androgen in an isolated system lends support to this interpretation(10).

Previous investigations have shown that anterior pituitary explants have a morphological influence on the isolated testis(5,11). The present study demonstrates that HCG has a similar effect on testicular explants and can stimulate production of androgenic hormone under *in vitro* conditions. Thus, as in the case of the ovary, the results obtained with the isolated testis are similar to those previously observed in experiments with the whole animal(12).

Summary. Testes from mice, 3 days of age, were cultured in an *in vitro* system for 7 or 14 days on a medium composed of agar, Tyrode's solution, synthetic medium 199 and chick embryo extract. Degenerative changes in control explants included a decrease in size of tubules and a reduction in cytoplasm of interstitial cells. Inclusion of HCG in the medium ameliorated these changes. Moreover, measurable amounts of androgen were produced by testes cultured on media enriched with HCG whereas no detectable amount was produced by those cultured on media enriched with lactogenic hormone.

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Studies on the Small Particle Complement-Fixing Antigen of Foot-and-Mouth Disease Virus. (25401)

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It has been well established that foot-and-mouth disease is associated with at least 2 specific particles(1,2). The larger particle, reported to be 23 mμ in diameter and having a sedimentation coefficient (s-rate) of 140, possesses all the viral properties of the infection together with about half of the complement-fixing activity; the smaller particle, 8-10 mμ in diameter and having an s-rate of 14 displays complement-fixing (CF) activity only (3,4). Preliminary chemical investigation of the smaller particle, hereafter to be called the CF-antigen, indicated a globulin nature for the material(5). This report presents the results of biochemical studies on the CF-antigen and purification procedures.

Materials and methods. Virus infectivity was measured by a plaque assay using primary bovine-kidney cell layers in 4-oz prescription bottles(6). *Complement-fixing activity* was measured by modification of the Traub and Möhlmann procedure(7). As used in this laboratory,* the test is a direct semi-quantitative one, using guinea pig complement with overnight fixation at 4°C. The endpoint was read visually from 0 to +4 on 2-fold serially diluted samples. Readings from 0 to +2 were counted as belonging to the next lower dilution; readings of +3 or +4 were counted as belonging to the dilution used. Thus, a sample having a reading of +4 at a dilution of 1:256 and a reading of +1 at a

* Savan, M., Edward, A. G., Martinsen, J., Poppensiek, G. C., unpublished.

dilution of 1:512 was considered to have a CF titer of 256. *Protein* was determined spectrophotometrically by the ultra-violet adsorption method of Warburg(8), and also with Folin-Ciocalteau reagent(9), using crystalline bovine plasma albumin as a standard. *Source of complement-fixing antigen*: bovine tongue epithelium, infected with foot-and-mouth disease virus, type A, strain 119 (FMDV-A119), which had been stored in dry-ice chest for extended periods ranging up to 3 years, was homogenized in a Servall Omnimix as a 33% suspension in phosphate-buffered saline (0.02 M phosphate buffer, 0.10M sodium chloride), pH 7.5, in the presence of Genetron 113 (trifluoro trichloro ethane), and small amounts of toluene and octyl alcohol. The homogenate was stirred overnight at 4°C, and the aqueous extract was separated by centrifuging for 1 hour at 900 × g. The aqueous phase was then centrifuged in a Spinco Model L with a #30 rotor at 30,000 rpm for 2½ hours to sediment out infectious virus particles and other large particles. The supernatant fluid, whose virus titer was reduced by at least 5 log units, was centrifuged for 22-24 hours at 30,000 rpm. The supernatant liquid was discarded down to the level corresponding to the top of the sedimented pellet. The pellet plus this residual volume of liquid (about 3 ml) were resuspended in phosphate-buffered saline, and the clear aqueous CF-antigen concentrate was the starting material used in all experiments. One gram of tongue tissue yielded about 200 CF units (CF titer × ml). The CF titers of the aqueous concentrates ranged from 256 to 512 per ml, each ml containing about 50 mg of protein.

