important when analyzing sera from diseased individuals. The reflection of disease in a tuberculous cow was best demonstrated by the new procedure while the standard procedure revealed little except a change in the concentration of bands.

The differential system yielded greater separation of serum proteins from a number of different animals. However, apparently this combination of pore size would not be suitable for all protein mixtures, since the separation of mouse, dog, chicken and pig serum proteins did not improve. Therefore, it is unlikely that any one combination of separating gels will adequately separate all the fractions of a complex mixture.

Summary. A polyacrylamide electrophoretic procedure, in which a complex ionic mixture migrates, through both a large pore and a small pore size gel on a single column is described. The procedure is simple, flexible and results in better separation and resolution of serum proteins. Human serum separated into 44-50 components; and bovine serum into over 30 bands. An economical apparatus for the rapid staining and destaining of disc gels is described which eliminates handling of the gels during the staining and destaining processes.

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Amino Acids and Ammonia of Fetal Calf Serum During Storage.* (31393)

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The addition of fetal calf serum to tissue culture media to promote the growth of cells has received general acceptance since its introduction by Fisher(1). In spite of the widespread use of fetal calf serum in tissue culture, there is a lack of information on stability and changes that fetal calf serum may undergo during storage. Proteolysis, with liberation of amino acids, occurs in pooled human serum(2). This type of enzymatic reaction may also occur in fetal calf serum with loss of the glycoprotein, fetuin, which is believed to be responsible for much of the activity of fetal calf serum(1). These considerations suggested a study of the stability of the growth-promoting properties of fetal calf serum and an analysis of the changes in amino acids during storage as an indication of proteolytic activity.

Methods and materials. Unfiltered, fetal calf serum was obtained from Reheis Chemical Co., Division of Armour Pharmaceutical.† The serum was divided into several portions, one of which was stored at −30° to provide a control for the storage tests. The other portions were stored at 5° and 37°. Sterility tests were conducted on all of the serum samples during and after incubation at 5° and 37° by adding a 0.1 ml sample of serum to

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† Rehatuin, supplied by Mr. Van Ness, Reheis Chemical Co., Chicago, Ill.

5 ml of trypticase soy broth (Baltimore Biological Laboratories). If the samples were contaminated, they were not used in the study.

The free amino acids of the stored fetal calf serum were determined with a Spinco Amino Acid Analyzer after deproteinization with picric acid by the method of Spackman (3).

To determine the growth-promoting activity of the fetal calf serum after storage, Stock Strain L cultures (NCTC clone 929, clone of Strain L) were maintained in a modified Waymouth's medium MB752/1(4). The medium was modified by replacing the salt solution with the tris-citrate salt solution described by Paul(5,6). To 100 ml of the modified medium were added 5 mg of neomycin sulfate and 0.2 mg of amphotericin B and fetal calf serum (10% v/v). The pH of the medium was adjusted to pH 7.6 in all experiments.

Stock cultures were grown in the above medium for 5 days in Blake bottles. The medium was removed and the cells detached from the flask by scraping and suspended by agitation. The cell suspension, after counting in a Coulter counter, was adjusted to 1×10^5 cells/ml. After washing once by centrifugation, the cells were suspended in the modified Waymouth's medium containing fetal calf serum (10% v/v) which had been stored at −30°, 5° or 37°. Two milliliters of the medium containing 1×10^5 cells/ml were pipetted into Leighton tubes. After incubation for 48 hours and 96 hours, the cells were detached from the tubes with 0.25% trypsin (1:300, Baltimore Biological Laboratories) in a citrate buffer(7).

Results. Growth of Strain L cells in fetal calf serum stored at 37° and 5°. The samples stored at 5° gave growth equal (92-110%) to the control (−30°) samples even after 3 months' storage. For this reason, all subsequent analyses were confined to the fetal calf serum samples incubated at 37°. The data for the 37° samples (Table I) indicate that, after 6 days of storage, significant changes in the quality of fetal calf serum occur. Considerable variation exists in the loss of activity (5%-62%) after 6 days' incubation. Since each of the 4 lots represents serum from a different fetal calf, these differences may be a result of variation in the age of the fetal calf. However, information on the size or approximate age of the fetal calf was not available from the supplier.

