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QUALITY CONTROL STUDIES ON FETAL BOVINE SERUM USED IN TISSUE CULTURE

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Summary

The quality of the fetal bovine serum (FBS) produced for tissue culture purposes by eight commercial suppliers in the United States was tested over a period of 1 year. The results were compared with tests on some special FBS produced during the same period under conditions which included maximal sterile precautions, freedom from whole cells, and rapid processing in the cold. The findings were that: (a) the specially produced FBS had demonstrably better cell growth-supporting capacity, (b) commercial FBS had a significantly higher free fatty acid content compared to the specially produced FBS, (c) higher free fatty acid content was correlated with poorer cell growth-supporting capacity, (d) extremely wide variations among the different commercial suppliers were found in some of the test results, (e) roughly 10% of commercial lots of FBS were contaminated with bacteria and/or fungi, and (f) at least three different bacteriological culture media, including blood agar plates, were required for adequate sterility testing of FBS. The need for better quality control of FBS is discussed, the method for producing FBS with better cell growth-supporting capacity is described, and both "minimal" and "stringent" ranges of acceptable values for some chemical tests suitable for quality control are given.

This report presents the results of a 1-year study conducted between 1968 and 1969 on the quality of commercial fetal bovine serum (FBS) produced for tissue culture purposes in the United States, and of some specially produced FBS which was procured and processed by methods aimed at obtaining highest possible quality in regard to cell growth-supporting capacity.

The goals of the study were (a) To select and evaluate a panel of tests which could be used by commercial suppliers of FBS to monitor the quality and uniformity of their product. (b) To use the panel to evaluate the quality of FBS produced by major commercial sources in the United States. (c) To evaluate the quality of some special FBS produced under conditions of maximal asepsis, freedom from whole cells, and rapid processing in the cold.

The panel of quality control tests was as follows: (a) Cell growth-supporting capacity (human fetal lung cells): Following initial plants of 10,000 and 20,000 cells per cm^2, cell counts were made 30 and 72 hr later. (b) Chemical measurements: Specific gravity, total protein, γ-globulin, hemoglobin, lactic dehydrogenase, and lipids (total lipids, neutral lipids, phospholipids, cholesterol, free fatty acids) were tested. (c) Contamination with microorganisms: Tests were done for bovine viruses, mycoplasma, bacteria and fungi.

The logistics of the study is shown in Fig. 1. A special FBS Production Facility‡ was set up to produce FBS according to a set of specifications described in "Materials and Methods." One hundred eleven lots of special FBS, 20 liters or

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‡ United States Public Health Service Contract NIH 43-66-539 to Hyland Laboratories, Inc., Los Angeles, Calif.

more each, were prepared by this facility. In addition, a total of 125 lot samples of FBS were obtained from eight different commercial suppliers by purchasing 100-ml bottles through conventional channels. This included the commercial product of Hyland Laboratories, an item separate from the special FBS which they produced under contract to the National Cancer Institute. The volume of the lots from which each of these samples came was 20 liters or more. The lot samples of "special" and "commercial" sera were sent to a Coding Laboratory,* which processed them into coded "unknowns" that were completely unrecognizable as to original source. These were then shipped to a Testing Laboratory,† which performed the panel of quality control tests described above. The distribution of lot samples from the different suppliers of commercial FBS is shown in Table 1.

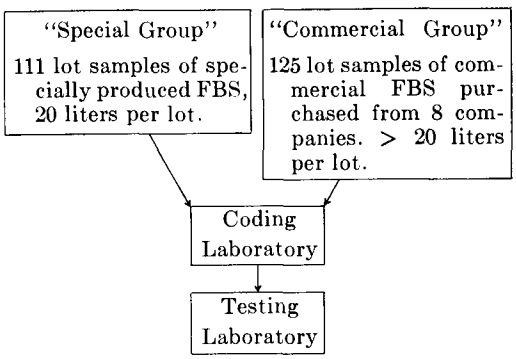

FIG. 1. Logistics of the fetal bovine serum study.

TABLE 1

SOURCES OF COMMERCIAL FBS LOT SAMPLES

Code No. of Supplier*	No. of Lot Samples
S1	21
S2	10
S3	21
S4	20
S5	9
S6	10
S7	16
S8	18
Total	125

* In alphabetical order, which was not necessarily the order of code identification, the commercial suppliers were: Armour, Baltimore Biological Laboratories, Flow Laboratories, Grand Island Biological Company, Hyland Laboratories, Industrial Biological Laboratories, Mazur, and Microbiological Associates.

MATERIALS AND METHODS

Specially Produced Unfiltered FBS

A detailed description of the method used for procuring the special FBS will be published subsequently (1). In summary, the steps were: (a) aseptic bleeding of the fresh fetus by cardiac puncture and drainage into a 500-ml blood donor bottle; (b) centrifuging of the clotted blood in the donor bottle at 0°C; (c) aspiration of serum into a 500-ml Fenwall plastic blood donor bag and centrifugation at 5,000 × *g* to sediment residual red and white cells; (d) transfer of the clarified serum to sterile bottles, removal of a sample for sterility testing, and frozen storage; (e) thawing and pooling of sterility-checked unit bleedings to form lots of 20 litters; and (f) coarse filter clarification to remove fibrin particles, removal of a final sample for sterility testing, and bottling. The main features of the special method were as follows:

1. Fresh fetuses. Fetuses were processed within 30 min after the mother was killed.

2. Aseptic collection procedure. Blood was obtained by cardiac puncture and drainage into a completely closed, sterile collection system.

3. No sterile filtration step. This requirement was included because it provided proof that the most careful sterile techniques were used.

4. Removal of residual red and white cells before freezing. This was done to prevent contamination of the serum with the products of hemolysis and particularly leukocytolysis. The prevention of leukocytolysis was considered important because proteases and other hydrolases released into the serum from leukocyte lysosomes could act on serum components either to lower their nutritional value or to produce toxic products, such as free fatty acids.

5. Rapid processing. A maximal time of 5 hr was permitted from the arrival of the fetus to freezing of the cell-free serum.