Results. Stability of the CF antigen was determined with respect to temperature, pH, enzymes and various methods of processing. Complement-fixing activity was completely retained for at least 3 months at 4°C, 1 week at room temperature (about 25°C), 4 hours at 37°C, and 30 minutes at 60°C. Activity was lost upon heating at 70°C. Adjustment of the CF-antigen solution to pH 5.0 for overnight at 4°C produced a precipitate having about 10% of the starting activity, with 50% of the starting activity remaining in the supernatant fluid. At pH 5.25, there was complete retention of activity, all in the supernatant fluid. Alkaline pH adjustment was accomplished by mixing the antigen solution with equal volumes of 4% sodium bicarbonate, 2% sodium carbonate, and 0.1N sodium hydroxide at final pH values of 8.3, 10.5, and 12.0, respectively. In the first 2 solutions CF activity was completely stable for at least 4 hours at room temperature, while there was a 75% loss of activity in the sodium hydroxide solution.

The effect of proteolytic and nucleolytic enzymes on the CF-antigen was determined. Trypsin and chymotrypsin (crystalline, Worthington) in concentrations as high as 5-10 mg per ml for 4 hours at 37°C had no effect on CF titer. Ribonuclease and deoxyribonuclease (crystalline, Worthington) acting for 24 hours at room temperature similarly produced no decrease in titer. The enzymes employed did not decrease significantly the total amount of proteins present in solution.

The CF-antigen concentrate was studied in the presence of the protein denaturant, urea, acting for 3 days at room temperature. A 2M urea solution did not affect CF activity, but with 6M urea, there was a loss of 75% of the starting activity. Trypsin did not have any effect on CF activity in these treated samples also.

Freeze drying of CF-antigen solutions in phosphate-buffered saline yielded powders retaining all the original activity. However, when precipitated preparations were dissolved in ammonium acetate solutions (no residue upon evaporation) and subsequently lyophilized, there were losses in activity ranging from 35 to 75%. The residual acidity of the acetic acid formed apparently was sufficient to cause this loss of activity.

Separation by precipitation of the CF-antigen was accomplished by various procedures. Exhaustive dialysis of CF-antigen concentrates against distilled water in the cold produced precipitates containing 75 to 90% of the total recovered activity which in turn ranged from 40 to 60% of the starting activity. The liquid within the dialysis membrane at the conclusion of the dialysis had a pH of 5.0 to 5.1 and this would account for the low

recovery. In order to avoid prolonged exposure of the CF-antigen to acid conditions, another deionization procedure involved shaking the CF-antigen solution with a prepared mixture of cation and anion exchange resins (Amberlite MB-3). However, this procedure also resulted in incomplete recovery of CF activity which was, in this case, partitioned fairly equally between precipitate and supernatant liquid. Isoelectric precipitation of the CF-antigen was studied over a pH range from 4.5 to 6.0 at intervals of 0.25 pH units. As indicated in the foregoing experiments on pH stability, there was a marked loss of activity at pH 5.0 and below. The precipitates formed contained very little to none of the remaining activity. Ammonium sulfate precipitations were made at pH 5.5 and pH 7.5 in the cold by adding that amount of a saturated solution of ammonium sulfate necessary to reach the desired percent of saturation in the CF-antigen solution. At both levels, activity was precipitated from 30 to 50% saturation, with less than 5% of the activity remaining in the supernatant fluid at the higher concentration of ammonium sulfate. However, recovery of activity from the precipitate at 50% saturation with ammonium sulfate was incomplete, sometimes being only half the starting activity. Paper electrophoresis of the re-dissolved precipitate showed, upon staining with bromphenol blue, a dark globulin area and a very faint albumin spot.

Ethanol fractionation was carried out at 2 different levels of ionic strength, the CF-antigen being either in a 0.01M phosphate buffer or in standard phosphate-buffered saline, with pH at 7.5 in both cases. Precipitations were done at 0°C with ethanol concentrations ranging from 10 to 40%. Recovery of CF-antigen was incomplete with activity spread between precipitates and supernatant fluids.

The CF-antigen was completely precipitated from aqueous solution by acetone in the cold, and the wet precipitate reconstituted in buffer with no loss of activity. Protamine sulfate and zinc ions precipitated part of the activity, while barium ions and sodium salts of ribonucleic acid and heparin had no effect.

