SERUM PROTEINS AND ANTIBODIES IN THE GERMFREE AND GNOTOBIOTIC PIG

Thesis for the Degree of M. S.

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ABSTRACT

SERUM PROTEINS AND ANTIBODIES

IN THE GERMFREE AND GNOTOBIOTIC PIG

by Robert A. Brooks

Serum proteins of germfree and gnotobiotic pigs changed during the first 8 days of life. Electrophoretic separation revealed a decrease in relative amounts of alpha globulin and an increase in albumin and beta globulin. These changes occurred in both germfree and Escherichia coli infected pigs. Immunoelectrophoretic analysis revealed that the serum proteins of neonatal germfree pigs gradually increased in their ability to form sharply delineated precipitin arcs with rabbit anti-pig serum antiserum. This ability was detected at a younger age and to a greater degree in animals exposed to E. coli by oral or subcutaneous inoculation. An immunoprecipitin arc in the gamma globulin range was detected in the serum from germfree and gnotobiotic pigs.

Antibodies to E. coli could not be detected by agglutination or immunodiffusion techniques in 8-day-old pigs which were inoculated at 1 day of age.

The effects of hemoglobin and nutrients absorbed from a milk diet on electrophoresis and immunoelectrophoresis of neonatal germfree pig serum were determined. Hemoglobin in concentration of 1 Gm./100 ml. resulted in a distortion of immunoelectrophoretic precipitin arcs and migrated with alpha and beta globulins during electrophoresis.

Robert A. Brooks

Absorbed nutrients had no detectable effects on either electrophoresis or immunoelectrophoresis of the serum.

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By

Robert A. Brooks

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To my colleague, James A. Osburn, I wish to express my thanks for assistance in establishing technical procedures used in this research.

I am grateful to the Department of Pathology, Michigan State University, for permitting a flexible schedule which allowed the completion of this work.

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INTRODUCTION

The development of serum proteins in neonatal pigs has been investigated by several workers (Lecce and Matrone, 1960; Lecce, Matrone and Morgan, 1961; Miller *et al.*, 1961; and Ramirez *et al.*, 1963). Generally the newborn pig has been described as having "incomplete" or "immature" globulins which gradually or quickly change, depending on the diet.

Extrinsic factors such as colostrum and antigenic stimulants in the environment are difficult to control and could differ from one investigation to another. A gnotobiotic or germfree pig would have fewer extrinsic variables so that the more precisely defined and controlled environment would offer a better basis for comparison between pigs and with other germfree animals as well. The development of serum proteins and the formation of antibodies could then be confidently associated with specific agents or events.

The objectives of this study were (1) to determine the normal serum protein profile of germfree pigs 1 to 8 days of age, as detectable by electrophoresis and immunoelectrophoresis, (2) to observe the effect of a single, well defined antigenic stimulus (*E. coli* 0 138:K 81 NM) on the developing serum proteins of gnotobiotic pigs from 1 to 8 days of age and (3) to determine whether antibody formation against *E. coli* 0 138:K 81 NM could be detected in the serums of gnotobiotic pigs under 8 days of age by using agglutination and immunodiffusion techniques.

REVIEW OF LITERATURE

The germfree pig is an expensive research animal because it requires specifically designed and constructed housing, it is difficult to obtain and requires meticulous care and handling. Therefore, most research with pigs has utilized a nonsterile environment, and this review will include investigations that utilized these animals.

The separation of serum proteins of newborn pigs and/or pig fetuses employing paper electrophoresis has been described by Rutqvist (1958), McCance and Widdowson (1959), Lecce and Matrone (1960), Miller et al. (1961), Lecce, Morgan and Matrone (1962), Waddill et al. (1962), Pirtle and Deyo (1963) and Ramirez et al. (1963). Waddill et al. (1962) and Ramirez et al. (1963) reported that gamma globulin comprised approximately 9% of the serum proteins in unfed, newborn pigs. Miller et al. (1961) found 6.5% gamma globulin, while Pirtle and Deyo (1963) gave a value of 5% of total proteins attributable to gamma globulin. All other workers cited have described the serum of newborn pigs as containing no gamma globulin detectable by electrophoresis. The alpha globulins have been shown to be the largest single protein fraction, comprising from 48% to 80% of the total. Albumin and beta globulin are considered minor fractions.

Lecce, Morgan and Matrone (1962) reported a protein with an electrophoretic mobility between albumin and alpha globulin in the serum of newborn pigs. This protein gradually disappeared and was undetectable in the serum of adult pigs.

It was noticed by Lecce and Matrone (1960) and Lecce, Morgan and Matrone (1962) that, as pigs matured, diets affected the proportions of electrophoretically separated serum protein fractions. However, the proteins detectable by immunoelectrophoresis were unaffected by diet and appeared with regular consistency at predictable times.