Cell Growth-supporting Capacity

Cell stocks. A large pool of third passage human fetal lung cells, explanted and grown in Eagle's minimal essential medium (MEM) with 10% FBS, was stored viably frozen in vials con-

* United States Public Health Service Contract 43-65-641 to Melpar, Inc., Falls Church, Va.

† United States Public Health Service Contract 43-67-1143 to Hyland Laboratories, Inc., Los Angeles, Calif.

taining 5×10^6 cells, 4 ml of medium, and 7.5% dimethyl sulfoxide (DMSO). These cells were used for all assays.

Preparation of test media. The sample of serum to be tested was added to Eagle's MEM to make a concentration of 10% v/v. This was called "test medium."

Preparation of cells for assay. Freshly thawed human fetal lung cells were diluted in 20 ml of test medium and centrifuged, and the supernatant was discarded. The cells were resuspended in 20 ml of test medium, counted with a Coulter electronic cell counter, and centrifuged again. They were again suspended in test medium, planted in glass prescription bottles in half-confluency, and grown to confluency using two changes of test medium. The cells were then trypsin-detached, counted, washed once by centrifugation in 20 ml of test medium, and planted in Falcon 60-mm Petri dishes in triplicate at two different inoculation densities: 10,000 and 20,000 cells per cm^2. At 30 hr and 72 hr after planting, the cells were trypsin-detached and counted. A standard serum sample of known cell growth-supporting capacity was tested in parallel with each test serum.

Chemical Measurements

Specific gravity. At constant known temperature, the density of serum samples was determined using a 25-ml glass pycnometer and a balance with a sensitivity of 0.0005 g. The specific gravity was calculated as the measured density divided by the density of water at the same temperature.

Total protein. Biuret method of Kingsley (2).

Hemoglobin. Spectrophotometric method of Wong (3).

Lactic dehydrogenase. Colorimetric measurement of Wroblewski (4). A reference standard containing LDH was prepared by the method of Henry (5).

γ-Globulin. This was determined by single radial diffusion in an agar-gel system. Immunodiffusion plates (Immuno-plate, Hyland) were prepared into which rabbit antiserum against bovine γ-globulin had been incorporated. Known quantities of bovine γ-globulin were introduced into the wells along with the test serum. After incubation at 37°C, the concentration of γ-globulin in the unknown was determined by comparing the diameter of the precipitation zone with that of the known standards. Linear standard curves could be prepared because of the relationship that the zone diameter is proportional to the logarithm of the concentration of γ-globulin.

Lipid panel. The following were determined according to the references given: total lipids (6), neutral lipids (6), free fatty acids (7), phospholipids (8), cholesterol (9).

Contamination with Microorganisms

Tests for contamination with bovine viruses. The procedures used to screen for bovine virus contamination are described in another publication in this journal (10).

Tests for contamination with Mycoplasma. Semisolid medium as formulated by Hayflick (11) was used. Test serum, 0.5 ml, was inoculated into semisolid medium and incubated anaerobically at 37°C for 1 week. Then 0.05 ml of the culture was plated on solid medium (same composition), and 1.0 ml was transferred to new semisolid medium and incubated for another 7 days. Again, 0.05 ml was plated on solid medium, and 1.0 ml was transferred to fresh semisolid medium and incubated for a final 7 days. Then 0.05 ml of the final culture was plated on solid medium for a third time. All solid medium cultures were observed for *Mycoplasma* species for 2 weeks.

Tests for contamination with bacteria and fungi. A 20-ml aliquot of test serum was incubated for 48 to 72 hr at 37°C, then inoculated into the following media (Difco formulation): brain-heart infusion broth, thioglycollate broth, trypticase soy agar, Sabouraud dextrose agar, Sabouraud dextrose broth, and blood agar.

All cultures were incubated at 37°C except one Sabouraud dextrose tube which was kept at room temperature. Observations were made on the 2nd and 14th days after inoculation.

Statistical Methods

Cell growth-supporting capacity and quality measurements. The test results on the FBS from the commercial suppliers fell into two categories. In the first category, analysis of variance showed no significant difference between suppliers. The mean of all suppliers (commercial group mean) could therefore be compared with the special group mean using a *t* test, and the conclusions

drawn were uniformly representative of all suppliers. In the second category, the means and/or variances of the commercial suppliers differed so widely among themselves that some suppliers were affecting the commercial group mean more than others. In this case a t test comparison of special and commercial group means was not done because the conclusions drawn would not have applied uniformly to all suppliers. Instead, vertical scattergrams of the individual test values from each supplier were compared with each other and with the special group.

Testing for correlations between quality measurements and cell growth-supporting capacity. From both the special group and the commercial group there were selected (a) a subgroup of 10 lot samples having the highest and (b) a subgroup of 10 lot samples having the lowest values of the mean cell count 72 hr after planting 10,000 cells per cm² and the cell count 72 hr after planting 20,000 cells per cm². The two subgroups of 10 lot samples each were then compared with respect to the various quality measurements by means of a t test.

Results

Cell Growth-supporting Capacity

The special group sera, i.e. the 20-liter lots procured and processed according to a special protocol aimed at producing the highest possible quality FBS, did indeed have significantly improved cell growth-supporting capacity (Tables 2, 3, 4, and 5). The number of lots tested was large (81 of the special group and 117 of the commercial group), and the levels of significance of differences in mean cell counts were as follows: 10,000 cells per cm² planted at 30 hr, $p < 0.01$; at 72 hr, $p < 0.05$; 20,000 cells per cm² planted at 30 hr, $p < 0.01$; at 72 hr, $p < 0.06$.

Chemical Measurements

Specific gravity and total protein content (Tables 6 and 7). The means of the special and

TABLE 2

Growth at 30 hr after Initial Incubation of 10,000 Cells per cm²

Data given in cells/cm²

	Special Group Sera	Commercial Group Sera								
		All suppliers	Individual suppliers							
			S1	S2	S3	S4	S5	S6	S7	S8
	cells/cm²									
No. of lot samples	81	117	21	8	15	20	9	10	16	18
Range	$4.7 \rightarrow 44.2 \times 10^3$*	$4.5 \rightarrow 30.0$								
Median value	12.4×10^3	11.5	12.4	15.0	9.4	9.7	12.5	11.0	12.7	11.6
Mean value	14.4×10^3	12.0	13.1	13.8	9.7	11.0	12.0	12.2	12.4	12.6
Variance	60.12×10^6	20.46	18.50	21.91	16.80	19.89	8.16	30.54	16.07	29.13
Standard deviation	7.8×10^3	4.5	4.3	4.7	4.1	4.5	2.9	5.5	4.0	5.4
Standard error of the mean	0.86×10^3	0.42	0.94	1.7	1.1	1.0	0.95	1.7	1.0	1.3

Comparison of the means (special group vs. commercial group): special group (14.4×10^3) > commercial group (12.0×10^3). Difference significant, $p < 0.01$ [$t = 2.8$].