TABLE I. Growth of Strain L Cells in Fetal Calf Serum Stored at 37°.

Lot No.	Days of incubation 6	12	21	28
1544	38	17	15	
2724	78	72	32	18
2723	68	55	28	
3559	95*	66	50	

Values are per cent of growth of cells grown in the same lot of fetal calf serum frozen at −30°. Cell counts after 4 days of incubation were used.

All values significant $P < .05$ by Mann-Whitney U Test(11) except sample marked with asterisk.

Changes in free amino acids and ammonia of fetal calf serum during storage at 37°. The fetal calf sera used in the growth studies were analyzed for amino acids on the fourth, sixth, twelfth, and twenty-first days of incubation at 37°. Only the amino acids hydroxyproline, proline, glutamine and glutamic acid changed significantly (Table II). Hydroxyproline, proline, and glutamic acid increased significantly during incubation. The instability of glutamine in serum is well known and, as expected, it decreased. The most apparent change was the sharp increase in ammonia concentration from 0.54 μmole/ml to 9.05 μmoles/ml. The pH of the fetal calf serum was 7.1-7.3 at the beginning of the experiments and was within this range at the end of the experiments.

The increase in ammonia suggested the possibility that the accumulation of ammonia

TABLE II. Effect of Storage at 37°C on Amino Acids of Fetal Calf Serum.

Amino acid	Days of incubation 0	6	12	21
Hydroxyproline	.05	.14	.24	.16
Glutamine	.73	.50	.28	.20
Proline	.14	.26	.62	.57
Glutamic acid	.83	1.04	1.28	.89*
Ammonia	.54	1.12	5.42	9.05

Values expressed as μmoles/ml of serum, average of 5 determinations.

* All values are significant $P < .05$ by Mann-Whitney U test except glutamic acid value at 21 days.

TABLE III. Effect of Ammonium Chloride on Growth of Strain L Cells.

Ammonium chloride added to medium (μmoles/ml)	2 days (cells/ml $\times$ 10^3)	4 days (cells/ml $\times$ 10^3)
.0	127.	283.
.1	*114.	207.
.5	72.	91.
1.0	74.	93.
2.0	37.	49.

Concentration of ammonia in culture medium before addition of ammonium chloride was .06 μmole /ml.

* All values significant $P < .05$ by Mann-Whitney U Test *except* .1 μmole at 2 days.

may be responsible for the decreased activity of the fetal calf serum. For this reason, ammonium chloride was added to the tissue culture medium containing fresh 10% fetal calf serum. The ammonium concentrations used (0.1 μmole/ml to 2.0 μmoles/ml) cover the ranges found in fetal calf serum on the fourth day of incubation to those found on the twenty-first day of incubation. The data in Table III show the toxic effect of ammonium chloride at this concentration. A progressive increase in toxicity is noted as the ammonium ion concentration is increased. Since this effect is produced within the concentration range found in stored fetal calf serum, it appears probable that the loss of activity of fetal calf serum may be due to an increase in ammonia during storage.

Discussion. The unfiltered fetal calf serum used in these experiments retains its ability to support the growth of Strain L cells for at least 3 months when stored at 5°C. There is considerable variation in the rate of loss of activity when fetal calf serum is stored at 37°. One lot lost 5% of its activity and another lot lost 62% after 6 days' incubation. This variation might also be apparent at the usual refrigerator temperature if storage tests at 5° were conducted for periods longer than 3 months. However, most laboratories probably do not store fetal calf serum at this temperature longer than 3 months.

