NUTRITIONAL AND IMMUNOLOGICAL EVALUATION OF COMMERCIAL ANIMAL PROTEIN PRODUCTS

by

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INTRODUCTION

Modern technology has produced a high degree of specialization in different fields of science with the consequent loss of a clearly defined common objective, frequently resulting in inefficient use of the potential and resources that diverse scientific disciplines can provide to solve many current problems.

The packing industries in the United States obtain a number of by-products, many of which are of biological value. Blood and its fractions, are among the by-products of commercial importance. These proteins are at present used only for some industrial processes, but their biological value has yet to be thoroughly examined on the basis of their natural function in the animal. The most obvious components of interest in this respect are the γ globulins since they contain protective antibodies. The use of specific antibodies to provide a passive immunity against disease has been for the most part superseded by the use of vaccines and chemotherapeutic drugs. Still, much of the recent research has been devoted towards the administration of γ globulins to prevent diseases in newborn animals using the physiological ability of these animals to absorb intact macromolecules through the gastrointestinal epithelium for a time after birth.

Another area of interest is the use of animal protein by-products as components of cell culture media. Serum

proteins have long been used in this respect to provide the necessary macromolecular requirements for cell growth. Tissue extracts and protein hydrolyzates have also been used extensively as a source of some of the micromolecular requirements for cell growth *in vitro*.

The present study was undertaken to evaluate: 1) a heat-desiccated bovine serum as a source of bovine antiviral antibodies and as a source of macromolecules for cell cultures, and 2) a collagen hydrolyzate as a source of amino acids for cell cultures. Both products are manufactured by the Rath Packing Company.

LITERATURE REVIEW

The Feeding of Serum to Neonatal Calves

The presence of antibodies against various common bovine viruses has been reported in serums of adult bovines in the United States. A serological survey conducted in the state of Massachusetts by Smith et al. (40), showed that of 589 random serum samples tested, 12.7 percent contained antibodies against infectious bovine rhinotracheitis virus (IBR) and 19.4 percent contained antibodies against bovine virus diarrhea agents (BVD), when tested by virus-neutralization. Hoerlein et al. (21) reported that 68.6 percent of the bovines that had suffered respiratory illnesses had significant myxovirus parainfluenza-3 (MP-3) antibody titer rises, measured by hemagglutination inhibition. Kunin and Minuse (26) isolated eight strains of bovine enteroviruses from a herd of dairy cattle. Of these animals 88.5 percent were serologically positive when tested with the LCR-4 strain of bovine enterovirus (BE). In a herd from which no virus could be isolated, 50 percent of the animals had virus-neutralizing titers.

The importance of feeding colostrum to prevent infectious diarrhea (calf scours) of newborn calves was originally pointed out by Smith and Little (41). Reisinger (37) has reviewed a number of later reports confirming these findings.

The etiology of this condition, according to the latter author, has only been partially elucidated. Although the role of E. coli seems to be well established in causing the disease some common bovine viruses may also be incriminated. This disease is considered to cause a 10 percent yearly calf loss according to estimates by Amstutz (2). Smith and Little (41) and (42) attempted to replace colostrum using specific immune serum to E. coli. They obtained almost the same degree of protection provided that it was fed to the calves in the first hours after birth.

The Use of Serum Macromolecules in Cell Culture Media

Evans and Earle (14) succeeded in cultivating animal cells on perforated cellophane using a fluid overlay. Puck et al. (35) obtained clones of mammalian cells on glass surfaces. These findings made available some systems where cell growth could be accurately quantitated. A number of methods to measure cell growth are now available. Several such methods are presented by Merchant et al. (30).

Complete studies of the requirements for mammalian cell growth in vitro have been made. Sato et al. (39), Fisher et al. (16, 17) and Fisher et al. (15) showed that the α globulin fraction of adult mammalian serum and fetuin, and α globulin present in fetal bovine serum contained a factor promoting attachment of cells to solid surfaces and subsequent growth. Serum albumin had a protective action for the

cell and together with other serum proteins served as a binder of cholesterol and other necessary micromolcular compounds. Phillips and Andrews (34) and Ham (19) made additional observations confirming these functions of serum albumin in cell culture media.

Disc Electrophoresis of Mammalian Serums

The disc electrophoresis technique was developed by Ornstein and Davis in 1959 and published by Ornstein (33) and Davis (10). These authors demonstrated the presence of more than 20 components in human serum. They also made a tentative identification of these bands by comparison with data obtained by Smithies (43) using starch gel electrophoresis. Narayan et al. (31) studied the components seen in rat serum by disc electrophoresis. They made a tentative identification of the components seen using the data reported by Beaton et al. (6) on starch gel electrophoresis of rat serum. Ashton (3, 4) described various genetic phenotypes in bovine serum, demonstrating variations in the β and slow α globulin bands by use of starch gel electrophoresis. Using the same technique, Giblett (22) observed the presence of transferrins in all the β globulin bands of human serum by Fe^{59} uptake.

The Use of Amino Acids in Cell Culture Media

Amino acids are widely used in cell culture media either

as protein hydrolyzates or as defined synthetic mixtures. Baker and Carrel (5) showed that embryo explants grown *in vitro* required embryo extract. Dialysis of the embryo extract resulted in reduction of its growth-promoting capacity partly due to the loss of amino acids.

Melnick (29) and Fogh and Lund (18) described various culture media containing 0.5 percent of an enzymatic hydrolyzate of lactalbumin. Neumann and Tytell (32) used a serum-less medium for cell growth containing 0.2 percent of lactalbumin hydrolyzate.

Levintow and Eagle (28) showed that as a rule, the requirements for amino acids are fairly uniform for many different cell lines. The inhibitory effects of excessive concentrations of amino acids were noted by Burrows and Neymann (8). These authors showed that while various peptones were nontoxic for embryo explants, the amino acids resulting from their complete hydrolysis were toxic. Synthetic dipeptides were also toxic but to a lesser degree than synthetic amino acids. Low concentrations of amino acids stimulated the contraction of heart muscle fragments without affecting growth. More recently, Eagle *et al.* (13) showed that high concentrations of certain amino acids (serine, glycine, ornithine) exerted an end-product feedback inhibition in cell lines of human origin.

MATERIALS AND METHODS

Cell Culture System

Two tissue culture systems were employed, one for mass cell culture methods and one for the plating of single cells.

Distilled water

The water used for cell culture media, solutions and for the rinsing of glassware was condensed from steam, demineralized[1] and redistilled in all glass distillery. Its conductivity was 0.3 PPM equivalents of sodium chloride when measured by a conductivity meter.

Glassware

Rectangular farming bottles of 5 in x 1-7/8 in x 1-7/8 in (200 ml) were used for the routine propagation of cell lines and primary cell cultures. Prescription bottles of 4 oz, 8 oz, 16 oz and 32 oz capacity with plastic screw caps were used for storage of cell culture media. This glassware was washed after use with a detergent solution (Microsolve[2]) and rinsed seven times with tap water, three times with steam condensed water and once with water distilled over glass.

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[1]Crystalab Deeminite, Crystal Research Laboratories, Inc. Hartford 4, Connecticut.

[2]Microbiological Associates, Inc. Washington 14, D. C.

Tissue culture plastic petri dishes[1] (15 x 60 mm) and 30 ml screw cap type plastic flasks[1] were used for all quantitative measurements. Disposable glass tubes[2] with rubber stoppers, held on stationary racks, were used for growth of primary bovine testicle cells.

Incubator

A water jacketed incubator was used, which maintained a 5 percent CO_2 atmosphere and a constant temperature with variations of not more than $\pm$ 0.2 C. The temperature was regulated by a safety thermostat located in the water jacket, in series with the air thermostat. The humidity was kept close to saturation by bubbling the air mixture through a water pan.

Cell culture media

A modification of the medium described by Melnick (29) was used for growth of cells in mass cultures. It consisted of a Hanks' balanced salt solution with lactalbumin hydrolyzate and yeast autolyzate. This mixture will be referred to as H-LaH medium. A medium described by Ham and Puck (20) referred to as F4 was used with slight modifications for the single cell plating experiments. Both these media were

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[1]Falcon Plastics Corporation, Los Angeles, California.

[2]Bellco Glass, Inc. Vineland, New Jersey.

mixed with 10, 15 or 20 percent of various serums and sterilized by filtration through a 0.2 μ Millipore filter[1].

H-LaH medium

Hanks' BBS	99.7 percent
Lactalbumin hydrolyzate[2]	0.1 percent
TC Yeastolate[2]	0.1 percent
Antibiotic concentrate[3]	0.1 percent

Medium F4

N16 solution[4]	200 ml
Saline F[4]	595 ml
NCTC 109[4]	40 ml
Antibiotic concentrate	1 ml
Cysteine HCl concentrate[5]	4 ml
Distilled water	10 ml

Media without amino acids were also prepared to be used in cell culture nutrition experiments with protein hydrolyzates. Hanks' BSS was used for experiments with mass cultures. F4 medium, prepared without amino acids was used for single cell

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[1]Millipore Filter Corporation, Bedford, Massachusetts.

[2]Difco Laboratories, Inc. Detroit 1, Michigan.

[3]Antibiotic concentrate: penicillin 10^5 u/ml, streptomycin 100 mg/ml in distilled water.

[4]N16, Saline F and NCTC 109 are commercially available from Hyland Laboratories, Los Angeles, California.

[5]Cysteine HCl concentrate: cysteine HCl 25 mg/ml in distilled water.

plating experiments. A modified N16 solution was used in preparing this medium.

Modified N16 solution This solution was made by mixing the series of stock solutions listed below:

Solution III (hypoxanthine) Five hundred mg of hypoxanthine were dissolved in 50 ml of distilled water alkalinized with two drops of concentrated ammonium hydroxide and taken to a final volume of 1000 ml with distilled water. The solution was stored at room temperature.

Solution IV (vitamins)

Thiamine HCl	0.5 g
Riboflavin	0.05 g
Pyridoxin HCl	0.05 g
Folic acid	0.01 g
Biotin	0.01 g
Choline	0.3 g
Ca Pantothenate	0.3 g
Niacinamide	0.3 g
i-Inositol	0.1 g
Distilled water to	1000 ml

This solution was stored at -20 C.

Solution V

NaCl (biological grade)[1]	148 g
KCl (biological grade)[1]	5.7 g
$Na_2 HPO_4 \cdot 7H_2O$	5.8 g
$KH_2 PO_4$	1.66 g
Distilled water to	1000 ml

Solution VI

$Mg SO_4$	1.54 g
$CaCl_2 \cdot 2H_2O$	0.16 g
Distilled water to	1000 ml

Solutions V and VI were kept at room temperature.

The modified N16 solution was prepared as follows:

Saline F	150 ml
Solution III (hypoxanthine)	50 ml
Solution IV (vitamins)	10 ml
Solution V	50 ml
Solution VI	100 ml
Glucose	1.1 g
$NaHCO_3$ (Biological grade)[1]	1.2 g
Distilled water	400 ml

The solution was sterilized by filtration through a 0.2 Millipore filter after having been slightly acidified by

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[1]Available under the Baker and Adamson trademark. Allied Chemical Company. New York, New York.

bubbling CO_2 through it. To prepare the F4 medium without amino acids (hereafter referred to as F4A medium), the modified N16 was mixed in the same proportions as shown for the F4 medium. Protein hydrolyzates were dissolved in saline F to give a final concentration of protein hydrolyzate of 1, 0.5, 0.25, 0.125, 0.10, and 0.05 percent in F4A medium.

Saline solution for washing and dilution of cells

A modification of the saline G of Ham and Puck (20) was used.

Glucose	1.1 g
Phenol Red, sodium salt	1.2 g
NaCl	8.0 g
KCl	0.4 g
$Na_2HPO_4 \cdot 7H_2O$	0.29 g
$MgSO_4 \cdot 7H_2O$	0.154 g
$CaCl_2 \cdot 2H_2O$	0.016 g
Lactalbumin hydrolyzate	2.0 g
Distilled water to	1000 ml

The lactalbumin hydrolyzate was dissolved in a small amount of water by heating to 80 C. The final solution was autoclaved at 120 C for 30 minutes. Once cooled, 1 ml of antibiotic concentrate was added.