Source of Variation	Analysis of Variance for the 8 Suppliers of Commercial Group Lot Samples			
	Degrees of freedom	Sum of squares	Mean square	
Between suppliers	7	160.6×10^6	22.94×10^6	$F_{7,109} = 1.13$
Within suppliers	109	2212.9×10^6	20.30×10^6	Variations between suppliers *not* significant.

* Multiplicative factors shown in this and the following tables apply to all remaining elements in the same row.

TABLE 3

GROWTH AT 30 HR AFTER INITIAL INCUBATION OF 20,000 CELLS PER CM²

	Special Group Sera	Commercial Group Sera								
		All suppliers	Individual suppliers							
			S1	S2	S3	S4	S5	S6	S7	S8
	cells/cm²									
No. of lot samples	81	117	21	8	15	20	9	10	16	18
Range	$5.6 \rightarrow 79.8 \times 10^3$	$4.8 \rightarrow 46.5$								
Median value	20.6×10^3	17.3	17.9	30.7	12.9	17.5	17.6	15.9	19.0	16.8
Mean value	22.9×10^3	18.5	19.5	20.4	15.2	17.9	18.2	18.0	20.6	18.0
Variance	127.6×10^6	73.2	82.36	118.67	67.48	52.27	50.04	68.23	76.89	93.04
Standard deviation	11.3×10^3	8.54	9.1	10.9	8.2	7.2	7.1	8.3	8.8	9.6
Standard error of the mean	1.26×10^3	0.79	2.0	3.8	2.1	1.6	2.4	2.1	2.2	2.3

Comparison of the means (special group vs. commercial group): special group (22.9×10^3) > commercial group (18.5×10^3). Difference significant, $p < 0.01$ [$t = 3.2$].

Source of Variation	Analysis of Variance for the 8 Suppliers of Commercial Group Lot Samples			
	Degrees of freedom	Sum of squares	Mean square	
Between suppliers	7	290×10^6	41.4×10^6	$F_{7,109} = 0.55$
Within suppliers	109	8165×10^6	74.9×10^6	Variations between suppliers *not* significant.

TABLE 4

GROWTH AT 72 HR AFTER INITIAL INCUBATION OF 10,000 CELLS PER CM²

	Special Group Sera	Commercial Group Sera								
		All suppliers	Individual suppliers							
			S1	S2	S3	S4	S5	S6	S7	S8
			cells/cm							
No. of lot samples	81	117	21	8	15	20	9	10	16	18
Range	$9.7 \rightarrow 17.3 \times 10^3$	$6.2 \rightarrow 10.8$								
Median value	23.9×10^3	19.4	18.6	28.6	14.0	15.7	19.4	18.4	27.4	19.9
Mean value	30.7×10^3	24.8	24.7	31.4	18.3	24.1	22.3	30.0	27.8	23.6
Variance	5.39×10^8	2.99	2.17	3.47	1.22	5.07	0.78	8.86	1.85	2.16
Standard deviation	23.2×10^3	17.3	14.7	18.6	11.1	22.5	8.9	29.8	13.6	14.7
Standard error of the mean	2.5×10^3	1.6	3.2	6.6	2.9	5.0	3.0	9.4	3.4	3.5

Comparison of the means (special group vs. commercial group): special group (30.7×10^3) > commercial group (24.8×10^3). Difference significant, $p < 0.05$ [$t = 2.1$].

Source of Variation	Analysis of Variance for the 8 Suppliers of Commerical Group Lot Samples			
	Degrees of freedom	Sum of square	Mean square	
Between suppliers	7	14.83×10^8	2.12×10^8	$F_{7,109} = 0.70$
Within suppliers	109	331.87×10^8	3.04×10^8	Variations between suppliers *not* significant.

TABLE 5

GROWTH AT 72 HR AFTER INITIAL INCUBATION OF 20,000 CELLS PER CM²

	Special Group Sera	Commercial Group Sera								
		All suppliers	Individual suppliers							
			S1	S2	S3	S4	S5	S6	S7	S8
	cells/cm²									
No. of lot samples	81	117	21	8	15	20	9	10	16	18
Range	$16.6 \rightarrow 230 \times 10^3$	$5.7 \rightarrow 195$								
Median value	39.5×10^3	32.0	27.1	49.1	22.9	30.4	40.9	26.1	42.9	29.4
Mean value	52.2×10^3	42.9	39.4	59.8	34.6	44.4	41.9	43.0	50.0	38.9
Variance	11.81×10^8	11.19	7.44	11.74	8.48	15.85	7.31	20.67	10.57	11.62
Standard deviation	34.4×10^3	33.5	27.3	34.3	29.1	39.8	27.0	45.5	32.5	34.1
Standard error of the mean	3.8×10^3	3.1	5.9	12	7.5	8.9	9.0	14	8.1	8.0

Comparison of the means (special group vs. commercial group): special group (52.2×10^3) > commercial group (42.9×10^3). Difference significant, $p < 0.06$ [$t = 1.9$].

Source of Variation	Analysis of Variance for the 8 Suppliers of Commercial Group Lot Samples			
	Degrees of freedom	Sum of squares	Mean square	
Between suppliers	7	47.58×10^8	6.8×10^8	$F_{7,109} = 0.59$
Within suppliers	109	1251×10^8	11.48×10^8	Variations between suppliers *not* significant.

