A sample of muscle fiber, suspended in air as we have described, can be observed for many hours. If the sample is allowed to dry slowly, it becomes less and less diamagnetic as the drying proceeds. Eventually, many of our samples became paramagnetic. During the drying, the magnetic anisotropy remains; this means that at a certain stage in the process the muscle fiber is paramagnetic in the direction of the fiber axis and diamagnetic normal to that axis.

Glycerated[5] muscle preparations, as well as air-dried and freeze-dried muscle fibers, showed strong magnetic anisotropy.

Both nerve and tendon taken from the rabbit showed asymmetric magnetic properties. In a sample of fresh beef liver no magnetic anisotropy could be detected with certainty.

SUMMARY

1. The magnetic susceptibility of muscle fiber is asymmetric.
2. The muscle fiber is more diamagnetic normal to its long axis than parallel to it.
3. We feel that magnetic measurements can be made to give useful information about the structure of biological tissues.

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Dr. Mueller's work was done during the tenure of a Research Fellowship of the American Heart Association.

The writers much appreciate Dr. Albert Szent-Györgyi's continued interest in this problem. Dr. Arnold is grateful to Drs. Weinberg and Hollaender, of the Oak Ridge National Laboratory, for permission to work in Woods Hole during the summers. Dr. Steele's new address is the Biochemistry Department, Tulane University Medical School.

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Since writing the above we have found a fine paper by E. Cotton-Feytis and Emmanuel Fauré-Frémiet, *Compt. rend.* **214**, 996–998, 1942 in which they made magnetic measurements on silk, hair, horn and tendon.

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*MOLECULAR GROWTH REQUIREMENTS OF SINGLE MAMMALIAN CELLS: THE ACTION OF FETUIN IN PROMOTING CELL ATTACHMENT TO GLASS**

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Communicated by Dr. George Gamow, October 23, 1957

In an earlier paper[1] it was shown that the growth requirements for small molecules exhibited by single S3 HeLa cells varied with the degree of dialysis of the

macromolecular fraction of serum used as a growth supplement[2] and a medium was defined which permitted good colony formation from single cells, even with extensively dialyzed serum protein preparations. Other experiments showed (*a*) that growth of S3 HeLa cells occurs only in media which render the cell capable of attachment and stretching when a nonagitated suspension is placed on a clean glass surface;[3] (*b*) that serum from different mammalian species[4] and from human subjects of different age groups produces markedly different degrees of stretching of glass-attached cells; and (*c*) that much, if not all, of this glass-attachment and stretching activity is associated with the macromolecular serum component. Fractionation of the macromolecular serum supplement revealed that at least two protein constituents play active roles in cell growth in vitro: serum albumin, which may function at least partly as a carrier of essential small molecules, and an α-globulin, which is important in the attachment of the cells to the glass surface.[1]

After these experiments were completed, a communication by Lieberman and Ove appeared[5] in which the cell-stretching activity of an α-globulin-containing fraction of normal serum was described, with properties similar to that of our preparation. In this paper we describe our experiments dealing with the isolation of an α-globulin-containing fraction from adult serum which promotes attachment and stretching of cells on a glass surface. Further experiments are presented, demonstrating that similar activity is possessed by the Fetuin system of fetal serum and that both Fetuin and the material isolated from normal serum exercise a strong antitryptic action, which may contribute to their biological effectiveness in promoting cell growth in vitro.

METHODS AND MATERIALS

The cells employed were the S3 clonal strain of HeLa, previously described.[4] Cells were trypsinized by our standard technique, plated in the desired number in Petri dishes containing test medium, and incubated at 38° C. in an atmosphere of 5 per cent CO_2. Attachment to glass was measured by microscopic counting on the glass surface either of viable cells or of fixed and stained preparations, a technique also utilized independently by Lieberman and Ove. The titers of the "cell-stretching factor" obtained by us are meaningful only under the specific experimental conditions employed, since the apparent activity of a solution varies greatly with the time of cell incubation and other factors.