Amino acid free saline solution (Saline A) This solution was used in the dilution of cells for single cell plating experiments. It was prepared as follows:

Glucose	1.1 g
Phenol Red, sodium salt	7.0 mg
NaCl (biological grade)	8.0 g
KCl (biological grade)	0.4 g
$Na_2HPO_4 \cdot 7H_2O$	0.29 g
KH_2PO_4	0.08 g
$MgSO_4 \cdot 7H_2O$	0.15 g
$CaCl_2 \cdot 2H_2O$	0.16 g
Bovine serum albumin, crystalline[1]	1.00 g
Distilled water to	1000 ml
Antibiotic concentrate	1.00 ml

The solution was sterilized by Millipore filtration.

Bovine serum albumin solution (BSA solution) This solution was used to fulfill macromolecular requirements in single cell plating experiments.

Bovine serum albumin, crystalline	3.5 g
$NaHCO_3$ (biological grade)	0.2 g
Saline A to	100 ml

The solution was sterilized by Millipore filtration. When used at a 10 percent level with culture media, it provided 3.6 mg/ml of BSA.

Overlay medium for plaque assay This medium was used

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[1]Nutritional Biochemicals Corporation, Cleveland, Ohio.

for virus plaque assays in plastic petri plates. The following ingredients were used:

Powdered Basal medium Eagle (Diploid) with L-glutamine[1]	9.62 g
$NaHCO_3$	2.2 g
Distilled water	500 ml
Antibiotic concentrate	1.0 ml

This medium was sterilized by filtration. Prior to use it was mixed with an equal volume of 2.2 percent Noble Agar[2] in distilled water. Horse serum was added to a 5 percent concentration.

Neutral red overlay solution This was prepared as a 0.01 percent neutral red in a 1 percent aqueous Noble Agar solution.

Trypsin solution A trypsin solution used to disperse primary cells was prepared according to the following formula:

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[1]Grand Island Biological Company. Grand Island, New York.

[2]Difco Laboratories, Inc. Detroit 1, Michigan.

Glucose	1.00 g
NaCl	8.00 g
KCl	0.4 g
$NaH_2PO_4 \cdot 7H_2O$	0.045 g
KH_2PO_4	0.03 g
$CaCl_2 \cdot 2H_2O$	0.016 g
Phenol Red	0.0012 g
Trypsin[1]	2.00 g
Distilled water	1000 ml

The solution was maintained at 37 C for 1 hour to dissolve the trypsin. This was followed by the addition of 1 ml of antibiotic concentrate to the mixture. It was sterilized by filtration through a Millipore filter.

EDTA - Trypsin solution This solution was used for the dispersion of cell line monolayers. It had the following composition:

NaCl	8.00 g
KCl	0.2 g
Na_2HPO_4	1.15 g
KH_2PO_4	0.2 g
Trypsin	2.00 g
Distilled water	1000 ml

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[1]Difco Laboratories, Inc. Detroit 1, Michigan.

After dissolving the trypsin at 37 C for 1 hour, 1 g of EDTA[1] (disodium dihydrogen ethylenediamintetra-acetate dihydrate) and 1 ml of antibiotic concentrate was added. The solution was sterilized by filtration using a 0.2 Millipore pad.

Cell cultures

Calf testicles were obtained through the courtesy of the Rath Packing Company, Waterloo, Iowa. The organs were removed from the slaughtered animals and shipped refrigerated at approximately 3 C. The testicles were processed upon arrival approximately 6 hours after recovery from slaughtered animals. The method used to obtain the testicular parenchyma has been described by Gratzek (24).

Cells from the parenchymal tissues were dispersed by agitation in a trypsin solution for 20 minutes at 37 C. The cells were then filtered through 4 layers of sterile gauze and centrifuged at 1000 rpm for 10 minutes in an International Model U centrifuge. The sedimented cells were resuspended in Saline G with 50 percent lamb serum and cell counts were made in a hemacytometer chamber. These bovine testicle cells were seeded at one million cells per ml in H-LaH with 10 percent lamb serum to obtain monolayers, unless otherwise specified. Five ml of the cell-medium suspension were added to each plastic petri plate and 1 ml was added to

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[1]Hach Chemical Company, Ames, Iowa.

each sterile disposable tube. Complete monolayers were obtained in 3 to 4 days.

An established bovine kidney cell line[1], was routinely farmed by dispersion of monolayers with warm EDTA-Trypsin solution. This was accomplished by washing the cell sheet twice with 3 ml of EDTA-Trypsin and then allowing 4 ml of the solution to remain in contact with the cells. After five minutes at 37 C the loosened monodisperse cell suspension was added to 45 ml of H-LaH with 10 percent lamb serum. The medium containing cells was then put in three farming bottles. A clone of this cell line that was able to grow with as little as 3 percent lamb serum, was obtained using the cloning methods described by Ham and Puck (20). This cloned cell line, designated as BK2, produced clones of very uniform morphology. It was used in single cell plating experiments.

In every case, the medium in petri plates, tubes or farming bottles was discarded at 24 hours and replaced with fresh medium. This eliminated a large quantity of cell debris and aided in the formation of uniform cell monolayers.

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[1]Madin strain. Obtained through the courtesy of Mr. M. F. Coria. United States Department of Agriculture. National Animal Disease Laboratory, Ames, Iowa.

Mammalian Serums

Both commercial prepared desiccated serum and various normal mammalian serums were used.

Heat desiccated serum

A heat desiccated bovine serum was obtained from a commercial source[1]. This material was obtained industrially by defibrination and centrifugation of bovine blood. Blood from slaughtered cattle was collected from a floor trough and mixed with an anticoagulant solution of sodium citrate. It was then defibrinated and centrifuged to separate the blood cellular components. Samples of liquid serum were routinely taken at this stage in the process for comparisons with corresponding desiccated batches. The serum was kept at low temperatures by continuous recycling through a refrigerating unit. The product was stored for 2 or 3 days and then dried with spray drying equipment similar to that used for the production of dehydrated milk. Samples of the dried serum were taken for testing purposes. One part of dried serum was reconstituted with 9 parts of distilled water. Both the liquid sample and the corresponding reconstituted dry sample were sterilized by filtration directly or diluted 1:4 with distilled water prior to sterilization. Either procedure re-

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[1]The Rath Packing Company. Waterloo, Iowa.

quired previous clarification through Whatman glass fiber filter pads or Selas 10 candles[1].

Flash pasteurization was sometime performed on the serum after the centrifugation step.

For some experiments, reconstituted dried serum was dialyzed against Hanks' BBS and then sterilized by filtration.

Normal mammalian serums

Bovine, horse and sheep serum were collected by venous puncture, allowed to clot and the serum decanted. The small amount of erythrocytes present in the serum were removed by centrifugation at 2000 rpm for 10 minutes. The serums were clarified by prefiltration and then sterilized by filtration through a Selas 02 candle[2]. These serums were all inactivated at 56 C for 30 minutes prior to use. Lamb serum and fetal calf serum for cell cultures were also obtained from commercial sources[3]. These serums were not heat inactivated before use. Serum was also obtained from 17 cattle owned by the Department of Veterinary Clinical Sciences. These samples were used to determine normal base values in disc electrophoresis studies.

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[1]Selas Corporation of America. Dresher, Pennsylvania.

[2]Selas Corporation of America. Dresher, Pennsylvania.

[3]Grand Island Biological Co., Grand Island, New York.

Protein Hydrolyzates

A collagen hydrolyzate[1], obtained by enzymatic hydrolysis of animal collagenous by-products was obtained. Lactalbumin hydrolyzate[2], also an enzymatic hydrolyzate, and gelatin hydrolyzate[3], a tryptophan-free acid hydrolyzate were used in comparisons with a collagen hydrolyzate. Stock preparations of all three hydrolyzates consisted of 2 percent solutions in either Hanks' BSS or in Saline F. The hydrolyzates were dissolved by heating to approximately 80 C at pH 3 (HCl 1N), filtered through glass fiber filter pads to obtain a clear solution and then alkalinized to pH 7.0 (NaOH 1 N). The 2 percent solution was sterilized by filtration through a 0.2 Millipore pad. Solutions of varying concentrations of hydrolyzates were prepared by diluting the stock 2 percent solution with sterile Hanks' BSS or Saline F.

The collagen hydrolyzate was solubilized with difficulty, leaving a certain amount of undissolved material that was retained by the filter pads. All three hydrolyzates were tested for amino nitrogen content by serial dilutions of the 2 percent solutions in distilled water. A drop of each dilution was placed on Whatman No. 1 chromatography paper, stained with 0.2 percent ninhydrin in acetone and dried at

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[1]The Rath Packing Company. Waterloo, Iowa.

[2]Difco Laboratories, Inc. Detroit 1, Michigan.

[3]General Biochemicals, Inc. Chagrin Falls, Ohio.

100 C. A 2 percent solution of lysine monoclorhydrate was also tested in parallel as a standard.

Disc Electrophoresis

Disc electrophoresis of bovine serums was performed using the technique described by Davis (10). The gel columns were formed by polymerization of the reactants in Pyrex glass tubes of 73 mm length, 7 mm outside diameter and 5 mm internal diameter. These tubes were washed prior to use in a boiling soap solution to which a few drops of concentrated ammonium hydroxide were added. They were thoroughly rinsed in distilled water and air dried. The tubes were fitted onto rubber stoppers with a central cylindrical well 5 mm deep and 6 mm wide.

The necessary chemicals were obtained from a commercial source[1] in the form of premixed solutions. The composition of these solutions was as follows:

Lower Gel A - 1

1 N HCl	24 ml
Tris (hydroxymethyl) aminomethane	18.15 g
N,N,N' Tetramethylethylenediamine	0.23 ml
Water to make	100 ml

[1]Canal Industrial Corporation. Bethesda, Maryland.

Lower Gel A - 2

Acrylamide monomer	30 g
N,N Methylenebiscrylamide monomer	2.5 g
Water to make	100 ml

Lower Gel B

Ammonium persulphate	0.14 g
Water	100 ml

Upper Gel

1 M H_3PO_4	3.5 ml
Tris (hydroxymethyl) aminomethane	0.71 g
Riboflavin	0.5 mg
Acrylamide monomer	0.25 g
N,N Methylenebiscrylamide monomer	0.625 g
Water to make	100 ml

Electrophoresis buffer

Tris (hydroxymethyl) aminomethane	6 g
Glycine	28.8 g
Bromphenol blue	5 g
Water	

Amido Black Stain

Naphthol Blue - Black	0.55 g
7 percent acetic acid in water	100 ml

A 7.5 percent polyacrylamide lower gel was prepared by mixing 2 parts of Lower Gel B, 1 part of Lower Gel A - 1 and 1 part of Lower Gel A -2. A column of 63 mm of liquid Low Gel was put in each tube and a 5 mm layer of water was placed on top. The lower gel was allowed to polymerize for 45 minutes and the water layer removed by inverting the tube. The upper part of the gel was rinsed once with spacer gel prepared by diluting the Upper Gel solution in an equal volume of water. A 6 mm layer of spacer gel was added and carefully overlayed with water. The spacer gel was allowed to polymerize for 20 minutes under a lamp provided with 2 fluorescent bulbs. The tubes were placed about 1 inch away from the lamp. At the end of this period, the water layer was discarded and 0.15 ml of the sample gel added. This sample gel was prepared by mixing 0.15 ml of upper gel with 4 lambdas of the serum to be run.

A 1 ml glass syringe with a 26 gauge needle and a PE 10 Intramedic[1] polyethylene tubing extension was used to overlay the gels with water. Faulty layering or dirty tubes resulted in gels with irregular surfaces that gave irregularly shaped discs resulting in a significant loss of fraction resolution.

Each serum sample was measured with a 10 lambda micro-

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[1]Clay-Adams, Inc. New York, New York.

pipette. The serum samples were run undiluted to obtain the largest possible number of bands and also diluted 1:2 and 1:4 in saline for better densitometric readings.

Electrophoresis was performed on the tubes positioned vertically between 2 plastic buffer reservoirs provided with platinum electrodes with the anode in the lower reservoir. A constant current of 2.5 mA per tube was applied and maintained until the albumin band of the serum was seen to migrate about 30 mm into the lower gel. A total of 10 tubes could be run simultaneously. The run was usually completed in 45 to 50 minutes and the voltage increased from 490 to 510 volts through the run. The bromphenol blue in the buffer acted as a tracer dye giving an easily observable line as it migrated through the gel. It also bound to albumin and gave a faint line that indicated the position of this protein during the run. As soon as electrophoresis was completed, the tubes were removed from the reservoirs and immersed in ice cold water. The gels were rimmed under water with a blunt end dissecting needle and then forced out of the tube by applying uniform pressure with a Clinac[1] propipette. The gels were then fixed and stained overnight with Amido Black stain. Destaining was accomplished in glass petri plates by frequent changes of a 7 percent acetic acid destaining solu-

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[1]LaPine Scientific Company. Chicago, Illinois.

tion. The destained gels were stored in stoppered tubes filled with destaining solution.