TABLE 6

SPECIFIC GRAVITY

	Special Group Sera	Commercial Group Sera								
		All suppliers	Individual suppliers							
			S1	S2	S3	S4	S5	S6	S7	S8
No. of lot samples	103	161	27	17	25	23	13	15	18	23
Range	1.0180 → 1.0269	1.0100 → 1.0300	1.016– 1.023	1.017– 1.022	1.019– 1.024	1.015– 1.030	1.019– 1.021	1.010– 1.028	1.018– 1.022	1.012– 1.025
Median value	1.020	1.020	1.021	1.019	1.020	1.020	1.020	1.019	1.021	1.021
Mean value	1.0205	1.0201	1.0204	1.0191	1.0203	1.0205	1.0198	1.0192	1.0204	1.0202
Standard deviation	1.4×10^{-3}	2.4	2.0	1.4	2.4	3.1	0.7	3.4	2.1	3.2
Standard error of the mean	0.12×10^{-3}	0.19	0.39	0.33	0.47	0.65	0.19	0.88	0.50	0.67

commercial sera were not significantly different for these two tests, nor did the suppliers differ significantly among themselves. One commercial lot sample, not included in the tables, had an extremely low specific gravity of 1.0044 and a total protein content of 0.87 mg per 100 ml. Cell growth in this lot sample was 16,200 cells per cm² 72 hr after plating 10,000 cells per cm², and 27,300 cells per cm² 72 hr after plating 20,000 cells per cm². A comparison of these values with those in Tables 4 and 5 shows that the cell growth-supporting capacity of this 50-liter lot was quite poor, especially after 30 hr in culture.

Hemoglobin content and lactic dehydrogenase content (Tables 8 and 9 and Fig. 2). Since the enzyme lactic dehydrogenase (LDH) is present

TABLE 7
TOTAL PROTEIN

	Special Group Sera	Commercial Group Sera								
		All suppliers	Individual suppliers							
			S1	S2	S3	S4	S5	S6	S7	S8
	g/100 ml									
No. of lot samples	108	164	27	17	26	24	13	14	19	24
Range	3.10 → 4.70	1.68 → 5.30	3.00–4.30	3.09–3.80	3.10–4.30	1.68–4.30	3.70–4.10	3.30–5.10	3.00–4.40	2.01–5.30
Median value	3.90	3.80	4.00	3.60	3.80	3.85	3.80	3.70	3.90	3.95
Mean value	3.91	3.81	3.94	3.51	3.79	3.73	3.83	3.76	3.86	3.93
Standard deviation	0.26	0.40	0.29	0.26	0.30	0.51	0.14	0.43	0.33	0.54
Standard error of the mean	0.025	0.031	0.056	0.063	0.059	0.105	0.038	0.114	0.076	0.111

Comparison of the means (special group vs. commercial group): special group (3.91) > commercial group (3.81). Difference nominally significant, $p < 0.05$ [$t = 2.3$].

Source of Variation	Analysis of Variance for the 8 Suppliers of Commercial Group Lot Samples			
	Degrees of freedom	Sum of squares	Mean square	
Between suppliers	7	2.564	0.3664	$F_{7,156} = 2.43$
Within suppliers	156	23.025	0.1476	Variations between suppliers marginally significant, $p < 0.025$.

TABLE 8
HEMOGLOBIN CONTENT

	Special Group Sera	Commercial Group Sera								
		All suppliers	Individual suppliers							
			S1	S2	S3	S4	S5	S6	S7	S8
	mg/100 ml									
No. of lot samples	108	164	27	17	26	25	13	14	19	23
Range	2.9 → 38.1	10.0 → 110.0	11.5–110.1	21.0–46.5	10.0–96.0	16.2–55.0	13.0–57.4	10.8–46.0	15.5–35.0	19.0–53.0
Median value	16.4	28.6	28.7	32.3	29.4	34.0	25.0	17.7	22.0	28.5
Mean value	17.3	30.6	35.7	31.9	33.9	35.3	27.4	19.8	23.1	29.2
Standard deviation	6.5	15.0	24.2	7.0	18.9	8.7	12.5	8.4	5.3	9.4
Standard error of the mean	0.63	1.17	4.65	1.69	3.71	1.74	3.48	2.25	1.23	1.95

in leukocytes as well as erythrocytes, it was included in the panel of chemical tests to see if it could be used as a measure of the contamination of FBS with the products of leukocytolysis as well as those of hemolysis. Leukocytolysis was deemed important to avoid because it results in the contamination of serum with activated lysosomal hydrolases which could degrade serum components and possibly produce toxic products, e.g. free fatty acids.

Both the hemoglobin and the LDH contents of the special group sera were predictably much lower than those of the commercial group sera because of the care taken to remove as many red and white cells as possible before freezing. Note the wide range of variation in hemoglobin and LDH contents of the commercial sera in Fig. 2, especially for suppliers S1 and S3. This is reflected in the wide variation in supplier variances in Tables 8 and 9. The ranking of suppliers

TABLE 9

LACTIC DEHYDROGENASE (LDH) LEVELS

	Special Group Sera	Commercial Group Sera								
		All suppliers	Individual suppliers							
			S1	S2	S3	S4	S5	S6	S7	S8
	LDH units									
No. of lot samples	96	96	11	12	17	15	5	11	12	13
Range	590 → 1,840	300 → 3,320	610–1,650	720–3,320	300–2,520	750–2,800	1,000–1,270	1,080–2,080	1,000–1,960	580–2,060
Median value	1,020	1,420	1,130	1,010	1,560	1,820	1,130	1,490	1,690	1,300
Mean value	1,067	1,478	1,068	1,362	1,516	1,997	1,128	1,540	1,602	1,250
Standard deviation	257	537	292	746	493	608	97	296	307	390
Standard error of the mean	27	55	88	215	120	157	43	89	89	108

