The quantitative nature of the reaction between aminoglycoside and polymyxin class antibiotics with polyanionic detergents

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Summary. Using classical tube and gel precipitation techniques aminoglycoside and polymyxin class antibiotics were found to quantitatively react with polyanionic detergents.

Polyanionic detergents are known to possess anti-phagocytic[1], anti-complementary[2], anti-protein[3], and anti-antibiotic[4] activity. Within biological systems, the most extensively studied member of this class of compounds is sodium polyanethol sulfonate (SPS). This substance is routinely added to blood culture media to inhibit the antibacterial activity of blood. SPS, as well as other polyantionic surfactants such as sodium dodecylsulfate (SDS) and disodium 4-dodecylated oxydibenzenesulfonate (Benax) appear to effectively obviate the biological activity of cationic compounds. Therefore, positively charged antibiotics, including aminoglycosides and polymyxins, and positively charged proteins, such as lysozyme and beta-lipoprotein, are selectively affected[5,6]. It is known that SPS, SDS, and Benax inhibit positively charged antibiotics by combining with them to form a precipitate, consequently eliminating their activity by removing them from solution[7]. The observation that large negatively charged detergent molecules can combine and form a precipitate with small positively charged antibiotic molecules prompted this study concerning the possible quantitative nature of this reaction.

Materials and methods. An investigation of the nature of the precipitation reaction was performed by both tube and gel techniques. The determination of equivalence zones using tubes was performed according to the classical Kabat and Mayer[8] method heretofore utilized for the quantitative measurement of antigen-antibody reactions. Detergents were prepared in distilled water in concentrations of 50,000, 25,000, 12,500, 6250, 3125, 1560, and 780 mcg/ml. SPS (Hoffmann-LaRoche, Nutley, N. J.), SDS (Mann Research Labs, New York, N. Y.) and Benax (Dow Chemical Co., Midland, MI) were the polyanionic detergents tested. Tween 80 (Atlas Chemical Co., Wilmington, DL) was utilized as a neutral detergent and benzalkonium chloride (Winthrop Labs, New York, N. Y.) as a cationic detergent. All antibiotics (Canalco, Rockville, MD) were obtained as standard reference powders without preservatives. Streptomycin, kanamycin, gentamicin and tobramycin representing the amino-glycosides; colistin and polymyxin B representing the polymyxins, and penicillin G were rehydrated in distilled water in concentrations of 10,000, 5000, 2500, 1250, 625, 312, 156, 78, 39, 20, 10 and 5 mcg/ml. Checkerboard titrations[8] was performed in 5 ml of liquid with the degree of precipitation measured by absorbance at 650 nm in a Coleman Junior spectrophotometer. In addition precipitates were centrifuged, weighed and plotted as described. The equivalence point was determined by the standard technique of plotting the

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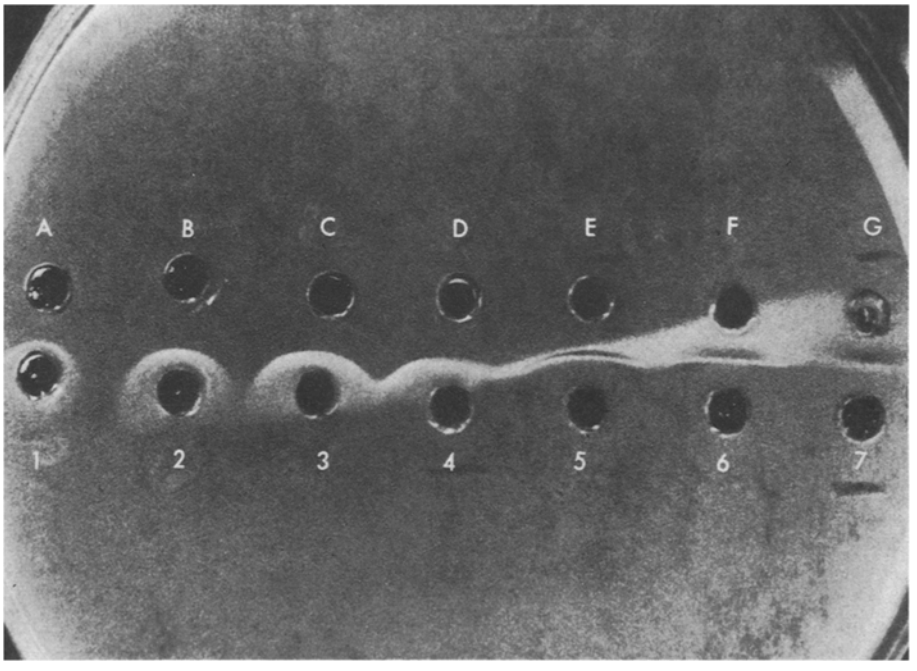

The quantitative precipitin test in gel: SPS and gentamicin. Below figure: Gentamicin concentrations: 2500 (A), 1250 (B), 625 (C), 312 (D), 156 (E), 78 (F) and 39 (G) mcg/ml. SPS concentration: 780 mcg/ml (1 through 7). The equivalence zone is between 156 mcg/ml gentamicin and 780 mcg/ml SPS. Lisegang bands switch sides on either side of the equivalence zone. From 1 through 3 antibiotic is in excess; from 6 to 7 detergent is in excess. The curvarure of precipitation away from well E at the equivalence zone indicates that its contents have the greatest diffusion rate.

amount of precipitation versus the concentration of the reactants. Incubation was at room temperature (21 °C) with measurements taken at 4, 8, 24, 48 and 72 h.