EXPERIMENTAL RESULTS

Trypsinized S3 cells pipetted onto a clean glass surface in the presence of normal human, bovine, or equine sera become attached to the glass within 2 hours at 38° C. and stretch out over an area of about 1,000 μ^2.[4] Good attachment and stretching, as illustrated in the photographs of Figure 1*A*, *B*, *C*, are obtained even with serum diluted to about 2 per cent of its original concentration. In the absence of an adequate concentration of the proper serum fraction, the cells either fail to attach or else become loosely and temporarily fixed to the glass as spheres. Such glass attachment and stretching do not occur with the micromolecular serum constituents alone but readily take place with the dialyzed macromolecular portion of serum resuspended in Hanks saline. Fractionation of the active, undialyzable material by

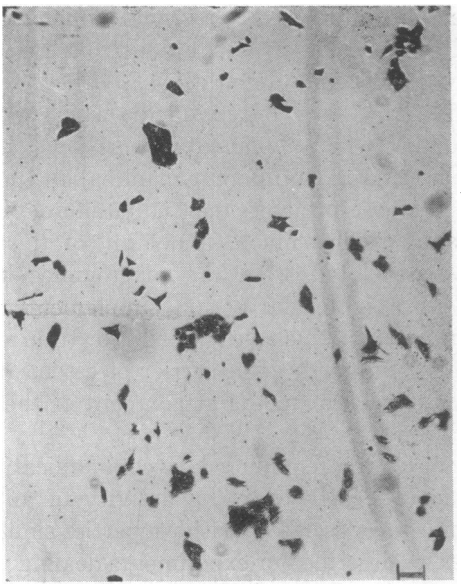

FIG. 1A. Photomicrograph demonstrating the stretched condition of the cells in an adequate concentration of purified Fetuin.

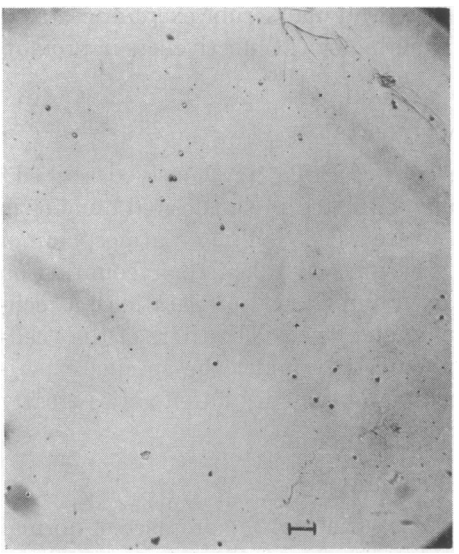

FIG. 1B. Photomicrograph demonstrating the rounded condition of cells, which will shortly be released from their bond to the glass in the absence of any "stretching factor."

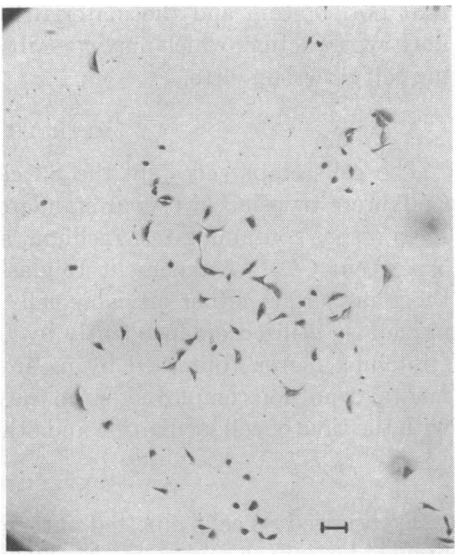

FIG. 1C. Photomicrograph demonstrating the stretched condition of the cells in an adequate concentration of normal serum. The black line represents a distance of 100 μ.

means of $(NH_4)_2SO_4$ was carried out as shown in Table 1, and the activity was found to follow the α-globulin fraction, precipitating in 35–50 per cent saturation of $(NH_4)_2SO_4$. Albumin and γ-globulin displayed no cell-stretching activity. In most of the following experiments, 5–10 mg/cc of purified albumin[6] were added to the medium, because it appeared to assist the nutritional state of the cells with-

out affecting appreciably their response to the "stretching factor." The active fraction was repeatedly precipitated with 45.0 per cent saturated $(NH_4)_2SO_4$ solution, resulting in an α_1-globulin-rich material, of which 70 μg/cc were sufficient to cause dispersed cells to attach to and stretch out on a glass surface and remain in this condition for some days.