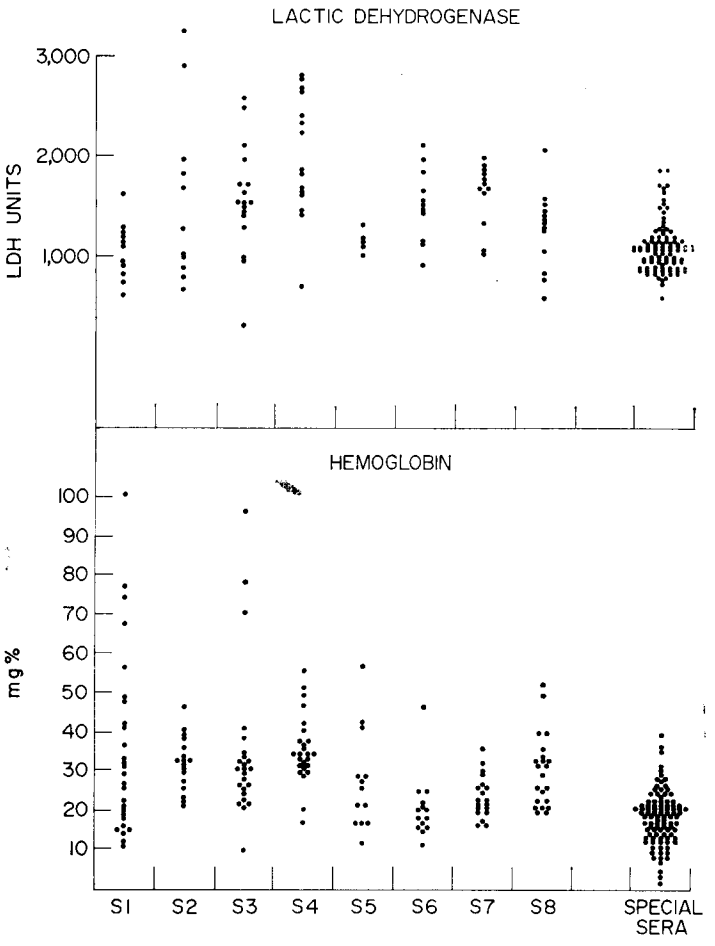

FIG. 2. Hemoglobin and lactic dehydrogenase content of fetal bovine serum produced by commercial suppliers (S1, S2, etc.) and by the special procedure designed to achieve highest quality in regard to cell growth-supporting capacity. Each point represents a lot of 50 liters or more in the case of the commercial sera, and 20 liters in the case of the special sera.

TABLE 10
γ-GLOBULIN

	Special Group Sera	Commercial Group Sera								
		All suppliers	Individual suppliers							
			S1	S2	S3	S4	S5	S6	S7	S8
	mg/100 ml									
No. of lot samples	90	163	28	17	25	22	12	18	19	22
Range	12 → 200	0 → 470	0–120	15–220	20–112	13–185	16–245	23–470	17–150	13–330
Median value	42	39	35	43	38	38	36	46	60	37
Mean value	49.0	60.9	40.3	52.4	45.1	50.4	53.1	129.2	63.7	68.2
Standard deviation	31.7	67.6	26.6	45.4	23.2	36.5	61.1	143.6	25.1	81.0
Standard error of the mean	3.3	5.3	5.0	11.0	4.6	7.8	17.6	33.8	5.8	17.3

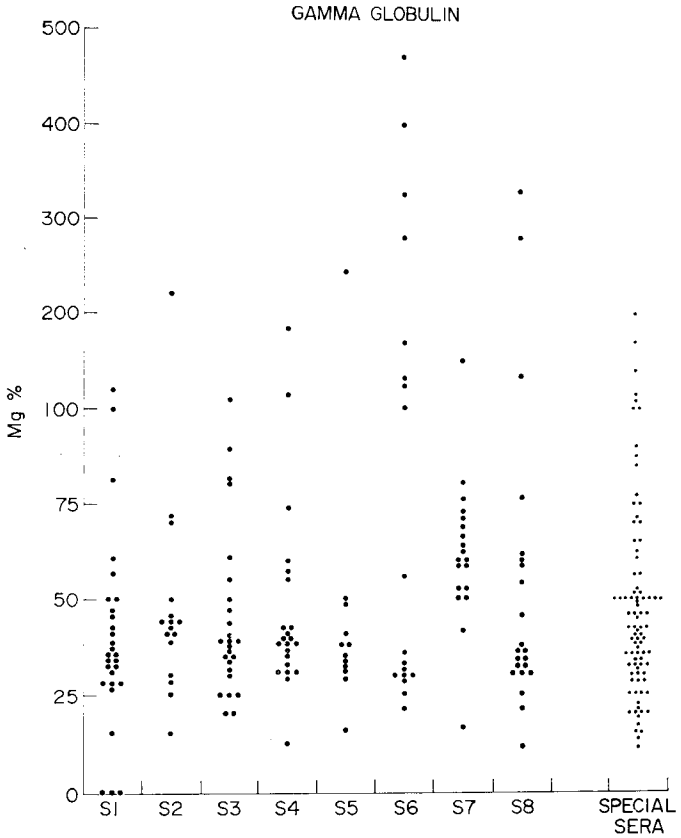

FIG. 3. γ-globulin content of fetal bovine serum produced by commercial suppliers (S1, S2, etc.) and by the special procedure designed to achieve highest quality in regard to cell growth-supporting capacity. Each point represents a lot of 50 liters or more in the case of the commercial sera, and 20 liters in the case of the special sera.

from highest to lowest average LDH values did not correspond to the ranking for average hemoglobin values, indicating a possible dissociation between the degree of red and white cell contamination.

γ-Globulin content (Table 10 and Fig. 3). This measurement was selected for evaluation because it could serve as a check on the adulteration of FBS with cheaper calf serum, and also because it might be correlated with higher levels

of antiviral antibodies which could act as inhibitors in virus-cell culture. Most noteworthy is the extremely wide range of variations of supplier S6 compared to the other suppliers. The variance in Table 10 reflects this.

Lipid panel (total lipids, neutral fats, cholesterol, phospholipids, free fatty acids (Tables 11 to 15 and Fig. 4). The tables and figure indicated show no significant differences between commercial suppliers, or between the means of the commercial group as a whole and the special group, for total lipids, neutral fats, cholesterol, or phospholipids. However, the variances for the commercial sera were always larger than for the special sera.

Both Table 15 and Fig. 4 show that *the mean free fatty acid content of the commercial sera was higher than that of the special sera ($p < 0.01$). This finding is of special importance because it is the only chemical measurement which correlated with cell growth-supporting capacity* (see below).