Brummerstedt-Hansen (1963) used immunoelectrophoresis to detect serum proteins of pig fetuses. The antiserum used was obtained from a rabbit which had been immunized with serum from adult pigs. Despite the fact that fetal serum was not used to produce the antiserum it was shown that the fetus developed 3 main protein fractions, i.e., albumin, alpha globulin, and beta globulin. The number of precipitin arcs increased from 4 at 25 days' gestation to 15 at 112 days' gestation. Using the same antiserum, adult pig serum was found to contain 20 precipitin arcs. In a separate study (Brummerstedt-Hansen, 1961) as many as 24 precipitin arcs were found in the serum of some adult pigs. Electrophoresis on agar showed 3 protein fractions in fetal pig serum having the same mobility as albumin and alpha and beta globulin. No gamma globulin was observed.

Sterzl et al. (1960) and Segre and Kaeberle (1962b) detected otherwise undetectable gamma globulin in the serums of unfed newborn pigs by employing immunoprecipitin techniques. A 50-fold concentration of serum was sometimes necessary before gamma globulin could be detected.

Although Jacobson and Moustgaard (1950) determined that the placental structure in the pig is not permeable to maternal gamma globulin, Myers and Segre (1963) reported evidence of transplacental transfer of gamma globulin in pigs.

The work of Ashton (1960), Kristjansson (1960a, 1960b), Brummerstedt-Hansen (1961) and Scopes (1963) illustrated that variations occur in the serum proteins of pigs. These variations have been attributed to genetic differences.

The immunologic capabilities of young pigs have been investigated by several workers. Hoerlein (1957) found that pigs deprived of colostrum did not form antibodies to injected antigens. Colostrum-fed pigs produced antibodies if the colostrum did not contain hyperimmune levels of antibody against the injected antigen. Segre and Kaeberle (1962) injected diptheria and tetanus toxoids into 3-week-old specific-pathogen-free pigs. They reported that feeding colostrum or mixing the toxoids with dilute, toxoid-specific hyperimmune serum resulted in an immunologic response first detectable 2 weeks after inoculation. Similar results could be obtained by mixing the antigen with large amounts of immune or normal serum from colostrum-deprived pigs. Injection of the toxoids alone resulted in little or no immune response. This substantiated the natural selection theory proposed by Jerne (1955, 1960) which described preformed antibody as a prerequisite for the formation of antigen-stimulated antibody.

Aiken and Blore (1964) reported that the newborn pig is capable of producing antibodies providing that the antigen is allowed to persist within the animal for several days after inoculation. This ability to produce antibodies is lost if large amounts of passively acquired antibodies combine with the antigen at the time of inoculation.

Miller et al. (1962) used a Salmonella pullorum antigen to determine the effect of the time of inoculation on the production of antibodies in the nursing pig. Other investigators cited by Miller et al. (1962) conducted similar studies using other antigens.

Olson and Wostman (1964), working with conventional and germfree guinea pigs reported that all major serum globulins were affected by antigenic substances.

MATERIALS AND METHODS

Source of specimens. Serum samples were obtained from 52 germfree and gnotobiotic Yorkshire pigs from 4 litters reared by the modified method described by Waxler et al. (1966). Samples were taken soon after birth and at varying times from 1.5 to 168 hours after subcutaneous or oral inoculation with Escherichia coli 0 138:K 81 NM. Litters 1, 2 and 3 were inoculated subcutaneously at 24 hours of age, and litter 4 was inoculated orally at 48 hours of age. The inoculum for litter 2 was obtained from Dr. D. K. Sorensen of the University of Minnesota. The other pigs were inoculated with organisms obtained from Dr. G. L. Waxler of Michigan State University. Germfree controls were maintained and sampled at periodic intervals (TABLES 1, 2, 3 and 4). Serum was also obtained from 6 young (4 to 7 weeks) and 8 adult, conventionally reared pigs. All serum was frozen at -70 C. until needed.

Preparation of rabbit anti-pig serum antiserum.

Antigen. The antigen used for the production of antiserum contained serum from the following sources: (1) 5 germfree pigs, (2) 4 monocontaminated (E. coli) pigs, (3) 6 conventionally reared young pigs and (4) 2 adult pigs. Serum from the germfree and monocontaminated pigs comprised approximately 1/2 of the pool. The adult pigs' serum was 1/3 of the pool and the young, conventionally reared pigs contributed 1/6 of the pool. Potassium aluminum sulfate was used as an adjuvant and the mixture of serum and adjuvant was prepared as follows (Hirschfeld, 1960):

45.0 ml. 10% $KAl(SO_4)_2 \cdot 12\ H_2O$

12.5 ml. pooled serum

40.0 ml. distilled water

The pH was adjusted to 6.5 with 5N NaOH, the solution was centrifuged, the supernatant fluid was discarded and the sediment was washed twice with 0.85% NaCl solution. The volume was then made up to 50 ml. with 0.85% NaCl solution.