TABLE 1

FRACTIONATION PROCEDURE EMPLOYED FOR THE α-GLOBULIN FRACTION OF NORMAL (AND FETAL) SERA

A. One-half volume of saturated $(NH_4)_2SO_4$ is added to 1 volume of serum and allowed to stand several hours at 5° C. and neutral pH. The precipitate is removed and discarded or may be further fractionated to yield purified γ-globulin.
B. The supernatant is adjusted to 50 per cent saturation of $(NH_4)_2SO_4$ by adding the proper volume of saturated $(NH_4)_2SO_4$ and again allowed to stand as described above. The supernatant is discarded.
C. The precipitate is resuspended in 0.85 per cent saline and dialyzed exhaustively against running tap water and finally against 0.85 per cent saline.
D. Steps A through C are repeated two more times as above.
E. Two final cycles of fractional precipitation by $(NH_4)_2SO_4$ are employed, wherein the fraction soluble at 40 per cent saturation and insoluble at 45 per cent saturation of $(NH_4)_2SO_4$ is recovered.
F. The final protein solution is filtered as a concentrated solution through a 0.02 porosity Selas filter and then its protein content determined by the quantitative Biuret reaction.

The properties of this protein material were reminiscent of Fetuin, the α_1-globulin component which comprises 45 per cent of calf fetal serum protein.[7] Therefore, study was initiated to determine whether fetal serum contains more cell-stretching factor than that of more mature animals. Sera were collected from normal and from fetal calves and titrated for their content of stretching factor by noting the highest dilution which would cause attachment and stretching of an S3 cell suspension under standard conditions. Typical results, shown in Table 2, demonstrated that the fetal calf serum contains approximately 10 times as much activity as that from the normal calf.

TABLE 2*

COMPARISON OF THE CONTENT OF STRETCHING FACTOR IN POOLED NORMAL AND FETAL CALF SERA

SERUM CONCENTRATION (Per Cent)	PER CENT OF ORIGINAL CELL INOCULUM REMAINING GLASS-ATTACHED AND STRETCHED AFTER 18 HOURS AT 37° C.	
	Normal Calf	Fetal Calf
10	66.8	105.0
3	34.2	105.0
1	0.3	107.0
0.3	0.0	35.2
0.1	0.0	1.0
0.03	0.0	0.0

* 5×10^4 cells of a monodisperse S3 suspension were pipetted into Petri dishes in a medium containing the indicated amount of serum, 40 per cent of the standard nutrient solution previously described, and Hanks saline to 100 per cent. The plates were fixed and stained after 18 hours of incubation at 38° C., and the number of glass attached, stretched cells on each plate was calculated from microscopic counts of 10 or more representative fields.

Calf fetal serum was fractionated in accordance with the procedure described by Pedersen for isolation of Fetuin.[7] The resulting preparation revealed a single electrophoretic component at pH 8.6, with a mobility of -4.4×10^{-5} cm^2 volt^{-1} sec^{-1} (Fig. 2*A*). Ultracentrifugal analysis resulted in a pattern in which 95 per cent of the material moves with a single boundary, with a sedimentation constant of 2.9 Svedberg units (Fig. 2*B*). Test with diphenylamine reagent[7] indicated the

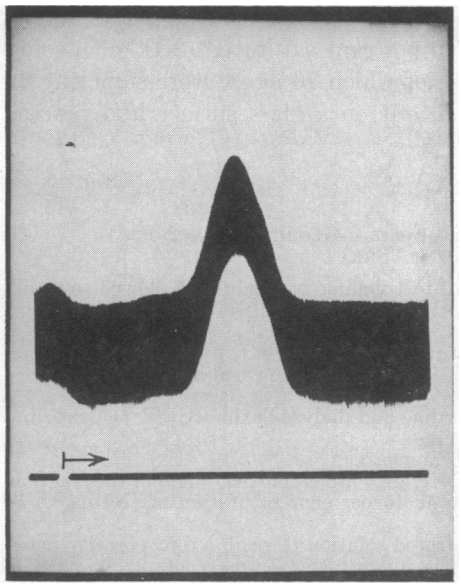

FIG. 2A. A descending electrophoretic pattern of purified Fetuin. The protein concentration is 1.1 per cent in diethylbarbiturate buffer, $\Gamma/2 = 0.1$, at pH 8.6. The slight shoulder indicates some heterogeneity. At pH 4.0 in acetate buffer and the same ionic strength, the material is about 95 per cent homogeneous electrophoretically with mobility indicated in Table III.