Tests for Correlation between Chemical Measurements and Cell Growth-supporting Capacity of FBS

From both special group and commercial group sera there were selected subgroups of 10 lot samples having the highest (subgroup *a*) and the lowest (subgroup *b*) values for the mean of the cell counts 72 hr after planting 10,000 and 20,000 cells per cm^2. The differences in growth-supporting capacity between subgroups *a* and *b* was significant at the $p < 0.01$ level in all cases. Groups *a* and *b* were then compared in regard to the chemical measurements discussed above. For both special group and commercial group sera, *no significant differences were observed between the two subgroups for specific gravity, protein, hemoglobin, LDH, or γ-globulin.*

TABLE 11

TOTAL LIPIDS

	Special Group Sera	Commercial Group Sera								
		All suppliers	Individual suppliers							
			S1	S2	S3	S4	S5	S6	S7	S8
	mg/100 ml									
No. of lot samples	34	60	9	6	12	11	4	4	5	9
Range	230 → 340	140 → 440	225–345	210–330	180–360	140–440	245–350	210–335	290–400	270–390
Median value	295	310	300	302	320	310	310	295	325	305
Mean value	294	308	296	291	312	311	304	284	331	319
Standard deviation	26.7	52.34	44.0	4.18	48.5	79.7	44.2	59.9	41.6	43.0
Standard error of the mean	4.6	6.8	14.7	17.0	14.0	24.0	22.1	30.0	18.6	14.3

TABLE 12

NEUTRAL FATS

	Special Group Sera	Commercial Group Sera								
		All suppliers	Individual suppliers							
			S1	S2	S3	S4	S5	S6	S7	S8
	mg/100 ml									
No. of lot samples	34	61	11	7	13	8	6	3	5	8
Range	145 → 235	90 → 305	150–245	120–215	120–295	90–280	100–215	145–240	205–305	175–260
Median value	198	205	195	200	230	202	195	160	215	202
Mean value	190	204	197	186	224	201	177	182	235	210
Standard deviation	22.8	42.5	27.4	34.1	43.7	57.5	45.5	51.1	41.4	30.7
Standard error of the mean	3.9	5.4	8.3	12.9	12.1	20.3	18.6	29.5	18.5	10.9

TABLE 13
CHOLESTEROL CONTENT

	Special Group Sera	Commercial Group Sera								
		All suppliers	Individual suppliers							
			S1	S2	S3	S4	S5	S6	S7	S8
	mg/100 ml									
No. of lot samples	34	60	12	7	12	8	6	3	5	7
Range	35 → 60	20 → 90	30–75	40–55	25–60	20–90	40–55	30–50	35–50	35–55
Median value	45	45	45	45	45	50	48	40	45	45
Mean value	45	46.8	47.1	45.7	46.2	51.9	48.3	40.0	45.0	45.7
Standard deviation	4.8	11.3	13.9	6.1	8.6	21.0	6.1	10.0	6.1	7.3
Standard error of the mean	0.8	1.5	4.0	2.3	2.5	7.4	2.5	5.8	2.7	2.8

TABLE 14
PHOSPHOLIPIDS CONTENT

	Special Group Sera	Commercial Group Sera								
		All suppliers	Individual suppliers							
			S1	S2	S3	S4	S5	S6	S7	S8
	mg phosphorus/100 ml									
No. of lot samples	34	62	10	7	13	9	5	4	5	9
Range	30 → 40	20 → 55	25–45	25–40	20–45	20–50	20–50	20–40	30–45	20–55
Median value	35.0	35	35	30	35	40	40	28	40	30
Mean value	34.0	34.8	35.0	30.7	35.4	38.9	38.0	28.8	38.0	32.2
Standard deviation	3.6	8.4	6.2	5.3	8.0	8.2	10.4	10.3	5.7	11.5
Standard error of the mean	0.6	1.1	2.0	2.0	2.2	2.7	4.6	5.2	2.5	3.8

The lipid panel was performed on fewer lot samples from both groups of sera: 33 to 34 of the special group and 44 to 47 of the commercial group. Nevertheless, a significant ($p < 0.05$) difference in cell growth-supporting capacity (mean of cell counts 72 hr after planting 10,000 and 20,000 cells per cm^2) was again found between subgroups *a* and *b*. The two subgroups were evaluated for total lipids, cholesterol, phospholipids, neutral fats, and free fatty acids. *The only significant difference noted was that of a higher fatty acid content for subgroup b ($p < 0.05$) in the commercial group sera.* Thus, fatty acid content correlated negatively with cell growth-supporting capacity. The toxic effect of free fatty acids on cultured cells has been reported (12).

Tests for Contamination with Viruses, Mycoplasma, Bacteria, and Fungi

Viruses. At the termination of this study, only 10 lots of special group and 13 lots of commercial group sera had been screened by the procedure of Molander et al. (10).

Of these, one lot of commercial serum was found to be contaminated with infectious bovine rhinotracheitis virus (13). Subsequently, Molander et al. (14), using the same screening procedure, found that approximately 10% of over 100 lots of FBS tested (approximately 50 liters per lot) were contaminated with bovine viruses. The most common viruses found were infectious bovine rhinotracheitis virus, bovine virus-diarrhea virus, and parainfluenza type 3 virus.

Mycoplasma. No mycoplasma organisms were found in 111 lot samples of special group sera, by the culture method of Hayflick (11). This finding is consistent with the statement of Barile (15) that all published attempts to isolate mycoplasma species from FBS have been reported as unsuccessful. However, recently Barile and Kern (16) found that, when they took 25-ml samples (instead of the conventional 0.5-ml samples taken in our study), bovine mycoplasma

TABLE 15
FREE FATTY ACIDS

	Special Group Sera	Commercial Group Sera							
		All suppliers	Individual suppliers						
			S1	S2	S3	S4	S5	S6	S7	S8
	mEq/liter									
No. of lot samples	33	60	11	7	13	7	5	5	5	7
Range	0.25 → 0.41	0.10 → 0.64	0.11–0.26	0.15–0.39	0.13–0.64	0.17–0.31	0.10–0.22	0.13–0.33	0.14–0.57	0.25–0.46
Median value	0.163	0.21	0.17	0.21	0.22	0.22	0.22	0.20	0.19	0.35
Mean value	0.176	0.242	0.185	0.227	0.266	0.220	0.190	0.222	0.264	0.359
Standard deviation	0.055	0.105	0.047	0.080	0.131	0.048	0.077	0.076	0.176	0.076
Standard error of the the mean	0.009	0.014	0.014	0.030	0.036	0.018	0.034	0.034	0.078	0.029

Comparison of the means (special group vs. commercial group): special group (0.176) < commercial group (0.242). Difference significant, $p \sim 0.001$ [$t = 3.38$].