The relative mobility of each serum band was calculated by obtaining the ratio between the distance travelled by the albumin band in that same gel and the distance travelled by the band in question, both measured from the cathodic end of the lower gel. Densitometric tracings were obtained with a Densicord densitometer[1] equipped with a Model 49 Integraph automatic integrator[1] attachment and a special disc electrophoresis carriage and photo tube slit.

A standard Versatol[2] human serum was also used in attempts to locate various protein bands and to calibrate the densitometer.

Virus Strains

Four bovine viruses were used to measure virus neutralizing antibodies in bovine serums. The NADL strain isolated by Gutekunst (25) was a cytopathic strain of bovine virus diarrhea routinely used in this laboratory. The ISU-2 strain of infectious bovine rhinotracheitis was isolated by Buening (7) and characterized as such serologically and biochemically in this laboratory. The LCR-4 strain of bovine

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[1]Photovolt Corporation, New York, New York.

[2]Warner-Chilcott. Morris Plains, New Jersey.

enterovirus was kindly supplied by Dr. Van der Maaten[1]. A strain of myxovirus parainfluenza - 3 isolated by Reisinger et al. (38) was also used.

All four viruses produced plaques under agar when using primary bovine testicle cells with a technique similar to the one described by Dulbecco and Vogt (12). The MP - 3 agent produced two plaque sizes that retained their size characteristics when purified by successive plaque selection. The two plaque variants also differed in their ability to hemagglutinate chicken erythrocytes. The large plaque variant, consistently produced more hemagglutinin than the small variant and was used in all hemagglutination and hemagglutination-inhibition tests.

A new recent isolate of bovine virus diarrhea obtained in our laboratory, was used as a challenge in an experimental animal. This strain, designated BVD-Lewis, was obtained from a mucosal disease case in a herd with no previous history of vaccination against BVD.

Antibody Assays

Antibodies against BVD-NADL, IBR-ISU-2 and BE-LCR-4 were detected using a standard constant-virus-decreasing-serum

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[1]Dr. M. J. Van der Maaten, Veterinary Medical Research Institute, Iowa State University. Ames, Iowa.

neutralization procedure. The indicator system employed was a plaque assay utilizing bovine testicle cell monolayers.

Liquid samples and reconstituted dry samples of Rath bovine serum were inactivated at 56 C for 30 minutes and serial four-fold dilutions were made in saline G. One ml of each serum dilution was mixed with 1 ml of a fixed dilution of virus. This virus dilution was calculated so as to provide a countable number of virus plaques per ml of neutralizing mixture. In general, 20 PFU of BE-LCR-4, 60 PFU of BVD-NADL or 60 PFU of IBR-ISU-2 per petri plate proved to be easily countable. The viruses were routinely diluted in saline G with 1 percent horse serum. The virus was allowed to react with the serum antibodies for 30 minutes at room temperature. The monolayers were washed twice with 3 ml of saline G and then 2 plates per serum dilution were inoculated with 1 ml of the virus-antibody mixture. Adsorption was allowed to proceed for 1 hour at room temperature. The fluid was discarded and the plates overlayed with 5 ml of overlay medium. The plates were incubated in a CO_2 incubator for 4 days and were overlayed with 4 ml of neutral red overlay. The plates were kept in the dark at room temperature for 4 to 5 hours. Virus plaques were then clearly visible and the results were recorded. End points were read at 100 percent neutralization of virus plaques.

Antibodies against MP-3 were tested by virus neutrali-

zation. Presence of unneutralized virus was detected by cytopathic changes observed in tubes with monolayers of primary bovine testicle cells. The serums were diluted as described above and the neutralization and adsorption conditions were also the same. Four replicate tubes per serum dilution were inoculated with 0.1 ml of the neutralization mixture containing a challenge dose of between 10 - 50 PFU of virus. After adsorption the liquid was discarded and 1 ml of liquid overlay medium containing 5 percent horse serum was added. The tubes were incubated at 37 C for 5 days and observed for cytopathic effects. A 50 percent end point was calculated by the method of Reed and Muench (36).

Serum samples from a caesarean derived, colostrum deprived calf fed reconstituted desiccated serum were also run in virus neutralization tests. Antibodies against BVD-NADL, IBR-ISU-2 and BE-LCR-4 were tested by plaque neutralization. Serial two-fold serum dilutions were used and a 50 percent plaque reduction end point was read. Hemagglutination inhibition (HI) tests were used to measure MP-3 antibodies. The large plaque variant of MP-3 was used to produce hemagglutinating antigen on primary bovine testicle monolayers. The HI test was performed in tubes, with chicken erythrocytes. The serums were diluted in serial two-fold steps with saline and 0.25 ml of each dilution was mixed with 0.25 ml of MP-3 antigen containing 2 hemagglutinating units. A volume of 0.25 ml of a 0.5 percent suspension of washed chicken erythrocytes

was then added. The tubes were left in a rack at 10 C until the erythrocytes sedimented in the control tubes. The HI titer of the serum was recorded as the reciprocal of the highest dilution of serum preventing hemagglutination.

Inhibitor Assay

To test for non-antibody viral inhibitors or interferon producing substances, liquid and desiccated Rath bovine serum samples from lot 4 were used. After being filtered and heat inactivated, serial dilutions were mixed with 100 PFU of vesicular stomatitis virus (VSV). A plaque assay was utilized to quantitate any inhibitory effect.

Bacteriological Assays

Qualitative and quantitative studies of the bacterial flora of desiccated bovine serums was undertaken.

Standard plate counts were performed on Bacto Plate Count Agar[1] plates. Plate counts of coliform bacteria were made on Bacto Violet Red Bile Agar[1] plates. Both counts were made according to the procedures described in the Standard Methods for the examination of dairy products (1).

Bovine blood agar plates were also inoculated with serial tenfold dilutions of serum samples in sterile distilled water. The identification of microorganisms was oriented

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[1]Difco Laboratories, Inc. Detroit 1, Michigan.

towards the detection of potential pathogens. This was done by observing colony morphology, cell morphology, gram staining characteristics, action on blood plates, and growth in media containing bile salts. Selected biochemical tests including fermentation of dextrose, lactose, sucrose, salicin, mannitol, raffinose, inulin, trehalose, sorbitol, arabinose, maltose, inositol, Simmon's citrate medium, gelatin, MR-VP medium, indol, peptone iron agar, nitrate media, litmus milk, urease and ability to grow in the presence of 6.5 percent NaCl, were performed on each of the strains of organisms isolated, once purified by repeated selection of single colonies.

Agar plates incubated under anaerobic conditions were also inoculated with the serum samples.

Effect of Desiccation on IBR Virus

It was of interest to know whether the temperatures used for desiccation of serum destroyed some of the common bovine viruses. Since the bovine serums had antibodies against these viruses, pig serum was used. The serum was obtained by collecting blood from the throat incision of a number of pigs. The blood was allowed to clot and the serum decanted. Previous to spray drying, 1.4×10^7 PFU of IBR-ISU-2 were added to 4 liters of pig serum. A sample of this serum was taken before and after the desiccation process. The first sample was tested for antibodies against IBR, the second

was tested for the presence of viable virus using a plaque assay.

Animal Experimentation

One cesarean-derived, colostrum-deprived calf was available for use to evaluate the absorbability of specific immunoglobulins present in commercially prepared desiccated serum.

Experimental animal

One Holstein calf was obtained from the Department of Veterinary Clinical Sciences by cesarean section and deposited aseptically in a previously disinfected 34 in x 60 in canvas bag. The calf was transported immediately after delivery to a disinfected isolation unit. Extreme caution was taken to prevent undue contamination. No colostrum was fed at any time. Serum samples were obtained from the dam and from the calf at birth.

Serum administration and feeding

The calf was fed 300 ml of sterile reconstituted desiccated serum mixed with 1 quart of autoclaved commercial milk replacer approximately 4 hours after delivery. This quantity supplied the animal with approximately 21 g of bovine plasma proteins. The calf was subsequently fed 1.5 quarts of autoclaved milk replacer supplemented with fat soluble

vitamins[1] and B complex vitamins[2] every 8 hours. After the second week, the calf was fed every 12 hours with 2 quarts of autoclaved milk replacer. The milk replacer was heated to 120 C in an autoclave and immediately cooled, obtaining a temperature of 90 C in the center of 1 gallon glass containers filled with the solution.

After 1 month of age, the calf was fed unheated milk replacer and alfalfa hay or beet pulp.

Collection of data

Serum samples were obtained daily by venous puncture for the first 8 days and subsequently at suitable intervals. These serum samples were inactivated and tested for antibodies against BE-LCR-4, BVD-NADL, IBR-ISU-2, and MP-3. The animal was later challenged separately with IBR-ISU-2 and BVD-Lewis strains. Both these viruses were purified by plaque selection. One million PFU of IBR-ISU-2 were inoculated in the left nostril of the calf and the alterations of the right nostril and general manifestations were recorded. One hundred thousand PFU of BVD-Lewis were inoculated intranasally and intravenously. Temperatures and clinical signs were recorded twice daily after each virus inoculation.

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[1]Cod liver oil from E. R. Squibb and Sons. New York, New York.

[2]Flavored yeast from E. R. Squibb and Sons. New York, New York.

Cell Growth Measurements

Two procedures were used to measure growth of cells in media containing either test serums or protein hydrolyzates.

Mass cultures with primary bovine testicles

Ten replicate plastic flasks per serum tested were seeded with 5 ml of H-LaH containing 20 percent serum, and 2 million primary bovine testicle cells per ml. The flasks were loosely capped and placed in a CO_2 incubator. At 14 hours five flasks were removed to obtain an initial cell count. The media in these flasks was discarded and the cells washed with saline. They were then fixed with neutralized formalin and stained with giemsa stain following the procedure of Ham and Puck (20). The flasks were then cut open and the flat plastic surface with the cells attached was observed under the microscope. By randomly placing 10 drops of immersion oil and focusing with the oil immersion objective of the microscope, one could obtain 10 random fields per flask. All the cells with normal morphology in each field were counted and averaged. This provided an estimate of the initial number of cells per oil immersion field, referred to as the initial value for that particular serum.

The other five flasks were processed using the same techniques at 44 hours of incubation, obtaining an estimate of the final number of cells per oil immersion field designated as the final value for the serum. Although these

values did not indicate the total number of cells per flask, they gave estimates that could be used for comparisons among serums. This method was also used to compare the growth promoting potentials of the protein hydrolyzates at various levels. In these experiments 10 percent lamb serum in Hanks' BSS was used, with 1.5 million primary bovine testicle cells per ml. Five replicate flasks were used per level since only the final number of cells per field was of interest. The flasks were fixed and stained at 40 hours.

Preliminary experiments showed that the use of 5 replicate flasks per treatment with 10 actual field counts per flask was more than enough to ensure the statistical validity of the comparisons.

Single cell plating

When testing serums, 5 or 10 replicate plastic petri plates were used per trial. Five ml of F4 medium containing 15 percent of test serums were added to each plate. These plates were preincubated and a measured amount of single dispersed cells in 0.2 ml volume was added. Clumps of cells were previously removed by low speed centrifugation at 200 rpm for 5 minutes.

The use of various cell types made necessary procedural alterations in the cell plating techniques. Second passage bovine testicle cells were seeded at 10^4 cells per plate to study the effect of the various serums used on the type of

clones that grew out. Bovine kidney line cells were seeded at approximately 800 cells per plate. The plates were incubated for 7 to 11 days in a CO_2 incubator with a minimum of temperature and humidity variations. They were then fixed and stained with the same procedure used for the plastic flasks. When using BK2 cells, 5 ml of F4A medium with 4 percent lamb serum and 10 percent BSA solution were added to each plate. This concentration of serum proved to be just slightly higher than the minimum required for growth. The BSA level was optimal, lower or higher levels gave less growth enhancement. Only 100 to 120 BK2 cells were added per plate, and the cell colonies allowed to propagate for 5 days. At this time they were fixed and stained.

In every case, the colonies of 4 or more cells were counted under a dissection microscope. Plating efficiencies were calculated by dividing the number of colonies per plate by the number of cells inoculated. Generation times were obtained by counting the number of cells per clone, and using the following formula:

$$G = \frac{T \times 0.301}{\log (\text{number of cells per colony})}$$

where G = generation time

T = incubation time.