Inoculation of rabbits. Six Dutch rabbits were inoculated intramuscularly according to the following schedule as suggested by Hirschfeld (1960):

1st day - 3 ml. of alum precipitated proteins per buttock (6 ml./rabbit)

14th day - 4 ml. of alum precipitated proteins per buttock (8 ml./rabbit)

28th day - 1 ml. of pooled serum per rabbit, intraperitoneally

On the 34th day blood was collected from the rabbits and the sera were checked for cross-reactions by mixing a drop of serum from each rabbit with a drop of serum from each of the other rabbits using a slide precipitin method to detect possible reactions. No precipitation occurred so the serum samples were pooled, dispensed in 1-ml. portions and frozen at -70 C. until used.

Electrophoresis. A Spinco Durham cell[a] electrophoresis chamber was modified to accommodate 1 x 3-inch glass slides (Figure 1). The slides were coated with 2.5 ml. of 0.7% Agarose[b] solution in 0.0375 ionic strength

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[a]Beckman Instruments, Inc., Fullerton, California.

[b]Bausch and Lomb, Inc., Rochester, New York.

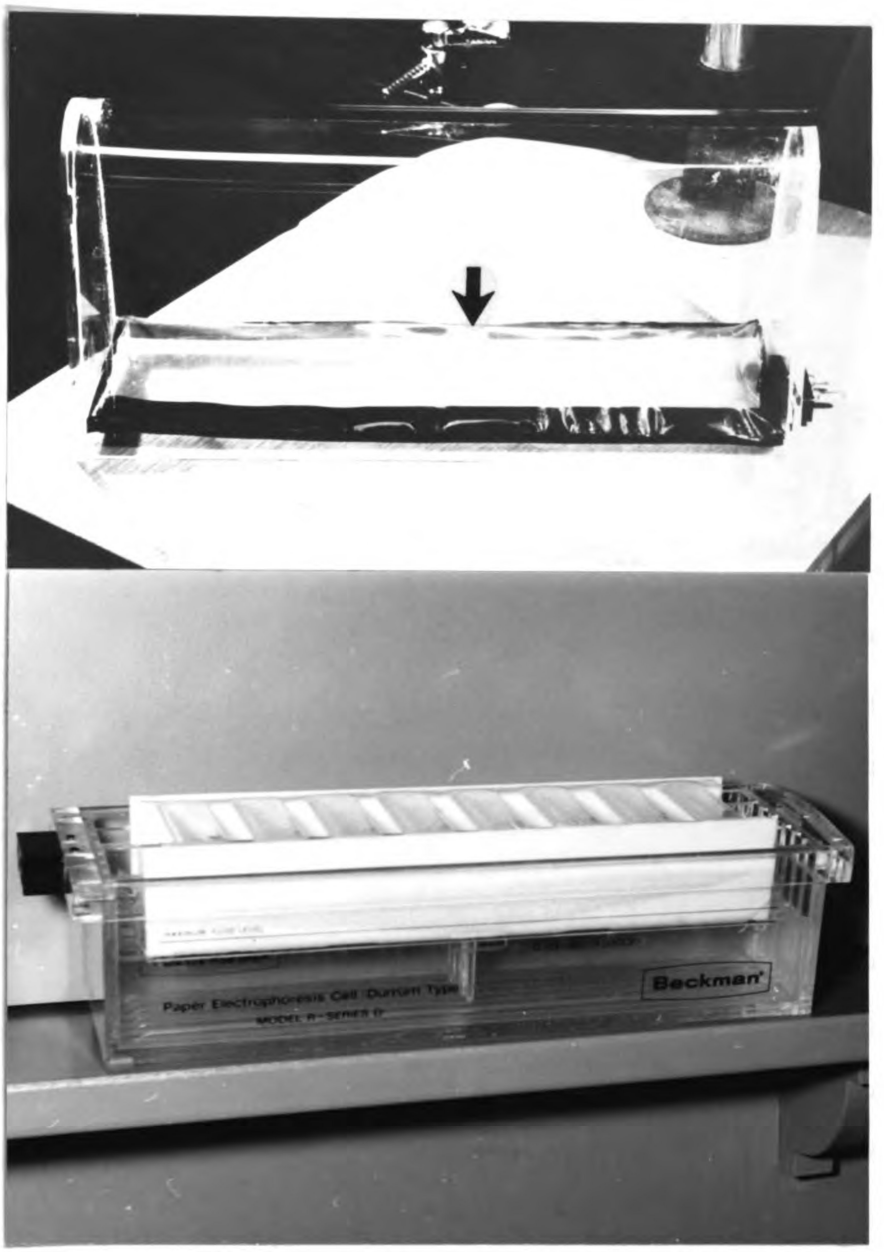

Figure 1. Electrophoresis chamber modified to accommodate 1 x 3-inch agar-coated glass slides. Top, cell cover with plastic sheet taped inside (arrow) to reduce air space. Bottom, cell showing paper wicks and slides in place.

veronal buffer, pH 8.5. Serum was applied directly to the surface of the agar and allowed to migrate for 80 minutes in 40 ma. of constant current. At the end of migration time the slides were immediately placed in absolute methyl alcohol for 15 to 30 minutes for fixation of the protein. They were then dried at 37 C. and stained with brom phenol blue for 20 minutes. Excess dye was removed in 2 baths of 5% acetic acid. The slides were then dried at room temperature. After exposing the slides to ammonium hydroxide vapors, densitometric tracings were recorded using a Beckman Analytrol.[a]

Immunoelectrophoresis. For a review of the theory and methods of immunoelectrophoresis the reader is referred to publications by Lawrence (1964), Jordan and White (1965) and Grabar (1965). Schiedegger's microimmunoelectrophoresis method (Schiedegger, 1955) was used with some modification.

