Schaefer (*4*) found that handling during the first 7 days produced the greatest reduction in adult emotionality measured in terms of behavior in an open field situation. Denenberg (*5*) reports that handling during the first 10 days of life resulted in avoidance learning superior to that found when handling was initiated later. In both of these studies, the period during which the treatment was initiated includes the critical period found in the experiment discussed in this report. Whether behavioral difference can be detected in experiments with such restricted age groups as were tested in this experiment remains to be determined.

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Nonidentity of Fetuin and Protein Growth (Flattening) Factor

Abstract. Fetuin, a fetal calf serum glycoprotein, appeared to possess activity with cultured mammalian cells similar to that of a protein growth factor partially purified from adult bovine and human sera. Column chromatography, however, yielded highly purified but inactive fetuin. These results leave open questions regarding the role of this interesting and readily purified protein.

Efforts to characterize the factors present in animal sera required for the growth of mammalian cells in culture led to the partial purification of a protein from bovine and human sera (*1*). The protein, studied most extensively with a culture of human origin, Appendix A 1 (*2*), causes adherence of cells to a glass surface; only in its presence do cells assume a flattened, epithelial-like appearance; and it is required for growth. Available evidence indicates that it is a glycoprotein.

Fisher, Puck, and Sato (*3*), working independently with a different cell culture, HeLa S3, reported some of these effects with fetuin, a glycoprotein from fetal calf serum. Their interesting results not only offered a rich source of the activity [fetuin represents about 45 percent of fetal serum protein (*4*)], but also suggested a possible in vivo growth-stimulating role for a molecule with flattening factor activity. The similar activity levels of fetal calf and beef sera, however, despite the high level of fetuin in the former, raised the question whether the activity of fetuin preparations might not result from contamination with the growth factor.

To test this possibility, highly purified fetuin was prepared. The protein was first precipitated from fetal calf serum with ammonium sulfate according to the initial step of the procedure of Fisher et al. (*3*). Electrophoretic analysis of this preparation showed that about 75 percent of the protein resided in a single peak whose mobility was the same as that of fetuin. After dialysis for 24 hours against sodium phosphate buffer (0.01*M*, *p*H 7.1), the ammonium sulfate fraction was applied to a column of DEAE-cellulose (type 20, height 10 cm, diameter 1.8 cm) according to the procedure of Sober *et al.* (*5*). Elution was carried out with sodium phosphate buffer solutions containing increasing concentrations of NaCl. Protein was estimated by the method of Lowry *et al.* (*6*); flattening factor activity was estimated by determination of the lowest concentration of each fraction which caused Appendix A 1 cells to adhere to a glass surface and induced the attached cells to assume an epithelioid shape (*1*).

The results of the fractionation procedure are illustrated in Table 1. As can be seen from Table 1, eluates 1 and 2 contained 74 percent of the recovered protein but only 6 percent of the recovered activity. On the other hand, most of the recovered activity appeared in eluate 4, which represented less than 12 percent of the protein.

To show that the inactive, peak-protein fractions contained fetuin, one of them (eluate 2) was examined ultracentrifugally and electrophoretically. As is shown in Fig. 1, ultracentrifugation yielded a single, symmetrical peak and the sedimentation constant ($Sw^{20} = 2.73$) was in good agreement with that of fetuin [$Sw^{20} = 2.60$ (*7*)]. In confirmation, electrophoretic analysis revealed a peak whose area was greater than 90 percent of the total area and which showed a mobility of -5.1×10^{-5} cm^2 v^{-1} sec^{-1} [under the same conditions (*8*), the mobility of fetuin in fetal serum was found to be -4.9×10^{-5}].

Ultracentrifugal examination of the active fraction, eluate 4, was complicated by its low protein concentration. However, two well-defined peaks were revealed. The major peak, representing about 80 percent of the total protein, was asymmetric and sedimented at a rate ($Sw^{20} \approx 3$) similar to that of fetuin, while the minor component sedimented

Table 1. Chromatographic separation of fetuin and flattening factor. The eluents contained sodium phosphate buffer (0.025*M*, *p*H 7.1) except for eluate 4, which had instead 0.05*M* KH_2PO_4. In addition, the eluents contained 0.05, 0.075, 0.10, or 0.5*M* NaCl for eluates 1 to 4, respectively.

Fraction	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg of protein)
Fetal serum	600	29,620	49.4
Ammonium sulfate	175	10,580	60.5
Pass through	1.0	0	0
Eluate 1	41.8	0	0
Eluate 2	85.0	250	2.9
Eluate 3	25.2	525	20.8
Eluate 4	19.8	3,390	171

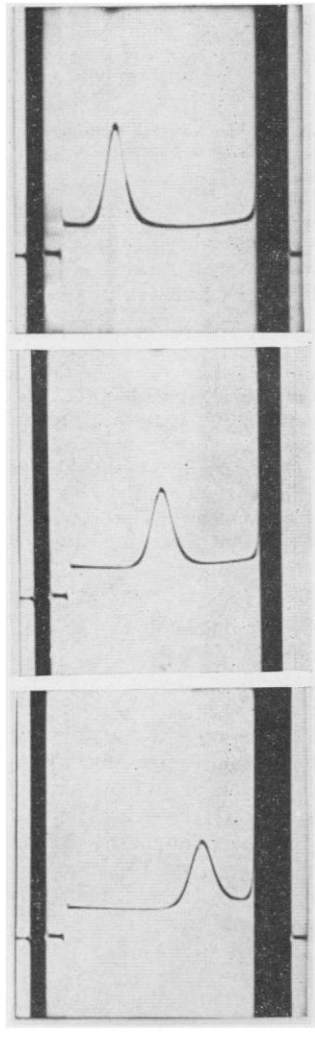

Fig. 1. Ultracentrifugal analysis of chromatographically purified fetuin. The protein, 10 mg/ml, was dissolved in sodium phosphate buffer (pH 7.1, $\Gamma/2 = 0.21$) and centrifuged at 59,780 rev/min. The photographs, from top to bottom, were taken 66, 126, and 186 minutes after maximum speed had been reached. The bar angle was 70 deg.

more rapidly. It was not determined which component contained the active protein.