RESULTS

Disc Electrophoresis

A maximum of 15 protein bands were seen when serum samples from 17 normal bovines were run. These bands would be characterized by their relative mobilities as shown in Figure 1. A tentative identification was attempted based on relative mobility and by comparison with published data on bovine serum electrophoresis by the starch gel method (3, 4). Figure 2 gives the relative mobility values for each band and a 5 percent confidence interval for these mean values. The number of animals that had a given protein band is also shown together with a tentative identification. All bovines had bands A (albumin), G (transferrin I), H (transferrin II) and M (slow α_2 globulin). The relative mobility of each band was very constant as shown by the small confidence intervals. Bands E and O, were seen in very few serums. Band O was tentatively identified as an α_2 macroglobulin.

Figure 3 presents results obtained when 10 different lots of reconstituted desiccated serums were run. The desiccation process did not alter the relative mobilities of the serum protein bands as shown by a Student's t test.

When compared to the normal bovine serums, the most marked changes seen in the desiccated serums were the fusion of bands G and H into one band which migrated with a

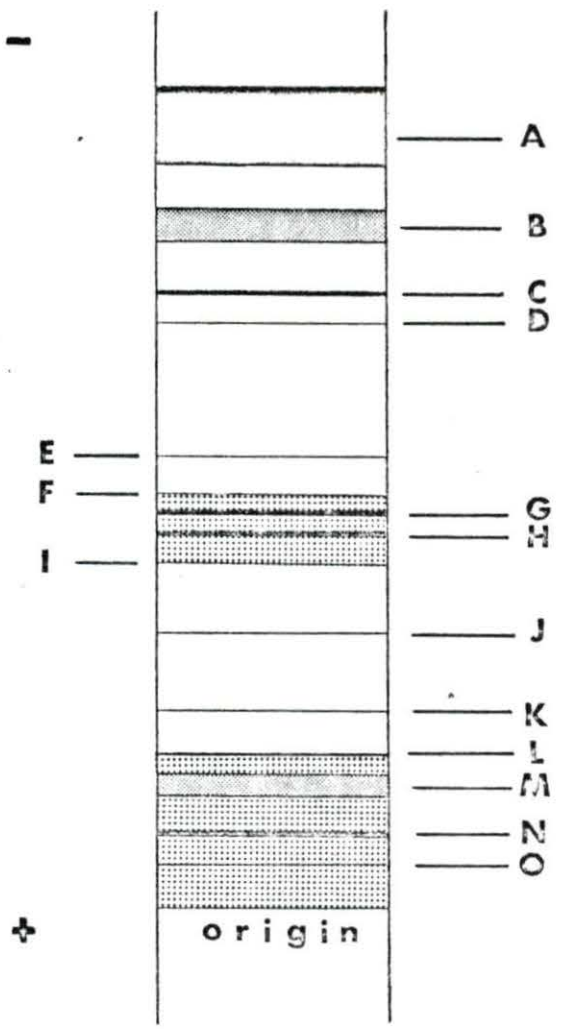


Figure 1. Diagramatic representation of protein bands of a normal bovine serum as separated by disc gel electrophoresis.

Protein band[a]	Average relative mobility	No. of bovines showing band	Identification of band
A	1.00	17	Albumin
B	0.89 ± 0.01	16	Postalbumin
C	0.80 ± 0.01	12	Postalbumin
D	0.76 ± 0.02	13	Fast α_1 globulin
E	0.59 ± 0.04	7	Fast α_2 globulins
F	0.54 ± 0.01	12	Fast α_2 globulins
G	0.52 ± 0.01	17	Transferrin I
H	0.49 ± 0.01	17	Transferrin II
I	0.45 ± 0.02	10	Transferrin III
J	0.36 ± 0.02	12	Transferrin IV
K	0.26 ± 0.02	12	Transferrin V
L	0.20 ± 0.02	11	Slow α_1 globulin
M	0.16 ± 0.02	17	Slow α_2 globulin
N	0.10 ± 0.02	12	γ_1 globulin
O	0.06 ± 0.02	5	α_2 macroglobulin

[a] See Figure 1

Figure 2. Disc electrophoresis characteristics of 17 normal bovine serums.

Protein band[a]	No. of desiccated serum lots showing band
A	10
B	6
C	9
D	5
E	8
F	3
G	1
H	10
I	8
J	7
K	6
L	4
M	9
N	7
O	5

[a] See Figure 1

Figure 3. Disc electrophoresis characteristics of 10 desiccated serum lots.

mobility characteristic for band H (transferrin II). Band E was very accentuated and nearly always present in the desiccated serums in contrast to the normal serums in which it occurred infrequently and always fainter. Bands F and L were frequently missing in the desiccated serums.

A typical normal bovine disc electrophoresis pattern is shown together with a standard human serum (Versatol) in Figure 4. Four samples from desiccated serums are shown in Figure 5. The fusion of the transferrin I band with the transferrin II band can be noted. The position of band E can be seen in Figure 6.

Densitometric tracings of the serum samples provided estimates of the percentage that each protein band represented of the total serum proteins. The percentages obtained for a given sample varied with the total amount of serum protein which was added to the gel. Since this quantity varied from sample to sample, it was not possible to make direct comparisons between the desiccated serums and the normal bovine serums. Five runs from desiccated serums were paired with an equal number of normal serum runs, choosing those with similar amount of protein as determined by the total number of integrator markings. The various bands were grouped in 5 major groups corresponding roughly to the paper electrophoresis bands to minimize the individual variations from animal to animal due to the absence of one or more bands.

Figure 4. Disc electrophoresis of a standard human serum (Versatol) and a typical normal bovine serum.

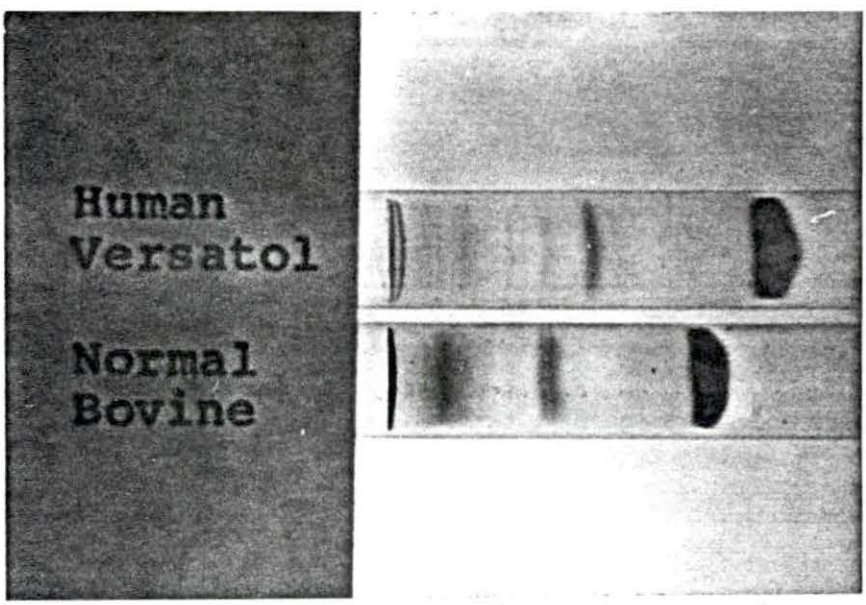

Human
Versatol
Normal
Bovine

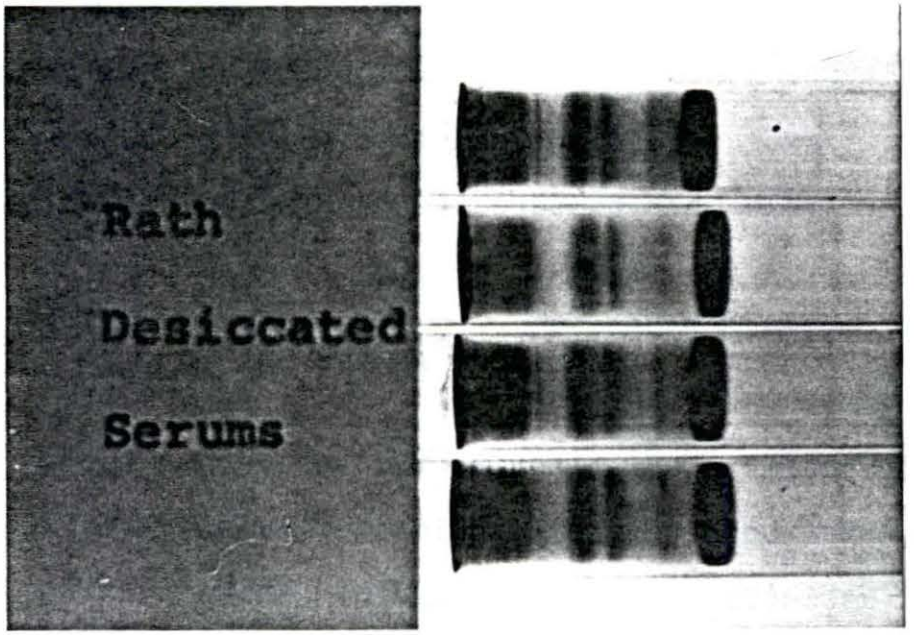
Figure 5. Disc electrophoresis of 4 samples of reconstituted desiccated bovine serums.

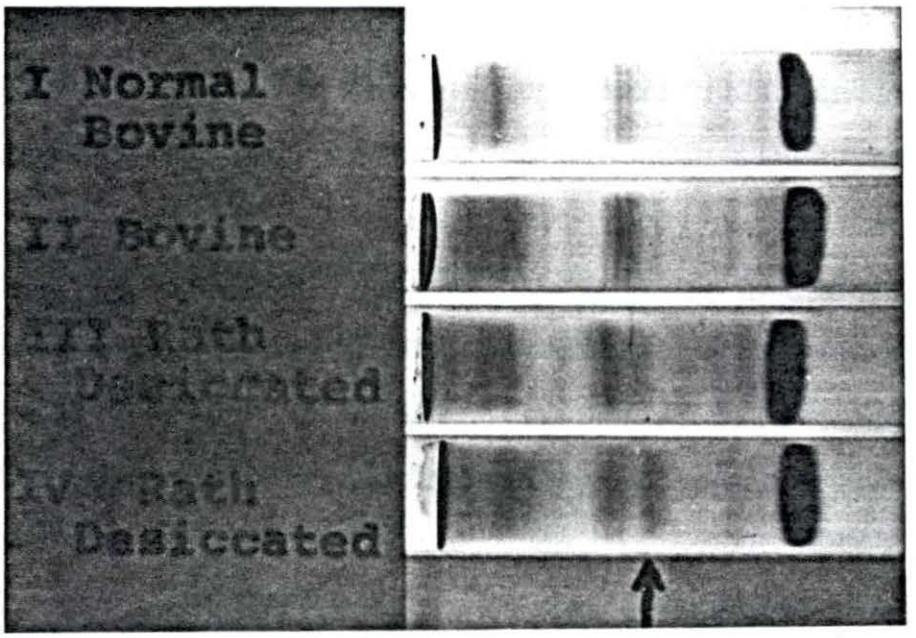
Figure 6. Presence of band E (arrow) in bovine serums.

I. Normal bovine without the band.

II. Normal bovine with band E present.

III. and IV. Desiccated serums with E bands.


Rath
Desiccated
Serums


I Normal
Bovine
II Bovine
III Rath
Desiccated
IV Rath
Desiccated

The differences in protein percentages between the corresponding band groups for each pair were statistically compared in a paired comparisons t test, as shown in Figure 7. The results show that the 5 desiccated serum lots tested had an increased amount of fast α globulins and lowered γ_1 globulin-α_2 macroglobulin levels when compared to 5 selected normal bovine serums.

Antibody Assays

Antibodies against bovine viruses were detected in the Rath serums, both before (liquid sample) and after desiccation (desiccated sample). Figure 8 shows that the antibody levels against BVD and MP-3 were usually high. The IBR antibodies were not always present or were present in low titers. The desiccation process did not significantly alter these virus neutralizing titers. A lot of serum that was flash-pasteurized before desiccation showed no major alterations in its antibody levels.

Sample lots of Rath serums were obtained over 3 seasons of the year. The distribution of antibody titers throughout this test period is shown in Figure 9. Not enough data is available for a definite conclusion but it can be tentatively assumed that there are no major variations in antibody levels throughout the year.

