if at

all, from normal serum amylase. They also suggest that the different K_m of Type II amylase may result from its attachment to a larger protein. Levitt *et al.* (4) identified the larger component of their two 11S size macroamylases as IgA immunoglobulin. Association of IgA globulin (mol wt approx 160,000) with normal serum amylase (mole wt approx 50,000) could be expected to yield a complex sedimenting at 10–11S and be precipitable by antiserum to IgA. Indeed, the latter occurrence was reported by Levitt and his co-workers (3, 4). On the other hand, we were unable to detect any change in the gel filtration pattern in one case (Case 3) with what would appear to be an 11S macroamylase after exposure of the serum to human globulin antiserum (2). It is possible, however, that a loose antigen–antibody complex did form in this case but became dissociated during gel filtration.

Levitt *et al.* (4) reported that their 3 patients with 7S macroamylases did not react with specific IgA, IgG, and IgM antisera to any greater degree than did normal human serum. We had earlier treated the serum of one patient with a 7S macroamylase (Case 1) with human globulin antiserum and antiserum to 7S gamma globulin (2). Subsequent filtration on dextran gel resulted in recovery of a macroamylase of even greater molecular size, thus suggesting that interaction of some type had occurred. However, if the 7S size macroamylases are also considered to be complexes containing amylase with a molecular weight of approximately 50,000 and some serum protein, candidates in normal human serum of the appropriate molecular weight (approx 100,000 or 4.5–5S) are singularly lacking. Some poorly characterized beta-globulins (13), haptoglobin (14), and transferrin (14, 15) are about the correct molecular weight, but no intact immunoglobulin would qualify on this score.

Although our attempts to locate a protein in serum which would combine with amylase were uniformly unsuccessful, Levitt and Cooperband (3) did succeed in demonstrating this in their case. The addition of isolated normal serum amylase or normal whole serum to protein fractions obtained after gel filtration of urea-treated sera from 4 of our macroamylasemia patients failed to result in recovery of a macroamylase. Similarly, no macroamylase formed when the Type I amylases resulting from urea treatment were added to the protein fractions derived from the same sera.

Soluble antigen–antibody complexes tend to dissociate at pH values between 3 and 4.5 (16). Based on this observation, the fact that the macroamylase dissociated at pH 3.4 in the single case we tested for the influence of acid pH may be taken as evidence suggesting that the macroamylase in this case consisted of an antigen–antibody complex. The same conclusion was drawn by Levitt and Cooperband (3) from similar observations made in their case.

These several observations lead us to conclude that the macroamylases in some patients with macroamylasemia may involve immunoglobulins but this would appear not to apply in all cases.

Summary. Observations on the behavior and nature of the macroamylase found in 12 patients with macroamylasemia lend further support to the earlier suggestion that the macromolecular amylase component is not homogenous. This was evidenced by variation in the relative amounts of macroamylase present in the serum and differences in the elution patterns obtained following dextran gel filtration. Disappearance or marked diminution of the macroamylase after exposure to urea would suggest that the macroamylase consists either of a polymer of normal serum amylase or a combination of normal serum amylase and a larger protein molecule. The active portion of the macroamylase complex appears to differ only slightly, if at all, from normal serum amylase regardless of the protein fraction with which it may be associated. Immunoglobulins may be involved with the complex in some but seemingly not in all cases.

Note added in proof. Three additional patients with macroamylasemia have been observed. The effect of acidification and possible protein binding were examined in one of these patients. Reduction of

the serum pH of this patient to 3.4 resulted in recovery of only Type I amylase after Biogel P300 filtration. When the pH of the amylase-free fractions isolated by such filtration was restored to 7.2, the fractions incubated with normal human serum, and the mixture then filtered on Biogel P300, Type II as well as Type I amylase were recovered.

1. Wilding, P., Cooke, W. T., and Nicholson, G. I., Ann. Int. Med. **60**, 1053 (1964).
2. Berk, J. E., Kizu, A., Wilding, P., and Searcy, R. L., New Engl. J. Med. **277**, 941 (1967).
3. Levitt, M. D. and Cooperband, S. R., New Engl. J. Med. **278**, 474 (1968).
4. Levitt, M. D., Goetzl, E. J., and Cooperband, S. R., Lancet **1**, 957 (1968).
5. Andrews, P., Biochem. J. **96**, 595 (1965).
6. Edelman, G. M., J. Am. Chem. Soc. **81**, 3155 (1959).
7. Lineweaver, H. and Burk, D. J., Am. Chem. Soc. **56**, 658 (1934).
8. Deutsch, H. F. and Morton, J. I., Science **125**, 600 (1957).
9. Flodin, P. and Killander, J., Biochim. Biophys. Acta **63**, 402 (1962).
10. Wilding, P., Clin. Chim. Acta **8**, 918 (1963).
11. Fischer, E. H. and Stein, E. A., *in* "The Enzymes" (P. D. Boyer, H. Lardy, and K. Myrbäck, eds.), 2nd ed., Vol. 4, p. 313 Academic Press, New York (1960).
12. Singer, S. J., *in* "The Proteins" (H. Neurath, ed.), 2nd ed., Vol. 3, p. 269, Academic Press, New York (1965).
13. Oncley, J. L., Scatchard, G., and Brown, A., J .Phys. Colloid Chem. **51**, 184 (1947).
14. Nyman, M., J. Lab. Clin. Invest. Suppl. **39**, 1 (1959).
15. Schultze, H. E. and Schwick, G., Clin. Chim. Acta **4**, 15 (1959).
16. Singer, S. J. and Campbell, D. H., J. Am. Chem. Soc. **77**, 3504 (1955).

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Protection of Neonates against Whole-Body Radiation by the Administration of a Single Emulsified Injection of a Lipopolysaccharide During Pregnancy (33828)

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In a previous publication it was reported that a purified endotoxin, a lipopolysaccharide (LPS) derived from *E. coli* could be given safely to mice if injected in a water-in-oil emulsion. Two to four times the LD_{50} dose was given with minimum lethality owing to the slow and prolonged release of the LPS from the mineral oil emulsion. The mice treated in this manner showed marked and prolonged resistance to challenges with a sarcoma—180 implant and a lethal infection with a staphylococcus (1). Experiments using whole-body radiation (WBR) as a challenge was, therefore, begun following the reports of Smith and her collaborators (2) and Zweifach and his collaborators (3), in which endotoxin was shown to be radioprotective. Additional observations by Smith *et al.*(4) and Ainsworth (5) and Hanks and Ainsworth (6) have confirmed and extended these observations.

In an experiment recently reported we obtained evidence suggestive of protection against lethal WBR in mice for approximately 1 month after a single injection of the LPS (7).

In other unreported experiments designed to see if the fetus could be protected against WBR the emulsified LPS was injected into CF_1 pregnant mice to determine their tolerance and it was found that 100 μg could be given without inducing abortion or killing the pregnant mouse which seems to be more vulnerable to the LPS. During the course of these studies a group of 11 neonates from a single litter became available and these were

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