Column chromatography, using several different adsorbents, was done at room temperature in 10 mm wide tubes, with column lengths ranging from 50 to 150 mm. The CF-antigen concentrate was loaded onto the column in a volume of 1-3 ml, with both charge and column having been equilibrated with the same buffer system. Elution was accomplished by stepwise increases in ionic strength of the eluting medium, at a flow rate of about 20 ml/hour. The eluate was collected in 4 ml fractions which were analyzed for protein content and CF-activity.

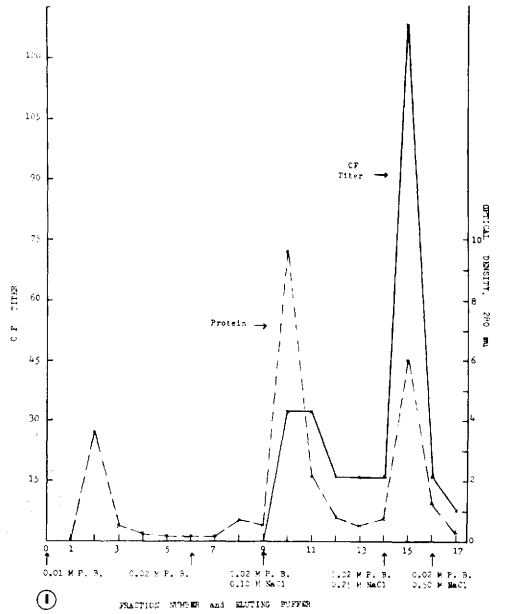
Results using a diethyl amino ethyl (DEAE) cellulose column buffered at low ionic strength are shown in Fig. 1. There was a 70% overall recovery of CF activity with a 40% recovery of proteins. The highest peak of activity contained 35% of the original charge and represented a 4-fold increase in purity. In another experiment, a DEAE cellulose column buffered at a higher ionic strength, 0.02M phosphate buffer containing 0.10M sodium chloride at pH 7.5, gave complete recovery of both CF activity and protein, with no significant purification.‡

Calcium phosphate columns prepared according to Tiselius(10), gave less than 1% recovery when equilibrated at low ionic strengths. Columns prepared in 0.02M phosphate buffer containing 0.10M NaCl yielded better recoveries of activity and fractions with purities comparable to those of the cellulose columns (Fig. 2).

The cation exchange resin, Amberlite CG-50, 400-600 mesh, equilibrated at pH levels ranging from 5.5 to 7.5 in isotonic buffers yielded unsatisfactory results, as did columns prepared of Dowex 50-x2, celite, and aluminum hydroxide (modified Willstatter Cv (11)).

Discussion. The small particle CF-antigen displays a markedly greater overall stability in comparison to the large particle having viral activity in the FMDV-A119 system. CF activity is retained after heating to 60°C for 30 minutes, while 90% of viral activity is lost after heating to 61°C for one-half minute

‡ While this paper was in preparation, Brown, F., and Cartwright, B., *Biochem. et Biophys. Acta,* 1959, v33, 343, reported the separation of crude suspensions of FMDV into two main fractions by chromatography on DEAE cellulose.



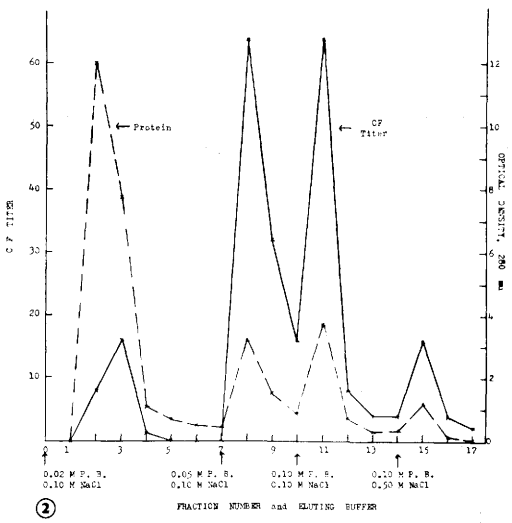


FIG. 1. Diethyl amino ethyl chromatography of complement-fixing antigen. Column and sample equilibrated with 0.01M phosphate buffer, pH 7.5. Column dimensions, 10 × 92 mm.

FIG. 2. Calcium phosphate chromatography of complement-fixing antigen. Column and sample equilibrated with 0.02M phosphate buffer containing 0.10M sodium chloride, pH 7.5. Column dimensions, 10 × 53 mm.