Source of Variation	Analysis of Variance for the 8 Suppliers of Commercial Group Lot Samples			
	Degrees of freedom	Sum of squares	Mean squares	
Between suppliers	7	0.1607	0.02296	$F_{7,52} = 2.46$
Within suppliers	52	0.4845	0.00932	Variations between suppliers nominally significant, $p < 0.05$.

species were indeed present in 17 of 139 lots of FBS from commercial sources.

Bacteria and fungi. Of 130 20-liter lots of unfiltered FBS produced for the special group, 19 lots were found to be contaminated with bacteria alone (4 lot samples) or with bacteria and fungi (15 lot samples); these were therefore excluded from the special group. *The success rate in producing 20-liter lots of unfiltered FBS was thus 85%.*

Table 16 displays the different culture media which were positive for each of the 19 contaminated lot samples obtained during the production of the special group sera. Note that less than a minimal group of three different media would not have detected all of the contaminated samples.

A significant number of commercial group lot samples were found to be contaminated with bacteria and fungi, even though these had presumably been checked for sterility before being placed on the market. Twelve out of 125 lot samples, or 9.6%, were contaminated with bacteria alone (8 lot samples), fungi alone (2 lot samples), or bacteria and fungi (2 lot samples).

Table 17 shows the distribution of contaminated sera among suppliers. Note that 6 of the 12 contaminated lot samples were from one supplier, 3 from a second, 2 from a third, and 1 from a fourth. These represented 29, 19, 10, and 5% of the total number of lots tested from these suppliers, respectively.

Table 18 displays the culture media which were positive for each of the 12 contaminated commercial lot samples. The pattern is strikingly different from that of the special group sera. For the commercial group, blood agar plates were positive with much higher frequency, with lower frequencies for the other media, including thioglycolate medium. An explanation for this could be that if a supplier used only thioglycolate medium for sterility checking, he would allow to pass as "sterile" lots which were contaminated with microorganisms that would not grow in thioglycolate but which would grow on blood agar or in other media. The conclusion must be drawn that a large battery of culture media, such as those used in this study, are necessary for adequate sterility testing of FBS. In particular, blood agar plates appear to be the most useful.

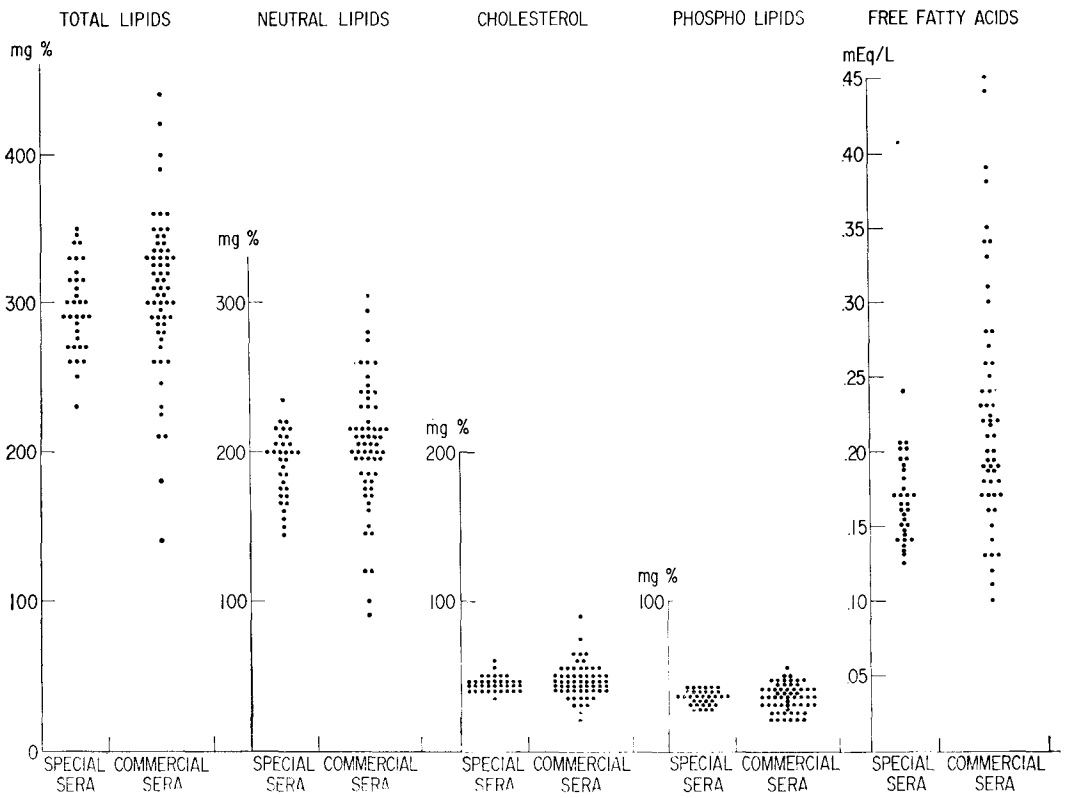

FIG. 4. Panel of tests for various lipids in fetal bovine serum produced by commercial suppliers and by the special procedure designed to achieve highest quality in regard to cell growth-supporting capacity. Each point represents a lot of 50 liters or more in the case of the commercial sera, and 20 liters in the case of the special sera.

DISCUSSION

1. A method has been developed for producing fetal bovine serum with demonstrably better cell growth-supporting capacity. Commercial producers of FBS should consider including the following items in their production methods: (a) the use of fresh fetuses, (b) bleeding by cardiac puncture into a sterile closed system, (c) complete removal of red and white cells from the serum before freezing, (d) keeping the time between bleeding and initial freezing as short as possible (less than 5 hr), and (e) keeping the temperature as close to 0°C as possible during processing.