Serum fraction	Bands included	Percent difference between desiccated seum and normal serum	t value
Albumin	A,B,C	0.17 %	0.056
Fast α globulins	D,E	4.26 %	3.080 *
Transferrins	F,G,H,I,J,K	-2.02 %	0.472
Slow α globulins	L,M	0.94 %	0.385
γ_1 globulin and α_2 macroglobulin	N,O	-3.86 %	3.015 *

* Significant at the 5% level

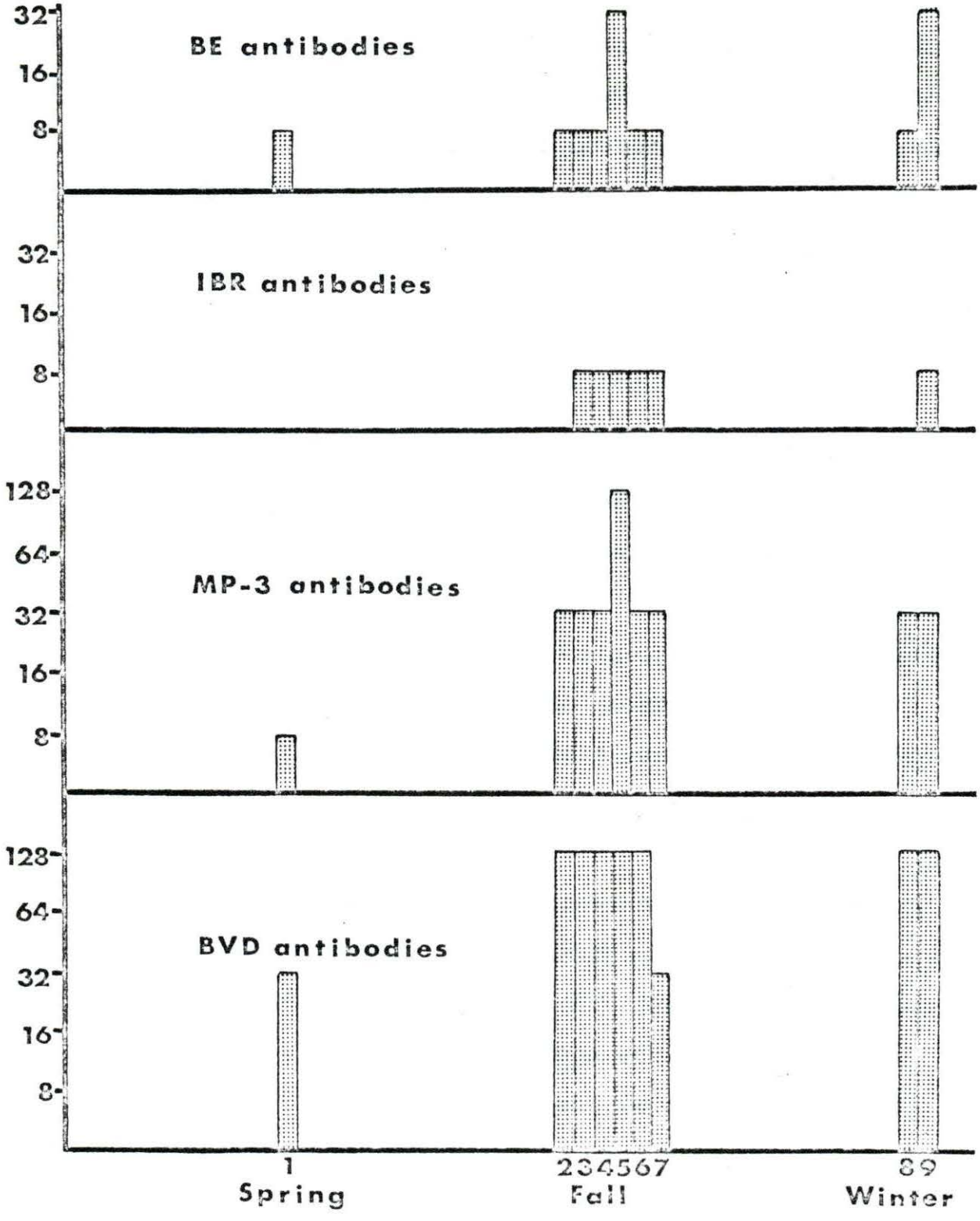
Figure 7. Differences between desiccated bovine serums and normal serums.

Figure 8. Levels of antiviral antibodies in Rath serums. Results are expressed as the reciprocal of the final serum dilution which inhibited 100 percent of the plaques.

Sample	BVD-NADL	IBR-ISU-2	BE-LCR-4	MP-3
Lot 1 Liquid	32	<8	8	8
Lot 1 Desiccated	32	<8	8	8
Lot 2 Liquid	128	<8	8	32
Lot 2 Desiccated	128	<8	8	32
Lot 3 Liquid	128	<8	8	32
Lot 3 Desiccated	128	8	8	32
Lot 4 Liquid	n.d.[a]	n.d.	8	32
Lot 4 Desiccated	128	8	8	32
Lot 5 Liquid	n.d.	<8	8	32
Lot 5 Desiccated	128	8	32	128
Lot 6 Liquid	128	8	8	32
Lot 6 Desiccated	128	8	8	32
Lot 7 Liquid	128	8	8	32
Lot 7 Desiccated	32	8	8	32
Lot 8 Desiccated (Pasteurized)	128	n.d.	8	32
Lot 9 Desiccated	128	8	32	32

[a] Sample not determined.

Figure 9. Levels of antiviral antibodies present in Rath desiccated serums throughout the year.


32
16
8
BE antibodies
32
16
8
IBR antibodies
128
64
32
16
8
MP-3 antibodies
128
64
32
16
8
BVD antibodies
1
Spring
234567
Fall
89
Winter

Inhibitor Assay

No reduction in the number of VSV plaques was seen when this virus was mixed with liquid or desiccated samples of Rath bovine serum. This indicated that, under the conditions employed in the test, these serums contained no antibody against VSV,nor any non-antibody inhibitors or interferon stimulating substances.

Bacteriological Assays

The results from standard plate counts and coliform counts indicate that the desiccated Rath serums contained a large quantity of bacteria. Figure 10 shows the standard plate counts and coliform counts per gram of desiccated powder. The types of colonies seen in blood agar plates is also indicated. The predominance of microorganisms of the genus Streptococcus is evident, as is the fact that the coliform counts were always considerably lower than the standard plate counts.

A flash pasteurized desiccated serum showed a marked reduction of bacterial contamination. Coliforms or Streptococci were not detected. The organisms present in this lot were either gram positive non-sporeforming rods or gram negative rods. Figure 11 lists the general or species of bacteria identified in some of the desiccated serum lots. Among the coliforms detected in various lots of desiccated

Figure 10. Bacteriological assays of desiccated Rath serums.

Desiccated Serum	Standard plate count per gram	Coliform count per gram	Colony types
Lot 2	49×10^4	9×10^2	-[a]
Lot 3	15×10^7	33×10^4	-
Lot 4	75×10^6	27×10^3	-
Lot 5	11×10^5	4×10^2	89% α hemolytic Streptococci 10% β hemolytic Streptococci 1% other
Lot 6	35×10^5	93×10^2	84% β hemolytic Streptococci 16% other
Lot 7	13×10^7	1×10^4	-
Lot 9	16×10^6	0	92% α hemolytic Streptococci 4% β hemolytic Streptococci 4% other
Lot 8 (Pasteurized)	5×10^3	0	no Streptococci

[a] Not determined.

Figure 11. Qualitative bacteriological assays of desiccated Rath serums.

Serum	Genera or species identified
Lot 2 Desiccated	Streptococcus equisimilis (β hemolytic)
	Citrobacter sp. (coliform)
	Flavobacterium sp. (bile resistant)
Lot 3 Desiccated	Streptococcus faecalis (α hemolytic)
	Streptococcus equisimilis (β hemolytic)
	Flavobacterium sp. (bile resistant)
	Alcaligenes sp.
	Micrococcus sp.
	Corynebacterium sp.
Lot 4 Desiccated	Streptococcus faecalis (α hemolytic)
	Streptococcus bovis (α hemolytic)
	Streptococcus equisimilis (β hemolytic)
	Aeromonas sp.
	Sarcina sp.
	Micrococcus sp.
	Corynebacterium sp.

serum were: Citrobacter sp., Proteus mirabilis, and Escherichia sp. In one lot tested very few anaerobic spore-forming bacteria were detected. During the industrial processing of the desiccated serum, the product was exposed to various potential sources of bacterial contamination. No studies were made to locate these sources of contamination.

Effect of Desiccation on IBR Virus

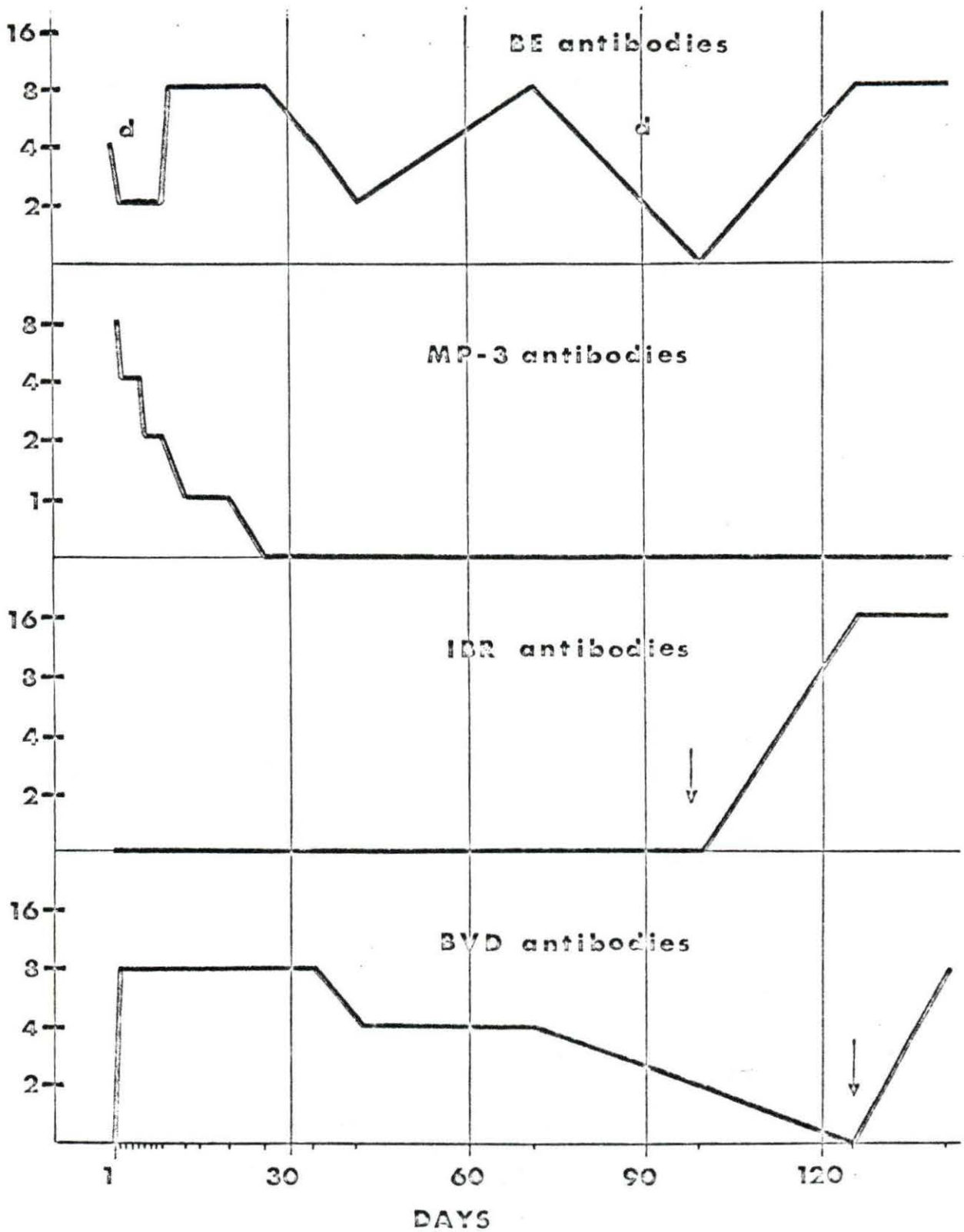
No virus was detected in desiccated serum to which IBR-ISU-2 virus had been added prior to drying. The pig serum used did not contain neutralizing antibodies against IBR-ISU-2.