The quantitative precipitin test in gel[9] was also performed since it provided the same information as the tube test, was somewhat less cumbersome and was amenable to large scale testing. Diffusion plates were made by pouring 12 ml of 1.5% agarose in deionized water into the bottom of 100 mm diameter petri plates. Parallel wells of 5 mm diameter and 15 mm apart from their centers were cut in the agar. As with the tube precipitin test one class of reactant was held constant during one test and the other diluted in a geometric series. The concentration range used in the tube test was employed in the gel test. The equivalence point was taken as the concentration of reactants which produced the sharpest line of precipitation without Lisegang bands.

Results and discussion. Tube precipitation studies produced the typical bell-shaped curves observed for the quantitative determination of antigen-antibody precipitation. Both pro-zone and post-zone phenomena were seen. The polymyxins, polymyxin B and colistin, yielded the greatest turbidity and precipitation with polyanionic detergents and had the widest equivalence zone of the tested antibiotics. The aminoglycosides, as typified by the kanamycin versus SPS study shown in the table, all produced bell-shaped curves, but with lesser amounts of precipitation than the polymyxins. There was no precipitation with any detergent tested and penicillin G, nor was there precipitation with tween 80 and benzalkonium chloride with any examined antibiotic.

Quantitative checkerboard precipitation tube dilution test: SPS and kanamycin

Kanamycin (mcg/ml)	SPS (mcg/ml)* 50000	25000	12500	6250	3125	1560	780
10000	−	+	+	+	+	+	+
5000	−	+	+	+	+	±	−
2500	−	−	+	+	+	−	−
1250	−	−	−	+	−	−	−
625	−	−	−	−	−	−	−
312	−	−	−	−	−	−	−
156	−	−	−	−	−	−	−

* The molecular weight of SPS is not known.

In gel (figure) the aminoglycoside and polymyxin class antibiotics reacted with the polyanionic detergents in the classical manner. In each, the equivalence point was determined as that area where the precipitation line was the sharpest and where precipitation bands switched position from antibiotic to detergent excess. The typical Lisegang bands seen in gel are representative of the prozone and postzone phenomena one observes during tube testing. In gel, the point of first precipitation is also the equivalence point. As a result the test can be accomplished within 4 h since first precipitation appears at this time. Consequently it is possible to rapidly determine the amount of antibiotic in solution utilizing a constant concentration of polyanionic detergent. The concentration of detergent needed to elucidate the equivalence point for any given antibiotic sample varies by the amount of antibiotic in solution. Several varied detergent concentrations can be set-up at the same time on different gel plates.

Based on classical precipitin testing by tube dilution and gel diffusion it would appear that the interaction of polyanionic detergents with polymyxin and aminoglycoside class antibiotics fulfills the requirements of a quantitative reaction. Accordingly, one may utilize either technique for measuring levels of these antibiotic classes and for the preparation of standards. Because the reactions can be read within 4 h these procedures should be useful in those instances where rapid measurements are needed.

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Growth-modulating serum tripeptide is glycyl-histidyl-lysine[1]

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Summary. The structure of a growth-modulating tripeptide from human serum and plasma has been determined to be H-glycyl-histidyl-lysine-OH.

We have previously reported the isolation of a small peptide from human serum which, in concentrations ranging from 20 to 200 ng/ml, promoted the growth of hepatoma cells in HTC and prolonged the survival of nonreplicating rat liver cells in low-serum (1%) monolayer culture[2,3]. Amino acid analyses of the purified active fraction suggested that the native factor was a tripeptide composed of glycine, histidine and lysine[2].

Among synthetic analogs tested, glycyl-histidyl-lysine (GHL) had bioactivities which were comparable to those of the serum factor in the hepatoma and normal liver cell systems[4]. Recently, GHL in nmole concentrations was shown to stimulate growth and differentiation of cortical neurons in vitro and to inhibit growth of glial elements[5]. Suppression of growth in fibroblast cultures by GHL at higher concentrations has also been reported[6]. At neutral pH ranges GHL has chelation properties toward several transition metals (Cu^{+2}, Zn^{+2}, Co^{+2}, Fe^{+3}) and seems to function synergistically with the metals to increase the adhesion of hepatoma cells (HTC_4) growing in low-serum (0.5–1.0%) to the monolayer support[7].

We present data herein that confirm that the native serum factor is a tripeptide with the structure: glycyl-histidyl-lysine. The serum tripeptide was isolated by methods detailed elsewhere[8]. In brief, the serum proteins are removed by heat coagulation and centrifugation. The supernatant which contains the tripeptide is passed through a molecular filter with a nominal cutoff at 500 daltons (Amicon, UM-05, Lexington, Mass.) then lyophilized. After reconstitution in 1% acetic acid, the frac-