(12). The CF-antigen is stable over the pH range 5.25 to 10.5, while viral activity is most stable only at pH 7.0 to 7.5 and loses 90% of its activity in 14 hours at 4°C at pH 6.5 and 10.0. CF activity is resistant to the action of proteolytic enzymes and nucleases while viral activity in the tissue culture FMDV-A119 system, at least, is sensitive to the action of trypsin.† These differences in stability are of particular interest in view of the suggestion that the small particle can be derived by disintegration of the larger infectious unit(13).

Our data indicate that the CF-antigen should be classified as a euglobulin because it is insoluble in salt-free solutions. Also, it is precipitated by half-saturated ammonium sulfate and shows a predominantly globulin-type electrophoretic pattern.

The incompleteness of fractionation of CF activity by the various experimental technics may indicate the presence of more than one small particle containing CF activity. As determined by column chromatography, Figs. 1 and 2, the so-called small particle CF-antigen does have more than the one peak of activity. The presence of impurities and changes in eluting technic can modify this pattern. Therefore, it would also be of interest to determine the chromatographic distribution of CF activity in virus-free vesicular fluid from FMDV lesions.

Summary. The so-called small particle CF-antigen fraction, free of infectious virus particles and larger components, was prepared by centrifugation from bovine tongue epithelium infected with FMDV, type A, strain 119. The CF activity of this fraction was stable through the pH range of 5.25 to 10.5, withstood heating for 30 minutes at 60°C, and was resistant to the action of proteolytic and nucleolytic enzymes. It was precipitated by dialysis and by ammonium sulfate, ethanol, and acetone. Diethyl amino ethyl cellulose and calcium phosphate columns prepared at different ionic strengths were used in chromatographic purification. The small particle CF-antigen displays the properties of a euglobulin and is appreciably more stable than the large infective particle with which it is associated.

† Unpublished data.

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Lipoprotein Lipase Metabolism in Experimentally Nephrotic Rats.* (25402)

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Our earlier studies(1,2) strongly suggested that deficiency of circulating albumin in experimentally nephrotic rats is in some manner responsible for abnormal intravascular retention of lipid and consequent hyperlipemia. The possibility of an abnormality of heparin or lipoprotein lipase metabolism as an associated causal factor in experimental nephrotic hyperlipemia also was suggested by earlier studies(3). Since it has been proposed(4,5) that in humans, nephrotic hyperlipemia results from a deficiency of lipoprotein lipase, the following studies were conducted.

Methods. Young, adult male rats of Long-Evans strain were used (average weight: 200 g). The nephrotic state was induced by injection of potent rabbit anti-rat kidney serum (6) 5 to 7 days prior to study and confirmed by demonstration of plasma albumin(7) content less than 1.2 g/100 ml and by presence of gross plasma lactescence and of marked hypercholesteremia(8). Disappearance of heparin from plasma of nephrotic rats was assayed, albeit crudely, by determining clotting time and by protamine titration of plasma obtained from groups of nephrotic and control rats bled at intervals following heparin injection. Each of a series of 10 nephrotic and 18 control rats was injected intravenously with heparin (0.5 mg/100 g). Five nephrotic and 12 control rats were bled with siliconized syringes, from the aorta 2 hours after heparin injection, and the remaining 5 nephrotic and 6 controls after 3 hours. Immediately after bleeding, 0.5 ml of each sample was placed in each of 3 serology tubes for clotting time determination and also in each of a 4-tube system containing serial dilutions of 0.01% protamine sulfate (prepared 24 hours previously). All tubes were kept in water bath at 37°C, and clotting times recorded as the time of average occurrence of a fast clot during gentle tilting at 30-second intervals. Lipoprotein lipase activity of nephrotic postheparin plasma was determined by ability to increase light transmission and to release unesterified fatty acids (UFA)(9) when added to a triglyceride substrate in normal rat plasma. For light transmission studies, plasma samples were obtained from 19 nephrotic rats, 10 minutes after injection of either 0.05 mg (5 rats) or 0.3 mg (14 rats) of heparin/100 g of body weight. Similar postheparin plasma samples were obtained from 16 normal, control rats. For further control purposes plasma

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