Animal Experimentation

Virus neutralization tests performed on serum samples from the dam showed that it had an antibody titer of 1/64 against BE-LCR-4, and no antibodies against BVD-NADL and IBR-ISU-2. No tests were run for MP-3 antibodies.

The calf was born with antibodies against MP-3 and BE-LCR-4 as shown in Figure 12. It was fed serum (lot 2), containing a 1/128 titer of BVD-NADL antibodies, a 1/8 titer of BE-LCR-4 antibodies, a 1/32 titer of MP-3 antibodies and no antibodies against IBR-ISU-2. Significant levels of BVD virus neutralizing antibodies were absorbed by the calf after feeding the serum. Bovine virus diarrhea antibodies decreased

Figure 12. Circulating antibody levels present in a calf fed desiccated Rath serum. Antibodies are measured by 50 percent plaque reduction with the exception of the MP-3 antibodies which were measured by HI. Arrows indicate date of challenge, (d) indicates periods of mild diarrhea.

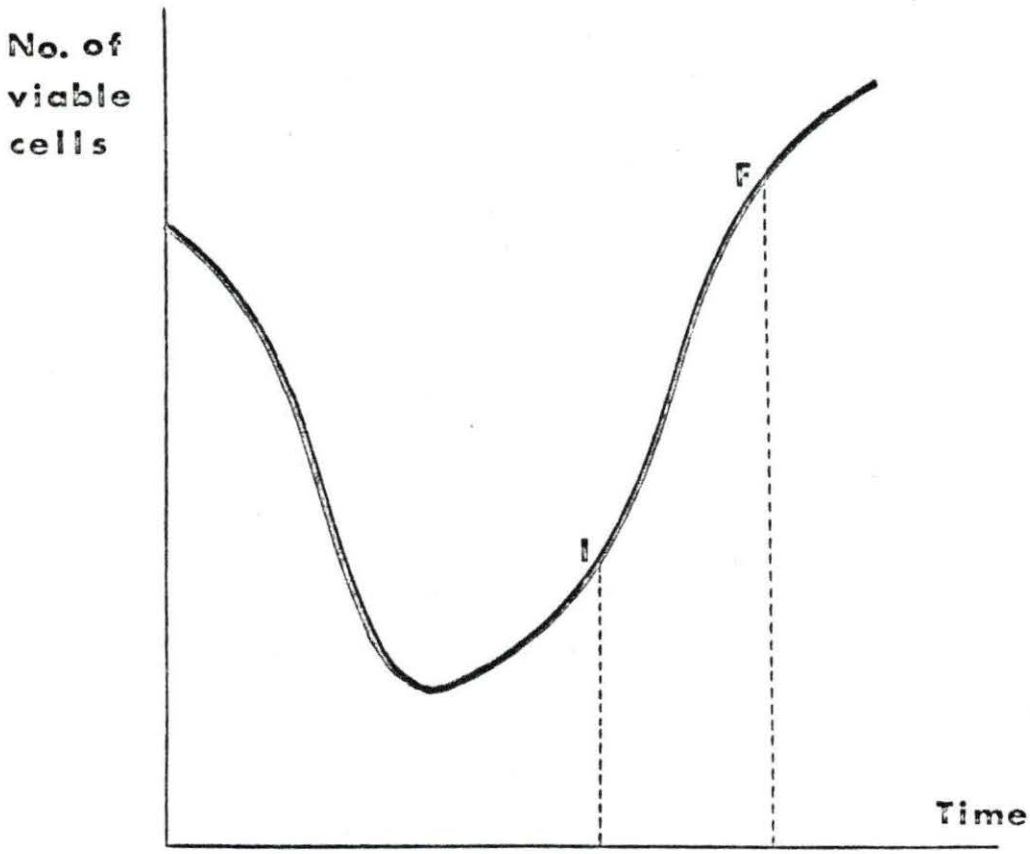

BE antibodies
16
8
4
2
d
d
MP-3 antibodies
8
4
2
1
IBR antibodies
16
8
4
2
v
BVD antibodies
16
8
4
2
v
1
30
60
90
120
DAYS

progressively until 4 months at which time they were undectectable. The MP-3 HI activity disappeared in less than 1 month. Antibodies against BE-LCR-4 appeared to fluctuate periodically. When the animal was challenged with IBR-ISU-2 at approximately 3 months of age, it showed no alterations of body temperature or white cell counts. It developed small necrotic focci in both nostrils from the fourth to the seventh day. The virus was reisolated from nasal swabs. A marked antibody response was noted.

At 4 months of age the calf was challenged with BVD-Lewis and developed a mild clinical disease. A first temperature peak was seen at 2 and 3 days postinoculation and a second one at 6 to 7 days. Leukopenia was observed concomitant with the first temperature peak. No diarrhea or lesions were observed and virus reisolation attempts were negative. A specific antibody response was also observed in this case.

Use of Desiccated Rath Serums in Cell Culture Media

In preliminary experiments a growth lag was observed. On closer examination it was seen that many of the cells which initially attached to the surface failed to divide and subsequently were released into the medium. Figure 13 is a schematic representation of this lag period and indicates the period of logarithmic growth when the field counts were made.



$$G = \frac{T \times 0.301}{\log F/I}$$

Figure 13. Schematic representation of the lag phase and logarithmic growth phase of heavily seeded bovine testicle cell cultures. Final (F) and initial (I) field counts were used to estimate generation times (G values).

Preliminary experiments showed that the desiccated Rath bovine serum did not support growth of cells as well as other normal mammalian serums. The product was dialyzed against Hanks' BSS for 24 hours at 10 C to eliminate any dialyzable toxic substance and also was supplemented with vitamins, amino acids and hypoxanthine. These nutrients were added to give a final concentration of 1 percent of solution IV (vitamins), 1 percent of a 100X solution of amino acids[1], and 7.5 percent of solution III (hypoxanthine). A typical microscopic field of bovine testicle monolayers observed with the oil immersion objective is shown in Figure 14.

Figure 15 shows the average number of cells per microscope field in initial and final observations and also the average G values obtained with different serums. Both the initial and final values obtained with horse serum were significantly higher than those obtained with any of the Rath serums. The G values obtained by this method were very variable and always longer than those obtained using other methods. This can be explained by the fact that in mass cell cultures of primary cells there are many cell types, some of which are incapable of duplicating in vitro but retain a normal morphology. Figure 16 presents the statistical treatment of results presented in the previous figure.

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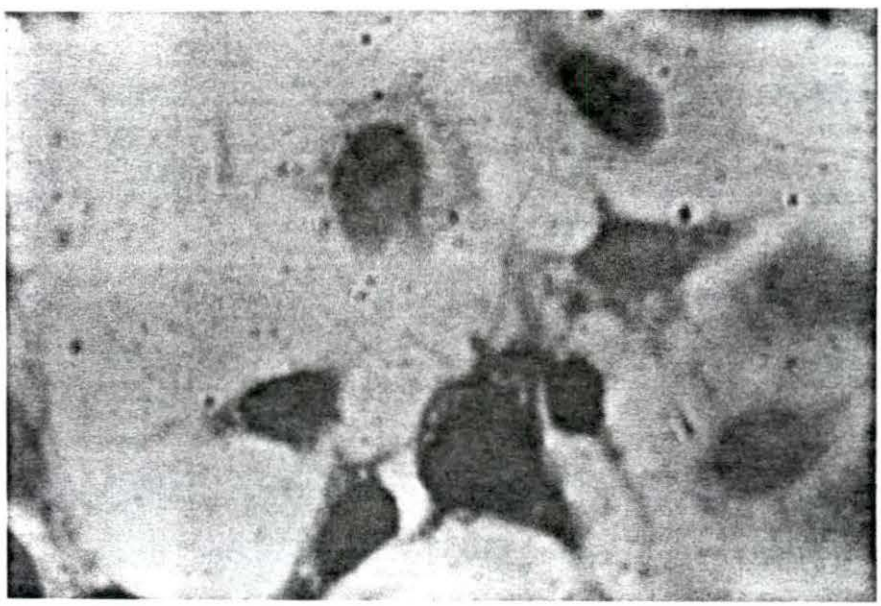
[1]Hyland Laboratories. Los Angeles, California.

Figure 14. Cells from a primary bovine testicle monolayer observed with the oil immersion objective.



Serum	Initial Values	Final Values	G Values (in hours)
Horse Serum	43.30	54.56	97.62
Desiccated Serum	27.06	45.58	41.16
Desiccated Serum with Supplements	34.26	44.86	87.88
Dialyzed Desiccated Serum	34.70	44.10	96.40
Dialyzed Desiccated Serum with Supplements	37.06	41.22	142.94
Liquid Rath Serum	33.26	48.70	54.78

Figure 15. Growth of heavily seeded primary bovine testicle cells using 20 percent levels of various serums. Average number of cells per microscope field in initial (14 hours) and final (44 hours) observations and G values of monolayers grown with various serums.

Comparisons	Degrees of Freedom	Tabulated F	Initial Values F Index	Final Values F Index	G Values F Index
Between all Serums	5	2.62	4.26 *	6.04 *	1.77
Horse vs. Liquid Rath Serum	1	4.26	7.71 *	4.84 *	1.25
Supplemented vs. Non-supplemented Serums	1	4.26	4.17	1.85	4.15
Dialyzed vs. Non-dialyzed Serums	1	4.26	3.49	1.32	2.97

* Significant at the 5% level.

Figure 16. Multiple comparisons between initial values, final values and G (generation time) values of various serums tested for growth of primary bovine testicle cells in mass cultures.

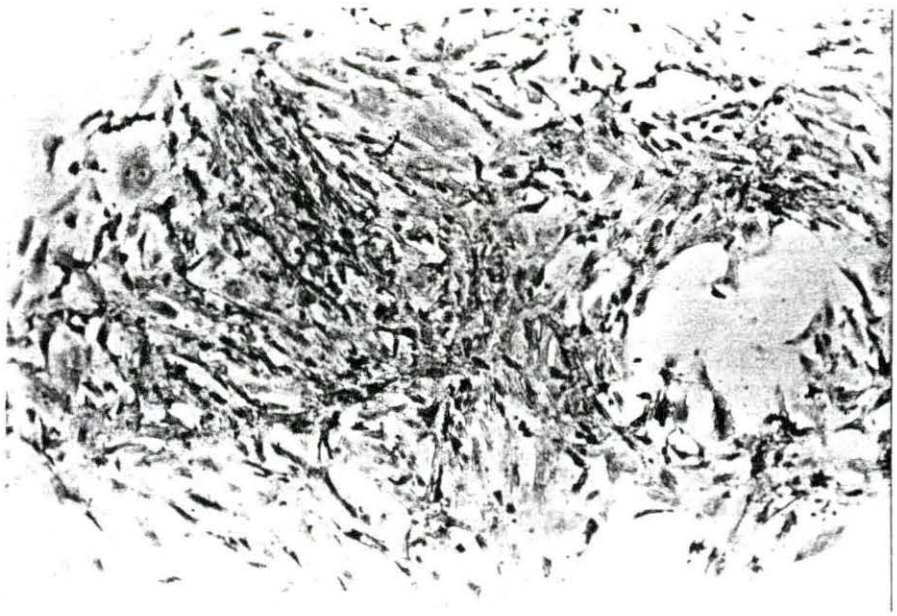
Multiple comparisons were performed showing only differences between the initial and final number of cells per field in cell cultures grown with horse serum as compared to a liquid sample of Rath bovine serum. No improvement was noted by dialysis or by the addition of supplementary nutrients.

Single cell plating experiments using primary bovine testicle cells yield 2 major types of clones composed of fibroblast-like and epithelial-like cells. The morphology of the fibroblastic type of clones varied when grown in different serums. A fibroblastic clone grown in media with lamb serum is shown in Figure 17. When fibroblastic clones developed in media containing horse serum they had a very large central area which was devoid of cells. A clone of this type is shown in Figure 18. In these experiments using both serums, 5 percent of the clones were epithelial-like. When using desiccated Rath serum, 100 percent of the clones were epithelial-like with a morphology as shown in Figure 19. The very low number of clones obtained indicated that only a few cells could grow under these conditions.

Very similar results to those obtained by mass cell culture methods were seen in single cell plating experiments with bovine kidney cell lines. The plating efficiency values in Figure 20 show that the liquid, desiccated and dialyzed desiccated Rath serums gave plating efficiencies that were much lower than those obtained from normal mammalian serums.