The same materials as described for electrophoresis were used for immunoelectrophoresis. There were some differences in technique. These differences were as follows: (1) the serum samples were applied to wells cut into the agar. A device was made for cutting reproducible patterns of wells and antiserum slots in the agar (Figure 2) as suggested by Jordan and White (1965), (2) migration of the serum proteins proceeded for 70 minutes in 40 ma. of constant current.

Immediately following electrophoresis the slides were removed from the migration chamber and the agar removed from each antiserum slot. Approximately 0.1 ml. of rabbit anti-pig serum antiserum was placed in

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[a]Beckman Instruments, Inc., Fullerton, California.

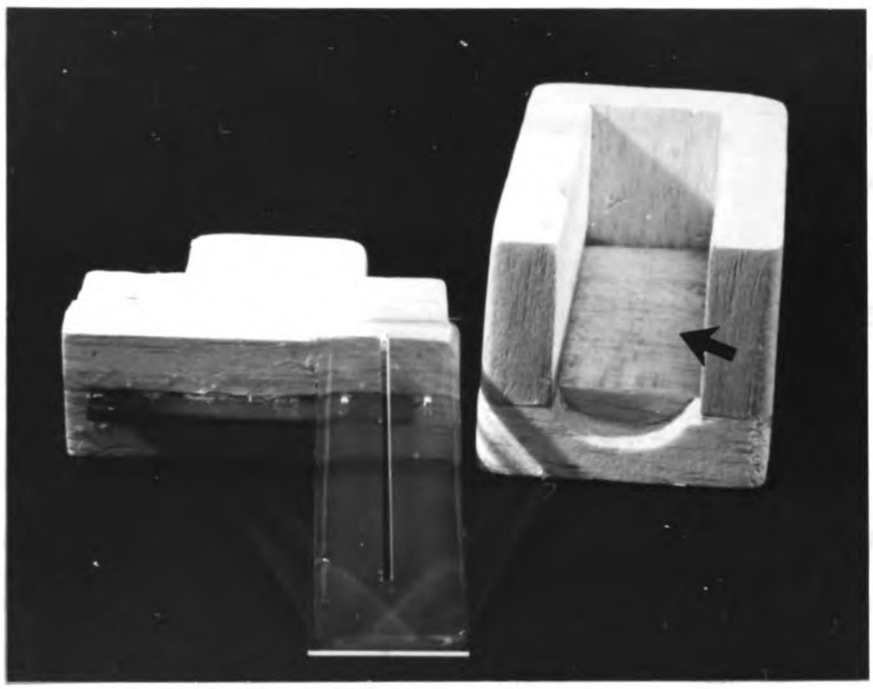

Figure 2. Agar cutter. An agar-coated slide (front, center) was placed in the chamber (arrow) and the plunger was pressed down to cut wells and slot for micro-immunoelectrophoresis.

each slot and the slides incubated for 18 hours at 37 C. in a moist chamber. Following diffusion and formation of precipitin arcs, the slides were "dialysed" against several changes of phosphate-buffered saline, 0.7 ionic strength, pH 7.2. This was completed in 24 to 48 hours at 37 C. Finally the slides were "dialysed" against several changes of distilled water at 25 C. for 2 to 4 hours. After drying at 37 C. the slides were stained with a trichrome stain described by Crowle (1961).

The effect of hemoglobin and chyle on serum immunoelectrophoretic patterns was determined by testing serum taken 30, 60 and 120 minutes after feeding 72-hour-old germfree pigs. Erythrocytes were added to a portion of each serum sample before freezing. The amount of hemoglobin present was determined by the cyanmethemoglobin method.

Immunodiffusion.

Antigen preparation. A culture of E. coli 0 138:K 81 NM was prepared by inoculating 1 liter of tryptose broth with the organism and incubating at 37 C. for 12 hours. The culture was centrifuged and the cells washed once with 0.85% NaCl solution. The organisms were then sedimented, resuspended in the 0.85% NaCl solution and subjected to ultrasonic vibrations[a] for 30 minutes. Disruption of the cell walls was obtained in approximately 98.5% of the bacteria as determined by a colony count of the suspension before and after exposure to ultrasonic vibrations. The cell walls and remaining viable cells were separated by centrifugation at 2,300 x g. for 30 minutes at 4 C. The supernatant fluid containing

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[a]Raytheon ultrasonic vibrator, type R-22-3, 9 kilocycle, 140 volts, Boston, Mass.

the soluble antigen was analyzed for protein content by a modified Folin phenol method (Daughaday et al., 1952) for dilute protein solutions. The protein content was 4.2 mg./ml.