Piez *et al*(2) observed marked increases in the free amino acids of dialyzed, pooled human serum. For example, hydroxyproline increased from 0.00 to 7.42 μmoles/ml and lysine from 0.00 to 15.75 μmoles/ml after 28 days at 37° and all amino acids increased. Incubation of fetal calf serum at 37° resulted in increases in hydroxyproline, proline, and glutamic acid, but none of the increases was of the magnitude observed in pooled, dialyzed human serum. The changes in amino acids observed in our experiments would suggest that proteolysis occurs to a very limited extent, if at all, in fetal calf serum.

The increase in ammonia in fetal calf serum is the most notable change that occurs during storage at 37°. Approximately 0.5 μmole/ml of the ammonia may result from glutamine conversion to glutamic acid. The source of the remainder is not apparent from this study. It is evident that the addition of ammonium chloride to the cells decreases the cell counts and the amount of ammonium chloride required to produce inhibition falls within the range found in the incubated fetal calf serum. The data suggest that the formation of ammonia may be responsible for the loss of activity observed during storage of fetal calf serum.

Fetal calf serum is used in media to support the growth of cells for virus production. Therefore, it is of considerable interest that Eaton and Scala(8), Eaton *et al*(9) and Furusawa and Cutting(10) have demonstrated the inhibition of influenza virus and Columbia SK virus by ammonia. The use of fetal calf serum containing ammonia could result in a decrease in virus synthesis in tissue culture.

Summary. 1. Unfiltered fetal calf serum undergoes no change in growth-promoting activity during storage at 5° for 3 months. Six days' storage at 37° results in a loss of activity. 2. Incubation at 37° results in a significant decrease in glutamine and significant increases in hydroxyproline, proline, glutamic acid and phenylalanine. 3. The most notable change was an increase in ammonia from 0.54 to 9.05 μmoles/ml after 21 days' incubation. Addition of ammonium chloride to the Strain L cells at concentrations equivalent to those found in the incubated fetal calf serum produced inhibition of the growth of Strain L cells.

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A New Method for Isolation and Fractionation of Complement Fixing Antigens from *Plasmodium knowlesi.** (31394)

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Although development of methods for effective separation of plasmodia from infected erythrocytes and isolation of purified parasite antigens have been the subject of many investigations during the past 50 years(1), certain major problems have persisted. A variety of methods, including use of enzymes, immune hemolysis, saponin, or other chemical agents, have been employed in efforts to free plasmodia from host red cells(2). Although these agents effectively mediated rupture of the erythrocytes, complete separation of the parasites from host blood components rarely was achieved(3). Moreover, questions have been raised concerning the possibility of a concomitant chemical alteration or loss of antigenic components of the parasite(1,4). Nonchemical lytic methods such as hypotonic lysis, mechanical grinding or cryolysis likewise have been of limited value. These latter methods often resulted in marked destruction of the parasite, or failed to alter the red cell membrane sufficiently to allow effective separation from the parasite. In addition, antigens obtained from parasites processed by these methods frequently exhibited a low order of specificity and sensitivity(1), and in our experience often were anticomplementary.

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Preliminary observations on the properties of host red cells and parasites in malaria infections revealed that the mechanical fragility of nonparasitized and parasitized erythrocytes was considerably greater than that of the parasites. The parasites appeared to be quite stable to many of the forces causing rupture of the host red cells. As a result of these observations, it was felt that the precisely controlled conditions obtainable with a French pressure cell(5) (American Instrument Co., Inc., Silver Spring, Md.) might provide a means whereby the host cells could be selectively fragmented without significant alteration of the parasite. The present studies revealed that preferential fragmentation could be achieved if the pressure was carefully regulated. The method which eventually evolved effectively destroyed the red cell membrane with little or no alteration of parasite morphology, and overcame the need for use of lytic agents which could alter or remove parasite antigens.

Materials and methods. Collection of parasitized cells. Rhesus monkeys were infected by intravenous transfer of fresh *Plasmodium knowlesi* infected blood. Upon reaching the terminal cycle, at which time parasitemias usually ranged from 25-30% or greater, the animals were exsanguinated by cardiac puncture. The blood was collected either in modi-