Figure 17. Clone of fibroblast-like bovine testicle cells grown in medium containing 15 percent lamb serum.

Figure 18. Clone of fibroblast-like bovine testicle cells grown in medium containing 15 percent horse serum.



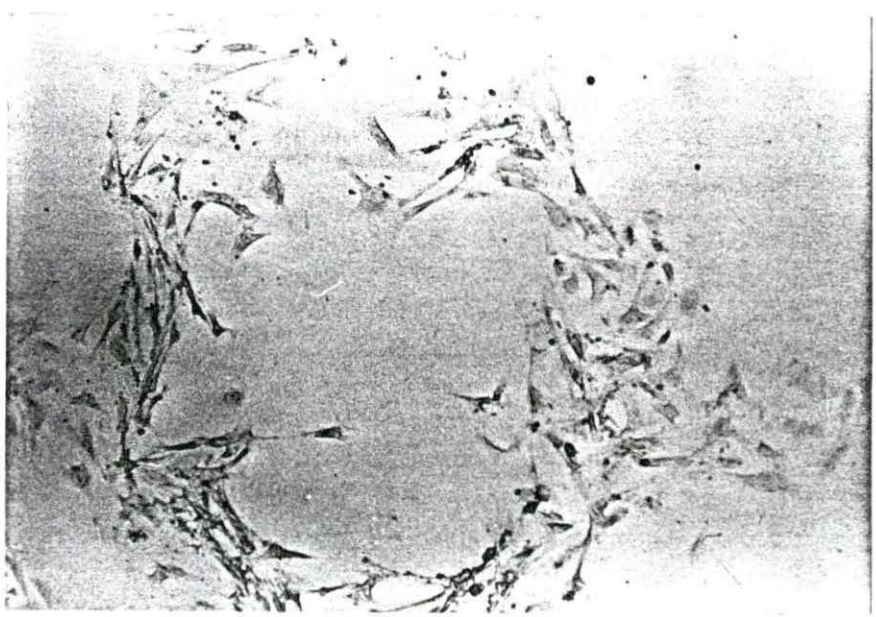
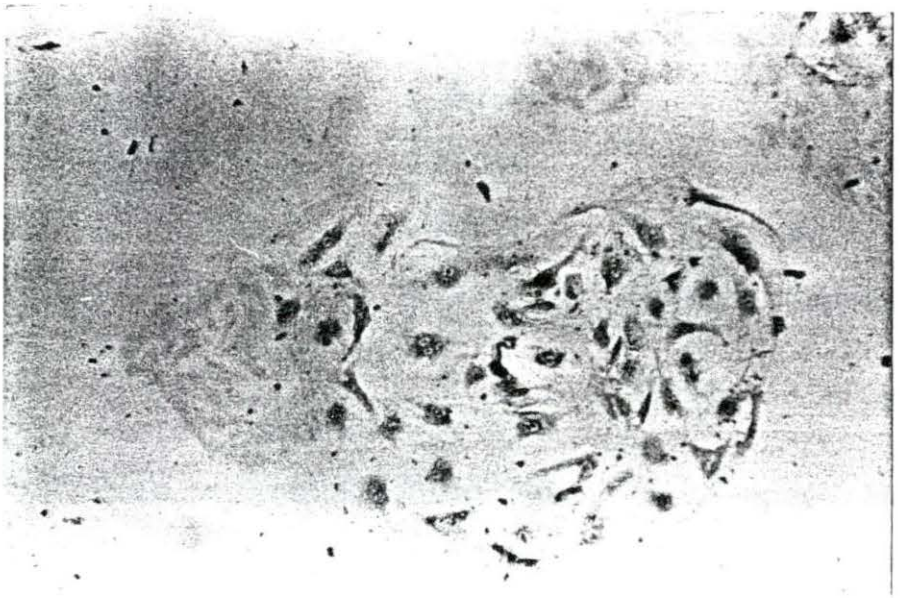


Figure 19. Clone of epithelial-like bovine testicle cells grown in medium containing desiccated Rath serum.



Serum	Plating Efficiency	Generation time (in hours)
GIBCO Lamb Serum [a]	24.0%	36.3
GIBCO Fetal Calf Serum	21.7%	39.7
Horse Serum	17.1%	47.4
Liquid Rath Serum	2.05%	54.1
Desiccated Rath Serum	0.29%	120
Dialyzed Desiccated Serum	0.087%	120

[a] Grand Island Biological Co.

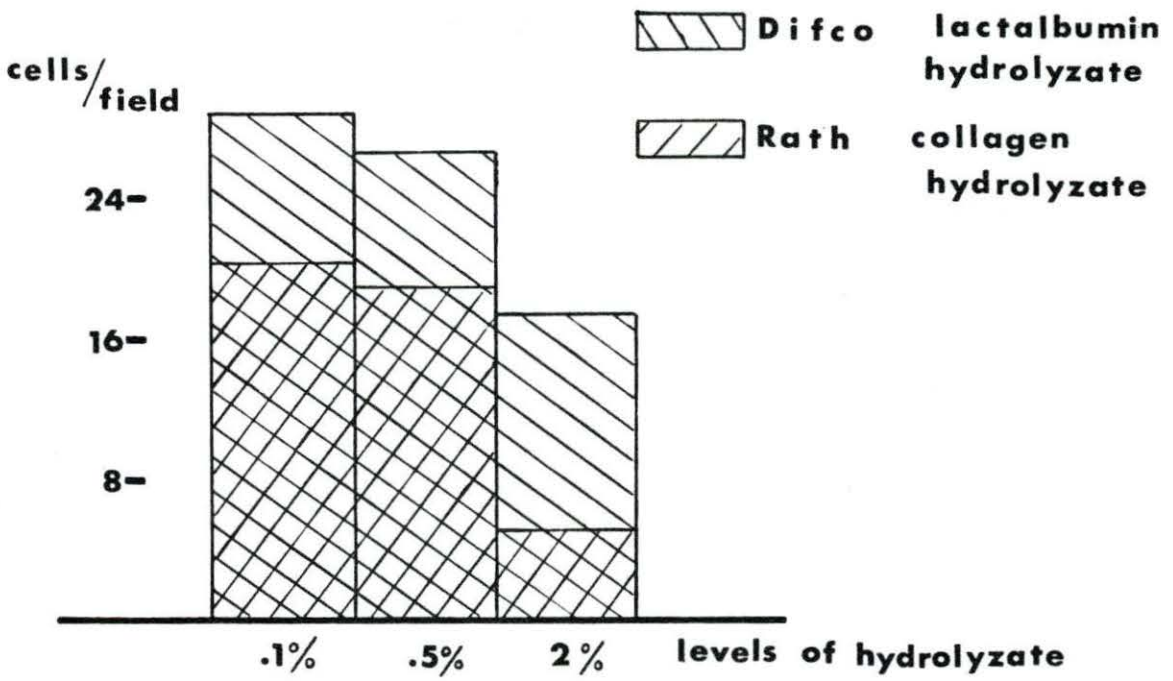
Figure 20. Influence of the type of serum on the growth of established cells derived from a bovine kidney.

The very long generation times obtained with the desiccated serums indicate that with these serums, cell division was almost completely impaired.

Collagen Hydrolyzate

When the Rath collagen hydrolyzate was tested as an amino acid source in mass cell cultures of primary bovine testicle cells, it showed a slight growth inhibitory effect at high concentrations when added to amino acid free Hanks' BSS with 10 percent lamb serum. Figure 21 also shows that this inhibitory effect was present when using lactalbumin hydrolyzate at the same concentrations. The statistical tests presented in Figure 22 show that the inhibitory effect of increasing concentrations of hydrolyzate could not be considered significant. There was a significant difference between both hydrolyzates. It was thought that this difference was due to the presence of saturated fatty acids in the collagen hydrolyzate acting as toxic factors. Experiments were undertaken using media which contained bovine serum albumin as a fatty acid binding agent and a cloned cell line to ensure a uniform population of cells. Figure 23 shows the typical clones produced by the bovine kidney cell line. The variation in morphology and size between the cell clones is readily apparent. Figure 24 shows clones produced by the BK 2 cell line. These clones were very uniform in size and

Figure 21. The effect of protein hydrolyzate on mass cell cultures of primary bovine testicle cells.



Growth of heavily seeded ($1{:}5 \times 10^6$ cells/ml) primary bovine testicle cells using varying levels of protein hydrolyzate

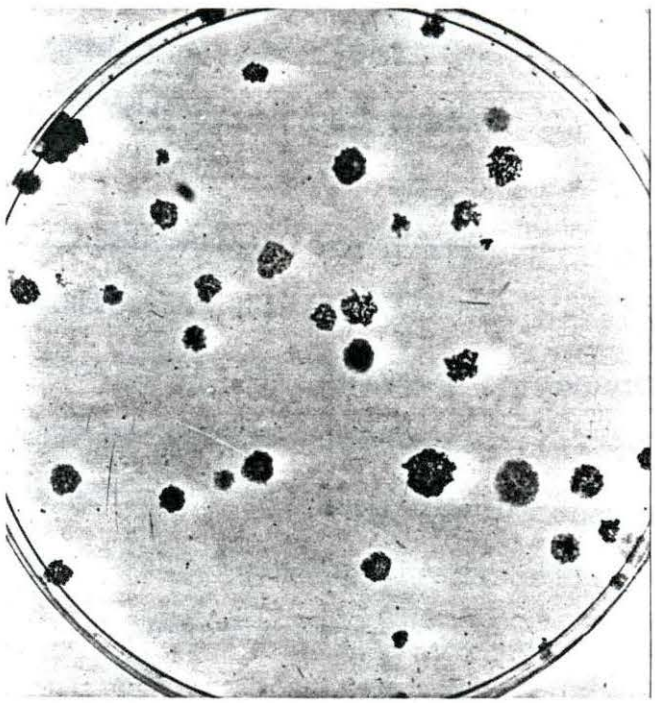
Source of variation	Variance	t value
0.1% Rath hydrolyzate	3.525	0.826
0.5% Rath hydrolyzate	5.697	
0.1% Difco hydrolyzate	7.257	0.933
0.5% Difco hydrolyzate	11.677	
0.1% Rath hydrolyzate	3.525	5.286 *
0.1% Difco hydrolyzate	7.257	

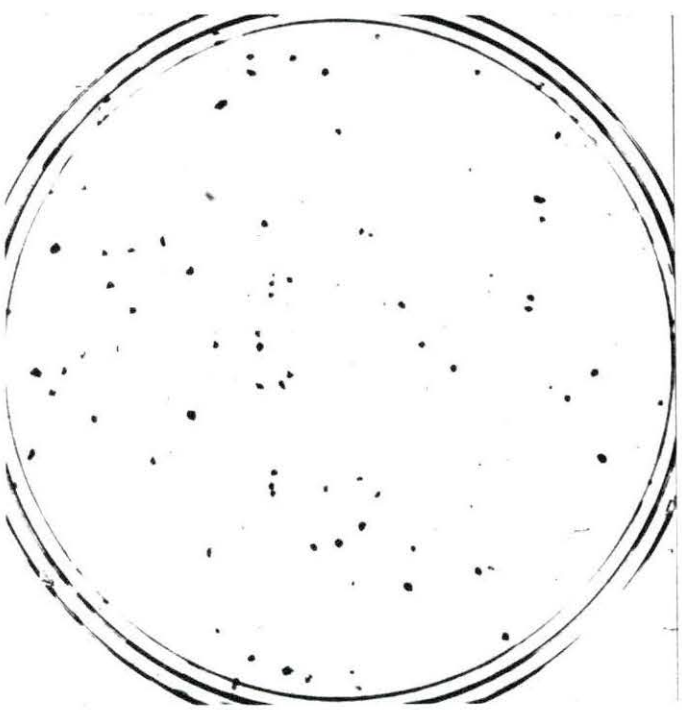
* Difference significant at the 5% level.

Figure 22. The effect of protein hydrolyzates on mass cell cultures of primary bovine testicle cells. Statistical tests.

Figure 23. Clones of cells from an uncloned bovine kidney cell line.

Figure 24. Clones of cells derived from the BK2 cloned bovine kidney cell line.