Agar diffusion. Two 0.7% Agarose solutions were made. Phosphate buffered saline, ionic strength 0.7, pH 7.2 was used for one and veronal buffer, ionic strength 0.0375, pH 8.5 for the other. Serum samples from gnotobiotic pigs exposed only to E. coli 0 138:K 81 NM were diffused against the E. coli supernatant fluid containing the soluble antigen. Diffusion was repeated using a 1:10 dilution of the E. coli supernatant fluid. Incubation during diffusion was for 24 hours at 37 C. in a moist chamber.

Agglutination. The culture of E. coli 0 138:K 81 NM was inoculated into 10 ml. of tryptose broth and incubated at 37 C. for 24 hours. The bacteria were then centrifuged at 2,500 rpm for 30 minutes and washed once with 0.85% NaCl solution. A drop of a heavy suspension of the bacteria was placed on the center of a glass slide. A drop of serum from gnotobiotic pigs exposed only to E. coli 0 138:K 81 NM was added and mixed with an applicator stick. The slide was tilted back and forth for 2 minutes at room temperature (23 C.) while the observation was made for clumping of the bacteria.

Total protein. Serum total protein was determined using a refractometer.[a]

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[a] AO 10401 TS meter, American Optical Company, Buffalo, New York.

Solutions. Formulae for buffers and stains may be found in the appendix.

RESULTS

Electrophoresis

Observations of the electrophoretic patterns of the serum protein of newborn pigs indicated that a major portion is alpha globulin with smaller amounts of albumin and beta globulin. Gamma globulin could not be detected. Similar results were reported by Rutqvist (1958), McCance and Widdowson (1959), Lecce and Matrone (1960) and Lecce, Morgan, and Matrone (1962).

A band of protein (Figure 3) which migrated between albumin and alpha globulin was noted in the serums of many newborn pigs, but after 3 to 5 days it could no longer be detected. This may have been the same phenomenon observed by Lecce, Morgan and Matrone (1962).

Similar changes occurred in the control pigs maintained germfree and in those given an inoculum of E. coli (Figures 4 and 5).

A proteinaceous residue at the site of application of serum to the agar surface stained with brom phenol blue and interfered with interpretations. It was shown to be small amounts of debris consisting mainly of erythrocytic membranes.

Immunoelectrophoresis

The most obvious change in immunoelectrophoretic patterns of young germfree and gnotobiotic pigs was in the alpha globulin fraction. As early as 1.5 hours after inoculation with E. coli the alpha globulins reacted with anti-pig serum antiserum to form more well defined precipitin

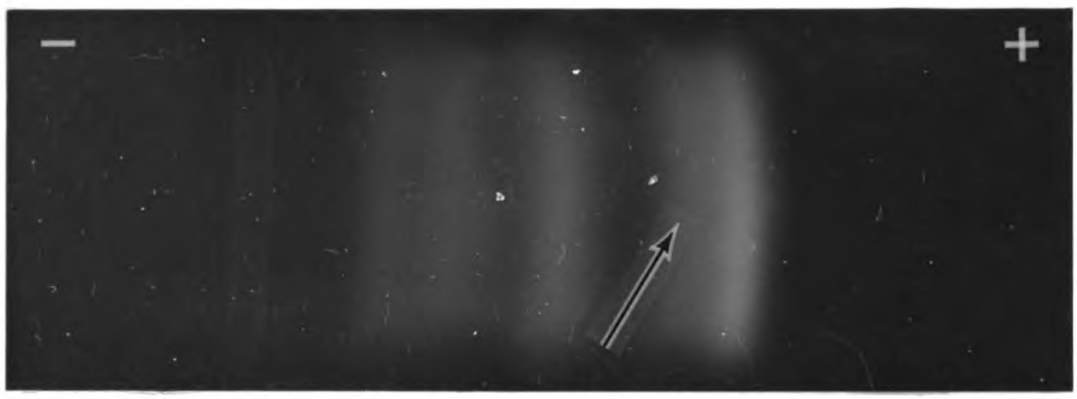
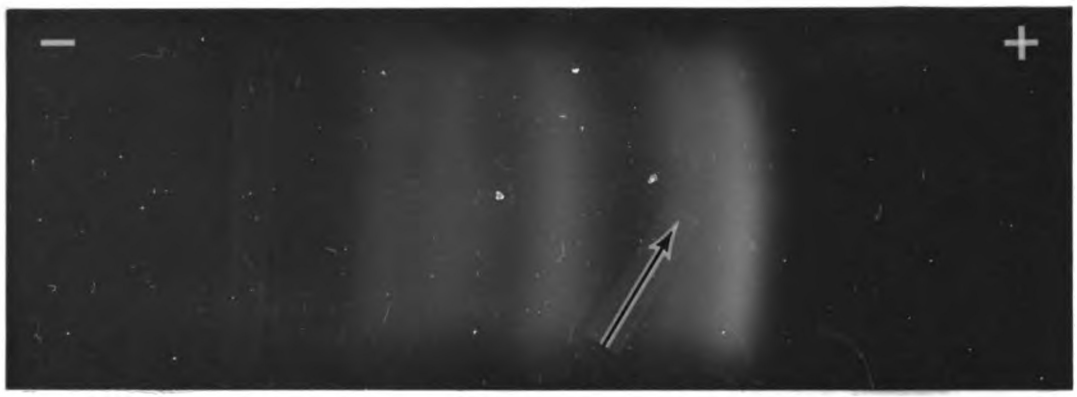


Figure 3. Electrophoretic separation of serum from a 24-hour-old, germfree pig. Arrow indicates protein which migrated between alpha globulin and albumin.