2. Commercial fetal bovine sera contained significantly higher free fatty acid levels compared to specially produced sera.

3. Higher free fatty acid levels were correlated with lower cell growth-supporting capacity. In view of findings 1 and 2 above, it is evident that at least one of the reasons why the commercial FBS had a poorer cell growth-supporting capacity was because of its higher average content of free fatty acids. A logical source of free fatty acids in FBS would be those produced by the action of the leukocyte lysosomal lipases postulated by deDuve (19) on serum lipids. This possibility supports the recommendation that red and white cells be excluded from FBS before freezing. The bovine Herpesviruses and myxoviruses now known to contaminate FBS could well be concentrated within leukocytes or adsorbed to red cells; this provides an additional reason for the elimination of all cellular elements before freezing.

4. Extremely wide variation in chemical measurements was found among the different commercial suppliers of fetal bovine serum. The 50-liter lot sample with a protein content of 0.85% (average = 3.87%) associated with poor cell growth-supporting capacity and the extremely wide variation in γ-globulin among lot samples

TABLE 16

LOT SAMPLES REPORTED POSITIVE FOR BACTERIA OR MYCOTIC ORGANISMS DURING THE PROCUREMENT OF SPECIAL SERA

Lot Sample No.	Culture Media for Which Specimen Was Positive				
	Brain-heart infusion agar	Thio broth	Trypticase soy agar	Sabouraud agar	Blood agar plates
1		+		+	
2		+	+	+	+
3	+	+	+	+	+
4	+	+	+	+	+
5	+	+	+	+	+
6	+	+	+	+	+
7	+	+	+	+	+
8	+	+	+	+	+
9	+	+	+	+	+
10	+	+	+	+	+
11					+
12	+	+	+	+	+
13	+	+	+	+	+
14	+	+	+		+
15	+	+	+	+	+
16	+	+	+	+	+
17		+			
18		+	+	+	+
19	+		+		

from supplier S6 are two of many examples of the wide variations in test results which were found. This attests to the need for better quality control of FBS. It should be noted that one possible cause of low protein content is adulteration with physiological salt solution, and of high γ-globulin content, adulteration with less expensive postcolostrum calf serum.

5. Roughly 10% of commercial lots of fetal bovine serum were found to be contaminated with bacteria and/or fungi. Although contamination rates at the present time may be reduced because of the increased interest in better sterility checking shown by commercial producers in recent years, it is still reasonable to assume that unacceptably high levels of contamination may exist in commercial FBS. Probably the most dependable prophylactic procedure is that the individual users of fetal bovine serum perform another sterile filtration in their own laboratories. When this is done, contamination with bacteria and fungi is reduced practically to zero (20).

6. At least three different bacteriological culture media, including blood agar plates, should be used for sterility testing of fetal bovine serum. As this study shows, commercial suppliers who use at least three different culture media for bacterial sterility testing, including especially blood agar plates, can expect their final products to have a lower contamination rate.

7. There is recent evidence that commercial fetal bovine serum is frequently contaminated with Mycoplasma. All of the 0.5-ml aliquots of FBS lots in this study were found to be negative for the presence of *Mycoplasma* species. However, Barile and Kern (16) have recently shown that, when *25-ml* aliquots of commercial FBS were tested, roughly 10% of various lots of commercial FBS were found to be contaminated with bovine mycoplasma.

8. Unfiltered serum: the success rate in producing 20-liter lots of sterile unfiltered fetal bo-

TABLE 17

DISTRIBUTION OF CONTAMINATED LOT SAMPLES OF COMMERCIAL SERA AMONG SUPPLIERS

Supplier Code	No. of Lot Samples Tested	No. Contaminated with Bacteria or Fungi	Percentage of Lot Samples Contaminated
S1	21	6	29
S7	16	3	19
S3	21	2	10
S4	20	1	5

TABLE 18

LOT SAMPLES OF COMMERCIAL SERA REPORTED POSITIVE FOR BACTERIA OR MYCOTIC ORGANISMS

Lot Sample No.	Supplier Code	Culture Media for Which Specimen Was Positive				
		Brain-heart infusion agar	Thio broth	Trypticase soy agar	Sabouraud agar	Blood agar plates
1	S3	+	+	+	+	+
2	S3		+			
3	S7				+	
4	S1					+
5	S1			+	+	+
6	S1					+
7	S1					+
8	S7					+
9	S4					+
10	S1				+	
11	S1					+
12	S7					+

TABLE 19
95% RANGES OF CHEMICAL TESTS ON FETAL BOVINE SERUM

Chemical Test	95% of Lots Were:	No. of Lots Tested	Mean
Commercial Sera			
Hemoglobin, mg/100 ml	Less than 68	117	31.12
LDH, units	Less than 2,600	69	1,542
Specific gravity	Greater than 1.017	118	1.020
Total protein, %	Greater than 3.10	116	3.86
γ-Globulin, mg/100 ml	Less than 280	117	50.6
Free fatty acids, mEq/liter	Less than 0.440	46	0.229
Specially Produced Sera			
Hemoglobin, mg/100 ml	Less than 28	108	17.31
LDH, units	Less than 1,650	96	1,067
Specific gravity	Greater than 1.019	103	1.020
Total protein, %	Greater than 3.51	108	3.91
γ-Globulin, mg/100 ml	Less than 100	90	49.0
Free fatty acids, mEq/liter	Less than 0.22	33	0.176

vine serum was 85%. The reason for specifying unfiltered serum in the special FBS study was to provide unequivocal evidence that aseptic procurement and processing had been carried out. However, for commercial suppliers to produce unfiltered FBS would be costly and of no particular advantage. Instead of specifying unfiltered serum, the quality control of sterility can be simply handled by taking samples for sterility testing immediately prior to a final sterile filtration step.

9. The need for better quality control of commercial FBS is amply demonstrated by this study. The recommended tests for cell growth-supporting capacity and sterility are those described above. The panel of tests for chemical composition, with recommended ranges of acceptable values, is summarized in Table 19. Two sets of values are shown, those which apply to 95% of the commercial group samples, and those which apply to 95% of the special group samples. These two sets could well be the basis for "minimal" or for "stringent" specifications required by the purchaser of large quantities of FBS.

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