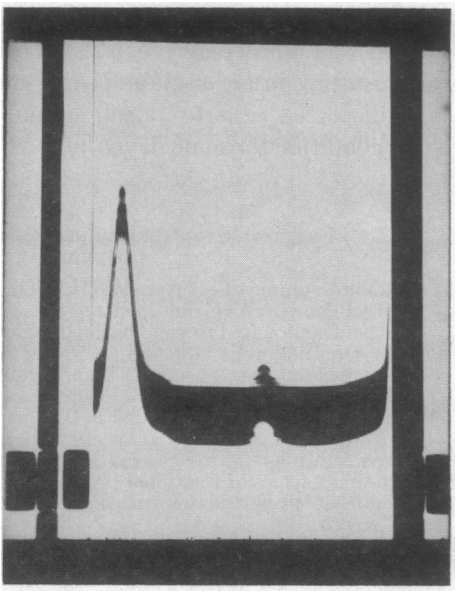

FIG. 2B. A sedimentation pattern of purified Fetuin. The protein concentration and buffer are as in Fig. 2A. The large peak at the left is the slower moving peak corresponding to a sedimentation constant of 2.9 Svedberg units, while the smaller one represents the material with a constant of 17.6 Svedberg units.

presence of sialic acid. These and other properties of the Fetuin prepared in the course of these experiments are summarized in Table 3.

TABLE 3

COMPARISON OF PROPERTIES OF OUR FETUIN PREPARATION WITH THOSE DESCRIBED BY PEDERSEN AND BY DEUTSCH*

Properties	Our Fetuin Preparation	Fetuin Prepared by Pedersen and by Deutsch
Water solubility	Soluble	Unknown
Per cent $(NH_4)_2SO_4$ saturation at which precipitation is initiated	38	38
Solubility in 5 per cent trichloroacetic acid (TCA)	Soluble	Soluble
Electrophoretic mobility ($cm^2\ volt^{-1}\ sec^{-1}$)		
pH 8.6	-4.4×10^{-5}	
pH 4.0	-0.9×10^{-5}	-0.9×10^{-5} (major component)
Sedimentation constant	95 per cent: 2.9 Svedberg units 5 per cent: 17.6 Svedberg units	96 per cent: 2.86 Svedberg units 3–4 per cent: 18.0 Svedberg units
Diphenylamine test for sialic acid of mucoprotein	Positive	Positive

* K. O. Pedersen, *J. Phys. and Colloid Chem.*, **51**, 164, 1947; H. F. Deutsch, *J. Biol. Chem.*, **208**, 669, 1954.

Our Fetuin preparations are highly potent in promoting cell attachment and stretching on glass, equaling in specific activity the most active fractions prepared from the serum of normal animals or adult humans. The total yield of the "stretching factor" from fetal serum is much higher than that obtained from normal adult

serum. This attachment activity of Fetuin requires the presence of divalent cations and can be prevented completely by 10^{-3} M versene. Mg in a concentration of 3×10^{-4} M is maximally active as the stretching cofactor. Ca is less active, while Zn, Cd, Co, and Cu are ineffective.

Fetuin is active as an inhibitor of proteolysis by agents like trypsin.[7] Since mammalian cells are known to be strongly proteolytic when grown in vitro,[8] the mechanism by which Fetuin promotes cell attachment and stretching on a glass surface might involve inhibition of the cellular tryptic action. Hence experiments were designed to test whether Fetuin can prevent the liberation of glass-attached cells by trypsin. Petri dishes were seeded with 2×10^4 S3 cells in complete nutrient medium[4] and incubated at 37° C. in 5 per cent CO_2 for 3–18 hours to insure excellent cell attachment and stretching on the glass. The medium was then removed, the cells washed three times in Hanks saline minus calcium and magnesium, and then resuspended in a trypsin-saline mixture, together with various concentrations of different serum fractions. The results are shown in Table 4 and demonstrate that both Fetuin and the less pure α_1-globulin isolated from adult serum are highly effective in preventing tryptic release of glass-attached cells, in concentrations at which albumin is without activity. Similar experiments showed that γ-globulin also is inactive.