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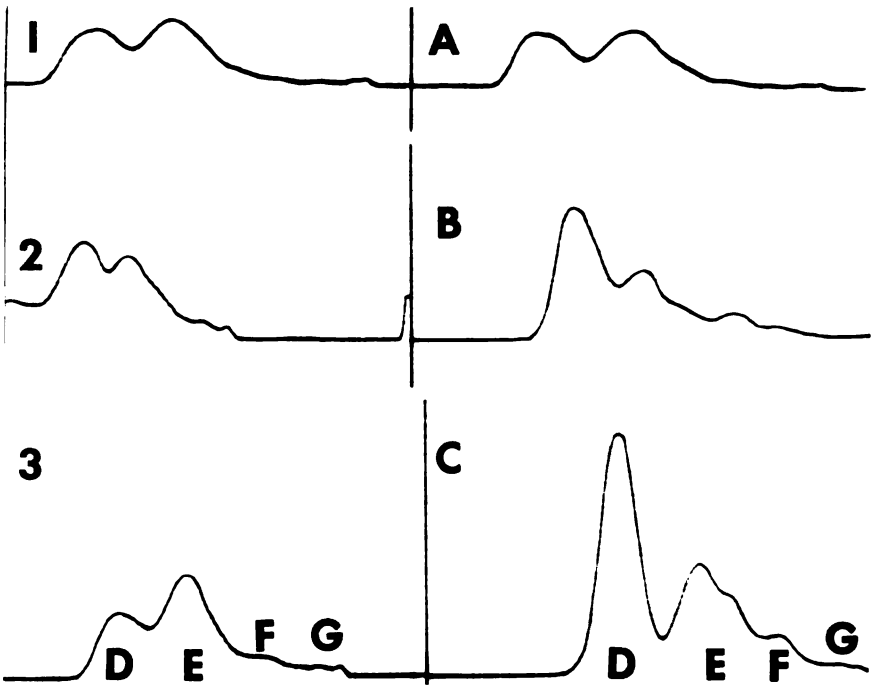

Figure 4. Electrophoretic patterns of 3 gnotobiotic pigs' serums. Left, all germfree at 24 hours of age. Right, (A) Pig 1, 20 hours, (B) Pig 2, 72 hours and (C) Pig 3, 121 hours after subcutaneous inoculation with viable culture of E. coli. Protein fractions are (D) albumin, (E) alpha globulin and (F) beta globulin. (G) denotes residue at point of application of serum to agar.

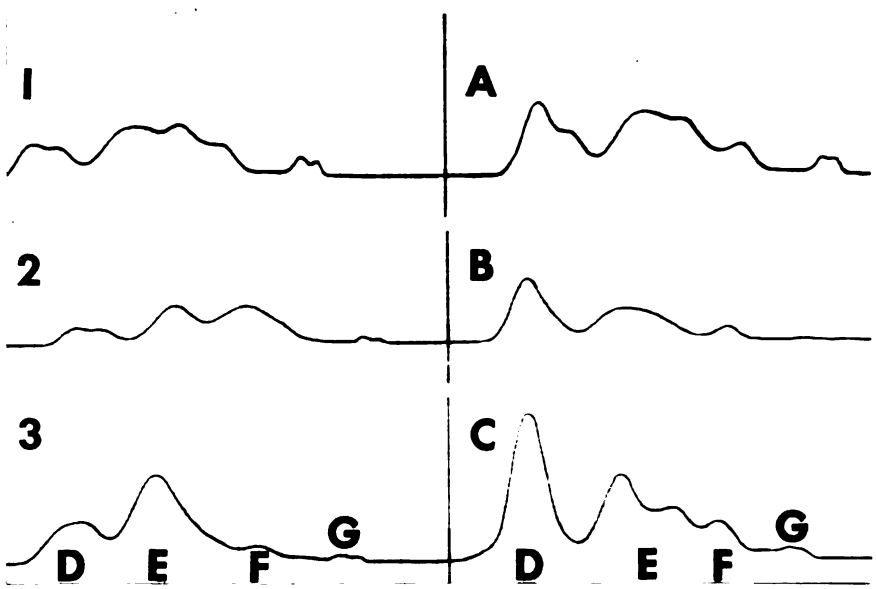

Figure 5. Electrophoretic patterns of 3 germfree pigs' serums. Left, all at 24 hours of age. Right, (A) Pig 1, 48 hours, (B) Pig 2, 170 hours and (C) Pig 3, 192 hours of age. Protein fractions are (D) albumin, (E) alpha globulin and (F) beta globulin. (G) denotes residue at point of application of serum to agar.