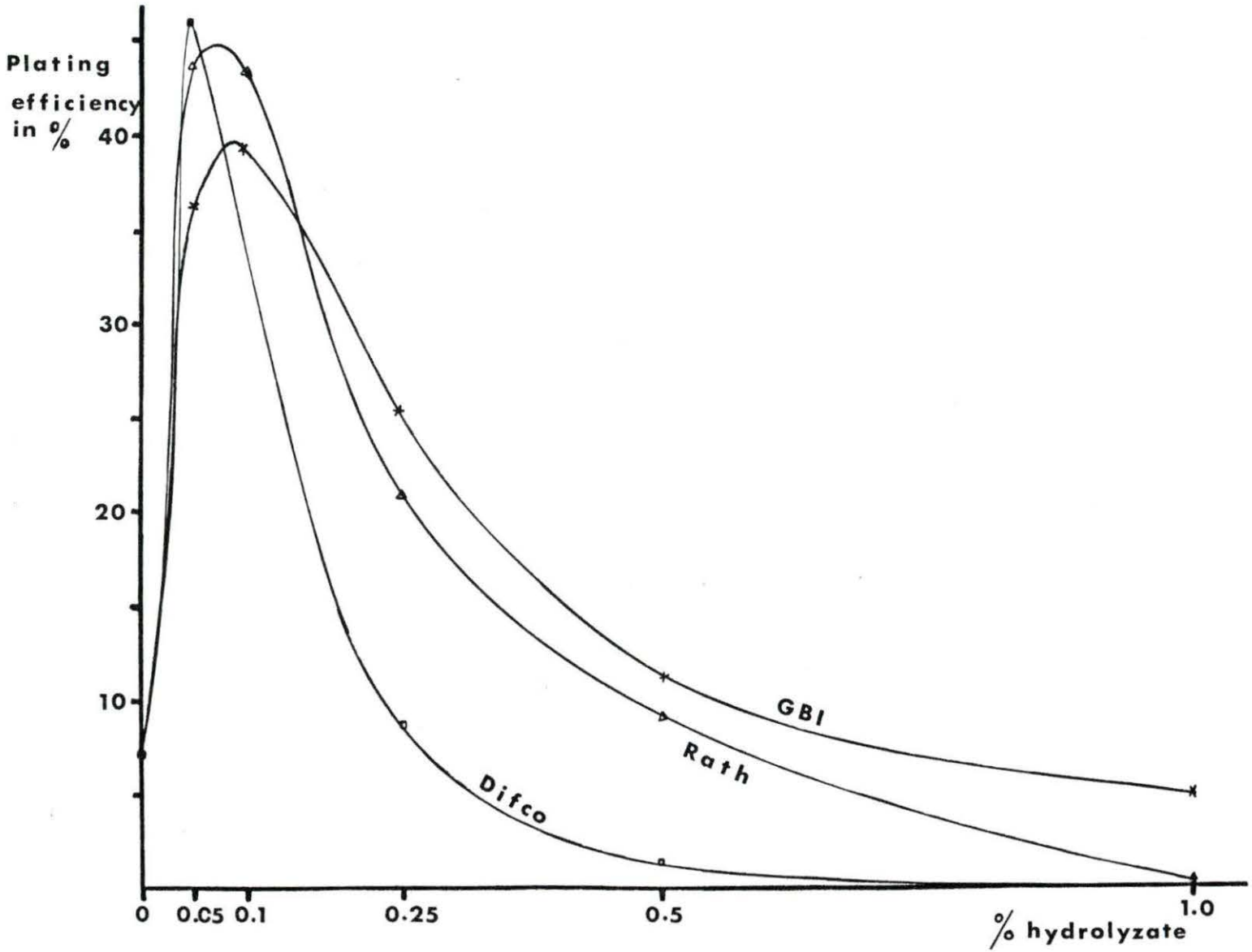


were all formed by the same morphologic type of cells. Figure 25 shows a typical BK2 clone after 5 days of culture. These cells, when grown in F4 medium with 10 percent BSA solution and 4 percent lamb serum, exhibited a plating efficiency of 37-40 percent and a generation time of 24.5 hours.

When the 3 protein hydrolyzates used were tested at various levels in F4A medium, the inhibitory effect of high concentrations of hydrolyzate was readily evident as shown in Figure 26. All 3 products showed an optimum at 0.05 - 0.1 percent, much lower than the commonly used 0.5 percent level. The difference in plating efficiencies at 0.01 and 0.5 percent was obviously significant. Some statistical tests run on the data are shown in Figure 27. No differences could be seen in the plating efficiency of the BK2 cells when using 0.05 percent of the hydrolyzates tested. At a concentration of 0.5 percent of hydrolyzate, some differences were detected.

Figure 25. BK2 clone of cells after 5 days of incubation.

Figure 26. The effect of various concentrations of protein hydrolyzates on the plating efficiency of BK2 cells.


Plating
efficiency
in %
40
30
20
10
0
0.05
0.1
0.25
0.5
1.0
% hydrolyzate
GBI
Rath
Difco

Source of variation	Variance	t value
0.05% Difco hydrolyzate	160	0.185
0.05% Rath hydrolyzate	66.9	
0.05% Difco hydrolyzate	160	0.762
0.05% GBI hydrolyzate	64.5	
0.50% Difco hydrolyzate	1.12	1.565
0.50% Rath hydrolyzate	85.8	
0.50% Difco hydrolyzate	1.12	11.29 *
0.50% GBI hydrolyzate	14.1	

* Significant at the 5% level.

Figure 27. The effect of various concentrations of protein hydrolyzates on the plating efficiency of BK2 cells. Statistical tests.

DISCUSSION

A calf fed a reconstituted desiccated serum during the first hours of life absorbed almost all the antibodies it contained, maintaining significant levels of these antibodies in its blood for several months. Similar results were obtained by Smith and Little (42) by feeding serum obtained from bovines hyperimmunized with E. coli cultures. The present experiments showed that desiccation of bovine serum did not affect the properties of the antibody containing globulin fraction of the serum, leaving them in a physico-chemical state that made them readily absorbable through the intestinal mucosa of the newborn calf. This suggests that the medication of newborn calves with a product of this nature may be of prophylactic value as well as being economically feasible, due to low manufacturing cost and ease of transportation.

Newborn calves are known to absorb large macromolecules, including antibodies through the duodenal mucosa during the first 24 hours after birth, according to Deutsch and Smith (11). These authors observed that the time at which antibodies ceased to be absorbed (closure), was not influenced by the feeding of colostrum, amniotic fluid, lactose, or by the parenteral administration of various hormones. These findings contrast with those found by Leece et al. (27) in pigs. They reported that the closure phenomenon in the

piglet was effected by a heat stable dialyzable component present in colostrum. Gillette and Filkins (23) reported that newborn puppies from bitches that had received large doses of ACTH or cortisone, could not absorb antibodies. Administration of these hormones to the puppies at birth did not alter the normal absorption. A more complete knowledge of the factors responsible for antibody absorption and the closure phenomenon in the gut of the newborn calf will be very helpful in understanding disease conditions in these animals, leading to better and more efficient animal management practices and preventive medications.

The rates of decrease of BVD and MP-3 antibody titers were markedly different. The disappearance of MP-3 antibodies in less than 1 month suggested that the HI action against MP-3 measured in the serum samples may have been due to non-specific inhibitory proteins. These inhibitors would be present at high concentrations in the serum of the neonatal animal and decrease progressively with age.

The levels of BE-LCR-4 antibodies exhibited a periodical variation with 3 peaks. Since BE isolation attempts were not made, a definite statement explaining this phenomenon cannot be made. The fact that mild episodes of diarrhea were associated with low levels of antibodies, may suggest that a bovine enterovirus infected this animal from birth.

Disc electrophoresis has been used extensively to study the characteristics of proteins. Serum proteins, in

particular the albumins, α and β globulins have been studied by using 7.5 percent polyacrylamide gels. Ornstein (33) calculated the average pore size of such gels obtaining values of 50 Angstroms, a size that would permit the migration of the 7S γ globulins (with a diameter of 44 Angstroms) but would only partially permit the penetration of the 19S globulins. This has to be taken into account when comparing a disc electrophoresis pattern of serum proteins with one obtained with starch gel electrophoresis, the latter having a larger pore size permitting the migration of 19S globulins.

Heat desiccation of bovine serum produced an increased amount of a band tentatively identified as a fast α_2 globulin. The nature or origin of this band could not be elucidated. Based on the observations made by Clarke (9) showing that free hemoglobin migrated with the same mobility as some of the transferrins in disc electrophoresis, this possibility can be ruled out, since there was no correlation between serum samples having large amounts of free hemoblobin and those that had a fast α_2 globulin band. The desiccation did not lower the amount of transferrins present but it did change the mobility of one of these bands, making it fuse together with a slower moving band. Desiccation also lowered the amount of γ_1 globulins and α_2 macroglobulins when considered together. Since the latter were present only in some serums, the reduction was mainly due to a loss in γ_1 globulins. This was not reflected in the antibody

titers, which remained unchanged after desiccation.

Considerable interest has developed in recent years in the production of desiccated media for tissue culture. There are now commercially available powdered media that include all the necessary micromolecular requirements for cell growth in vitro. These media have to be supplemented with serum before use. The use of spray dried serums represents a distinct possibility in making available a powdered medium that contains all the components necessary for cell growth. The Rath serums tested were not satisfactory for cell growth, but the available data indicates that the desiccation process itself was responsible for only a minor part of this alteration since samples of serum taken before desiccation did not prove to be acceptable. It was not possible to precisely determine the causes of this alteration in the serums, but some possibilities were excluded. Dialysis and addition of nutrients, including thermolabile vitamins did not improve the quality of the product, even when it ensured that all the citrate and excess calcium ions added during the defibrination process were eliminated.

It was thought that the Rath serums contained toxic nondyalizable proteins of bacterial origin, although other possibilities could not be excluded. Most cell culture media or glassware contain traces of heavy metals, which have a toxic effect on cells. The addition of proteins to the culture medium helps in neutralizing this toxic action if

the proteins are not in a denatured state. The data presented cannot exclude the possibility that denaturation of proteins binding heavy metals had taken place, especially since desiccation of the serum did produce alterations in the transferrin bands as seen by disc gel electrophoresis.

While it is generally recognized that amino acids are indispensable for the growth of cell cultures, only lately has attention been directed to the problem of feedback inhibition due to an excess of these nutrients. These phenomena are well known in bacterial physiology and recent reports by Eagle et al. (13) indicate that they also occur in mammalian cells. The use of lactalbumin hydrolyzate at 0.5 percent in growth media is common in many cell culture laboratories. While this will not produce drastic alterations on the growth of cells, it may be responsible for a slower rate of growth and discrete morphological alterations such as increased cellular granularity, due to endproduct inhibition. No attempts were made to find out which inhibition mechanism was operating or which were the biochemical reactions inhibited. Further studies on the endproduct inhibition of mammalian cells by amino acids could uncover important regulatory mechanisms present in cells and aid in obtaining a deeper understanding of various biochemical reactions.

SUMMARY

The number and electrophoretic mobility of normal bovine serum protein bands was determined using the disc electrophoresis technique. Ten lots of commercially heat-desiccated bovine serums were also tested detecting some variations in amount and mobility of protein bands. The deletion of one of the transferrin-containing β globulin bands was observed in almost all samples of heat-desiccated serum. An increase in fast α globulins and a lowered amount of γ_1 globulins was also detected.

All the lots of commercial serum obtained at various times during the year contained antibodies against 4 common bovine viruses. The heat desiccation process did not affect the levels of these antibodies.

The serums, as obtained directly from the industrial process, were heavily contaminated with bacteria. A flash-pasteurization before the desiccation of the material, decreased the bacterial flora to relatively low levels. The desiccation process itself, proved to be effective in destroying a bovine virus.

When a sample of sterilized desiccated serum was fed to a neonatal calf, it absorbed the antibodies present in the product. Levels of circulating antibodies were detectable up to 4 months of age. The animal was challenged separately with 2 bovine viruses once the circulating antibodies had

disappeared, and a mild clinical infection was induced in both cases.

The heat-desiccated serum did not support survival or growth of cells in culture as well as did serums routinely used for cell culture procedures. This was shown in both mass and single cell cultures. Dialysis and/or addition of vitamins and other nutrients to the heat-desiccated serum did not affect these results.

A collagen hydrolyzate obtained under industrial conditions was also tested in cell cultures with promising results. The product showed slight cell toxicity when used in cell culture growth media. Under appropriate non-toxic conditions, it served as an amino acid source for cell growth of comparable quality to other protein hydrolyzates presently on the market. All protein hydrolyzates tested showed an optimum growth promoting effect when used at 0.01 percent in cell culture media, and were definitely inhibitory at higher concentrations.

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