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Sterility in Female Guinea Pigs Induced by Injection with Testis

Abstract. Adult virgin female guinea pigs were injected with an emulsion of homologous adult testis and Freund's adjuvant before exposure to males. The fertility of this group was only 24 percent while the fertility of the control group was 84 percent. The testis-injected guinea pigs had also a high titer of antibodies against testis.

The production of sterility in the female by immunizing her to sperm of the same species is a possible solution to excessive fertility. Beginning with Landsteiner's work, in 1899 (*1*), antibodies to sperm have been repeatedly demonstrated by complement fixation, sperm immobilization, agglutination, and anaphylaxis (*2, 3*). However, that sterility is induced by immunization of animals with the sperm of the same species is open to serious doubt. A number of observers reported some success, but further work in the field was discouraged by a thoroughly negative report of Henle *et al.* (*4*) in 1940, and since then the subject has been largely neglected. The introduction of Freund's adjuvant for enhancing immunization encouraged us to reinvestigate this approach (*5*).

Female guinea pigs (550 to 700 g) were given three successive intradermal injections 2 and 3 weeks apart. Injections totaled 0.7 ml, distributed in seven sites over the back. A cellular suspension in saline of fresh guinea-pig testis (240 mg per injection) was added to equal amounts of Freund's adjuvant to give a water-in-oil emulsion. Six animals received saline, 13 received Freund's adjuvant plus saline, and 13 received pooled testis plus Freund's adjuvant. Two guinea pigs from each group were bled and sacrificed 38, 61, and 78 days, respectively, after the last injection. No histological change was found in the ovary, uterus, kidney, vagina, or adrenal.

Seven weeks after the last injection, the rest of the animals (7 in the second and third groups) were exposed to a male for 3 weeks, then exposed to a second male for 3 weeks, and then isolated. Nine weeks after isolation the testis-injected guinea pigs were exposed to a male again for 4 weeks and to another male for 5 weeks and then isolated. Eight additional animals were similarly injected with material prepared from a single testis of their partner. The first injection was of freshly prepared material and the second and third injections were kept frozen (193 to 270 mg of testis per injection). Seven weeks after the last injection these animals were exposed to the donor of the testis for 6 weeks. Eight weeks after isolation from the male they were exposed to a second male for 6 weeks and then isolated.

As a second series, 52 guinea pigs (450 to 650 g) were used. Eight were not injected; 14 received Freund's adjuvant only; 14, Freund's adjuvant plus guinea-pig testis (300 mg per injection); 7, Freund's adjuvant plus guinea-pig liver (300 mg per injection); and 8, Freund's adjuvant plus Sherman rat testis (300 mg per injection). The animals were injected in the same manner as those in the first series; the animals in the second series were exposed to a male 7 weeks after the last injection. The male was changed every 3 weeks, and the females were observed for pregnancy for 15 weeks after exposure. Animals injected with guinea-pig testis showed high circulating antibody titers against guinea-pig testis. This was proved by tanned hemagglutination test (titer: control, < 20; experimental, 20 to > 5000); by agar gel diffusion test (three lines with testis saline extract); by sperm immobilization test (all sera and some vaginal fluid from immunized animals immobilized sperm within 5 minutes, even though control sera permitted sperm to live more than 60 minutes); and by positive skin reaction.

All animals were observed for ovarian function by means of vaginal smears from 5 weeks before the first injection until 7 weeks after the last injection. No animal showed ovarian dysfunction. In the first series, six of the seven controls injected with Freund's adjuvant were fertile. Only one of the seven immunized with pooled guinea-pig testis became pregnant, and two of eight given the partner's testis became pregnant. After isolation for 9 weeks, reexposure of the last two groups resulted in the same animals becoming pregnant; all others remained sterile.

Table 1. Summary of fertility in female guinea pigs.

Preparation	Number	Fertile
Controls		
No injection	8	7
Freund's adjuvant	7	6
Freund's adjuvant	14	11
Guinea-pig liver plus Freund's adjuvant	7	7
Rat testis plus Freund's adjuvant	8	6
Totals	44	37 (84%)
Study Animals		
Guinea-pig testis plus Freund's adjuvant	7	1
Guinea-pig testis plus Freund's adjuvant	8	2
Guinea-pig testis plus Freund's adjuvant	14	4
Totals	29	7 (24%)

In the second series, fertility was found in seven of eight noninjected controls; in 11 of 14 given Freund's adjuvant; in seven of seven given liver preparation; and in six of eight given rat-testis preparation. Only four of 14 given guinea-pig testis plus Freund's adjuvant became pregnant. Thus, fertility among the controls was 84 percent (37 of 44), while it was only 24 percent (7 of 29) in the group injected against homologous testis.

There are two possible mechanisms of sterility: (i) cellular immunity, as in Freund's experiment in aspermatogenesis (*3*), and (ii) circulating antibodies which could appear in the vaginal fluid according to our observation or which could cause the uterus to contract on contact with sperm, according to Katsh (*2*). But the mechanism of sterility is still uncertain, and it should be studied in the future.

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