TABLE 4

DEMONSTRATION OF ABILITY OF FETUIN AND OF LESS PURE α_1-GLOBULIN FROM ADULT HORSE SERUM BUT NOT OF ALBUMIN TO PREVENT DETACHMENT OF CELLS FROM GLASS SURFACE BY TRYPSIN*

PROTEIN CONCENTRATION (MG/ML)	PROTEIN TESTED		
	FETUIN	α_1-Globulin from Adult Normal Horse	Human Albumin
2.0	115.%	172.%	37.%
0.4	58.%	4.%	0
0.1	1.%	0.%	0

* Each Petri dish received 5×10^4 S3 cells in complete growth medium containing 30 per cent whole serum. After incubation for several hours, during which all the cells become attached to the glass surface and highly stretched, the liquid was removed, the cells washed three times in saline lacking divalent cations, and 5.0 cc. of this saline, plus the indicated concentrations of proteins, were added. Then trypsin in a final concentration of 0.01 per cent was added to each plate, and all plates were incubated at 38° C. in 5 per cent CO_2 for 10 minutes, after which the number of cells remaining attached in each plate was determined by microscopic counting. The figures above indicate the percentage of cells which remain stretched on glass relative to a control without trypsin.

These experiments do not establish whether the antitryptic factor associated with the α-globulin of normal serum is identical with the much more abundant "stretching factor" present in the Fetuin fraction of fetal serum. The material isolated from normal serum by Lieberman and Ove exhibits two electrophoretic components at pH 8.6 and a pair of sedimentation boundaries, of which the principal one accounts for two-thirds of the total material. It contains 0.043 mg of sialic acid/mg of protein, as opposed to 0.028 mg/mg for our Fetuin preparation. Its solubility in $(NH_4)_2SO_4$ and trichloroacetic acid have not been described. It is noteworthy in this connection that our Fetuin preparations are soluble in water, whereas the α-globulin of Lieberman and Ove is water-insoluble.[5]

Experiments in further fractionation of Fetuin are under way to determine the molecular distribution of the cell-stretching activity among the components of this system.

Experiments which will be reported elsewhere have demonstrated that calf fetal

serum exercises a strong stimulating action on the growth of single mammalian cells which fail to produce good colonies in conventional media.

SUMMARY

Fetuin, which exhibits a single electrophoretic boundary at pH 8.6 and is ultracentrifugally homogeneous to the extent of 95 per cent, is fully active as the factor required for attachment and stretching of S3 HeLa cells on glass. The mechanism of this action of Fetuin may be a reflection of its ability to inhibit tryptic digestion of outer-cell-wall proteins.

* Contribution No. 63. This study has been aided by a grant from the Rockefeller Foundation and from the Commonwealth Fund.

† Candidate for the Ph.D. degree in the department of biophysics. Part of this material is taken from the doctoral thesis of Mr. Fisher.

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[3] Data to be published.

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[6] Thanks are due to Dr. J. T. Edsall and to Dr. D. M. Surgenor, of Harvard University, and to Drs. W. E. Ward and R. B. Houlihan, of Cutter Laboratories, for samples of highly purified albumin used in these experiments.

[7] K. O. Pedersen, *J. Phys. and Colloid Chem.*, **51**, 164, 1947; H. F. Deutsch, *J. Biol. Chem.*, **208**, 669, 1954.

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*THE MECHANISM OF ACTION OF ALDOLASE AND THE ASYMMETRIC LABELING OF HEXOSE**

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Communicated by H. B. Vickery, October 15, 1957

It has been suggested by Bidder[1] that the failure of methyl-labeled lactate to label carbon atoms 1 and 6 (C_1 and C_6) of glucuronic acid to an equal extent ruled out glucose as an intermediate in this interconversion, since it seemed reasonable that, in the guinea-pig liver slices they used, a symmetrically labeled glucose would have resulted from the operation of the Embden-Meyerhof scheme. Similar experiments were performed subsequently by Eisenberg,[2] in which the glucose obtained from the glycogen of liver slices was also examined, and it was reported that the glucose and glucuronic acid had similar ratios of radioactive carbon in the C_1 and C_6 positions ($C_6^{14}/C_1^{14} = 1.06$–1.2). Enzymic evidence now strongly supports the precursor role of a glucose derivative, uridine diphosphoglucose, in glucuronide synthesis.[3] However, the problem of the asymmetric labeling found is not resolved.

Schambye, Wood, and Popják[4] have reported greater labeling of C_3 compared to C_4 in the conversion of asymmetrically labeled glycerol to glucose isolated from liver glycogen of the rat. This discrepancy was greater with time following the