arcs (Figures 6 and 7). Other protein fractions had analogous changes. The albumin arc became broader, usually extending to the antibody slot (Figure 7). Similar changes were noted in germfree pigs, but they occurred more slowly and were not as pronounced as in the pigs inoculated with E. coli. Some sera possessed a protein fraction which formed a precipitin arc in the gamma globulin range on the cathodic side of the antigen well (Figures 6, 8 and 9). Often this arc appeared to increase in intensity with serum from pigs several hours old. One alpha globulin precipitin arc (Figure 6) increased, then decreased in clarity as the pigs grew from 1 to 8 days of age. Identification of major groups of serum proteins in a young germfree pig and a conventional adult pig was made (Figure 8).

Total serum protein

The total protein values of the serums changed only slightly during the course of the experiment (TABLES 1, 2, 3 and 4). However, there was no consistent pattern of change and serums of the control animals had changes similar to those in the inoculated pigs. These values are normal for newborn pigs.

Antibody production

Pigs 8 days old and younger which had been exposed only to E. coli organisms at 24 hours of age contained no detectable serum antibodies against E. coli antigens when microimmunodiffusion and agglutination techniques were used.

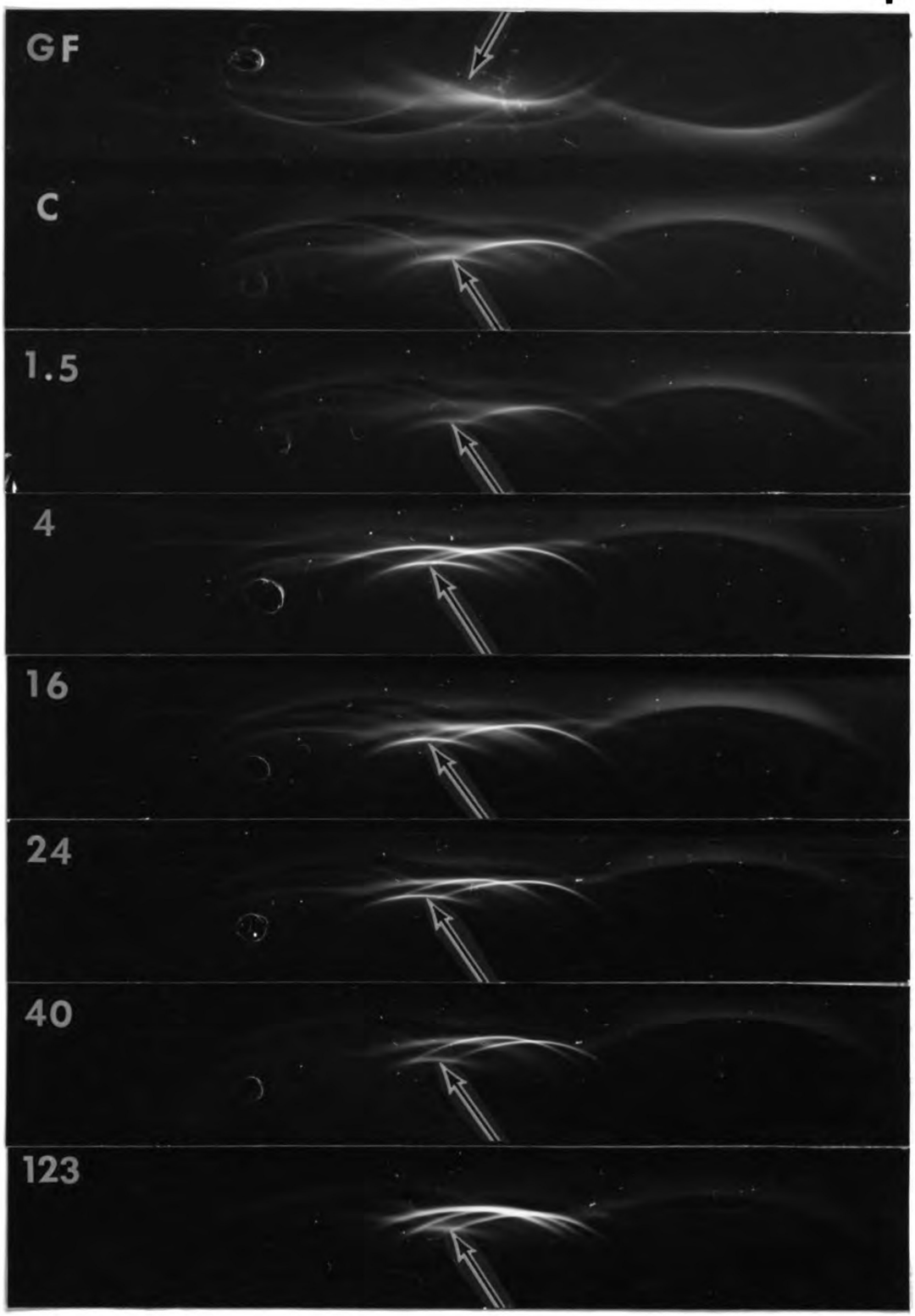

Figure 6. Serum immunoelectrophoretic patterns of 7 pigs from litter 4. (GF) initial serum at 48 hours of germfree life. (C) germfree control 24 hours later. Numbers indicate hours after oral inoculation of 48-hour-old germfree pigs with E. coli. Arrows indicate alpha globulin arc which increases, then decreases in clarity. Most alpha globulin precipitin arcs increase in clarity as pigs' age increases. Also note precipitin arc in the gamma globulin range on the cathodic side of the antigen well.

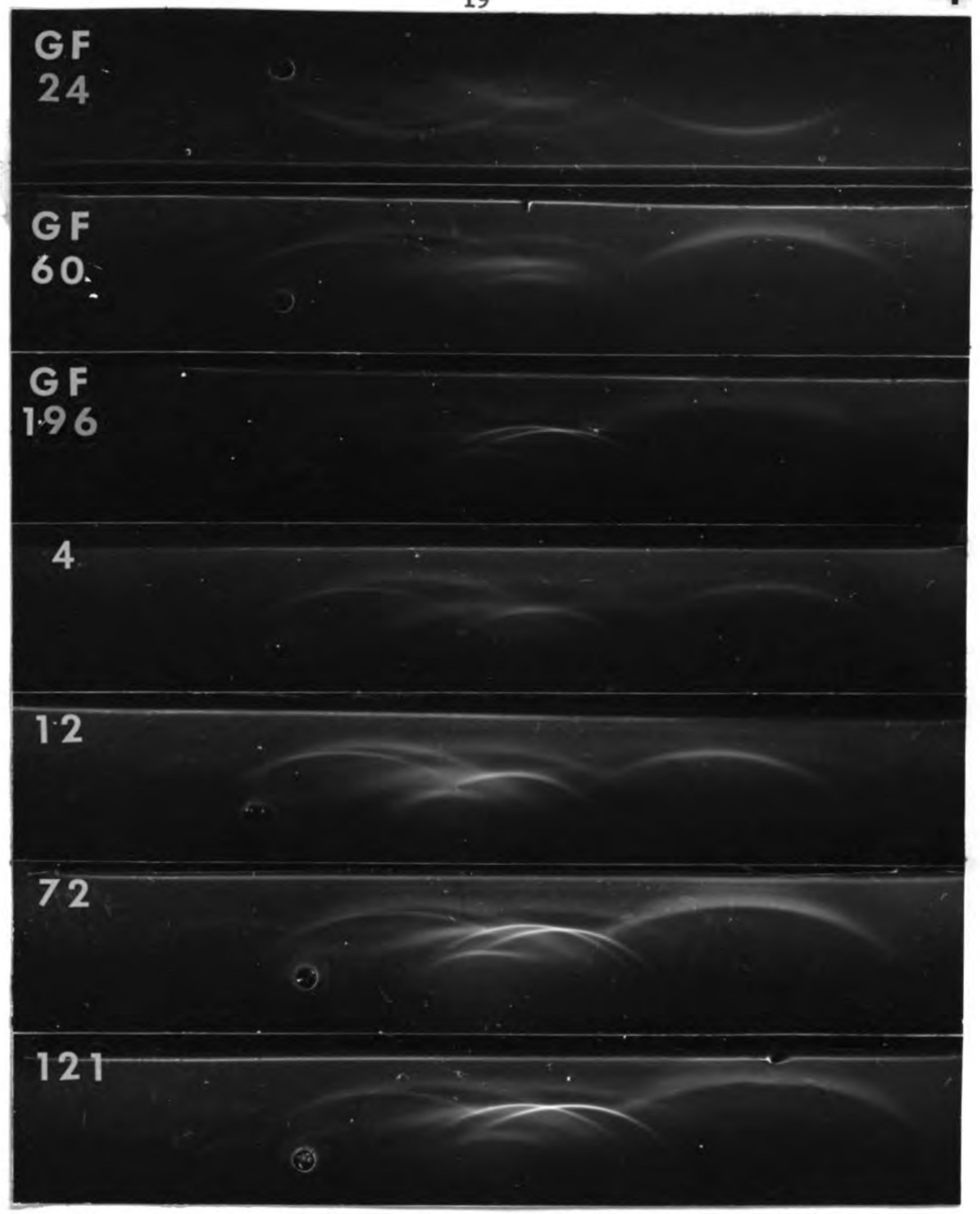

Figure 7. Serum immunoelectrophoretic patterns of 6 pigs from litter 1. Top 3 pictures show serum from germfree pigs. Numbers indicate age in hours. Bottom 4 pictures show serum from pigs inoculated at 24 hours of age. Numbers indicate hours after subcutaneous injection of E. coli. Therefore, the actual age of the bottom 4 animals is 24 hours plus the numbers given.

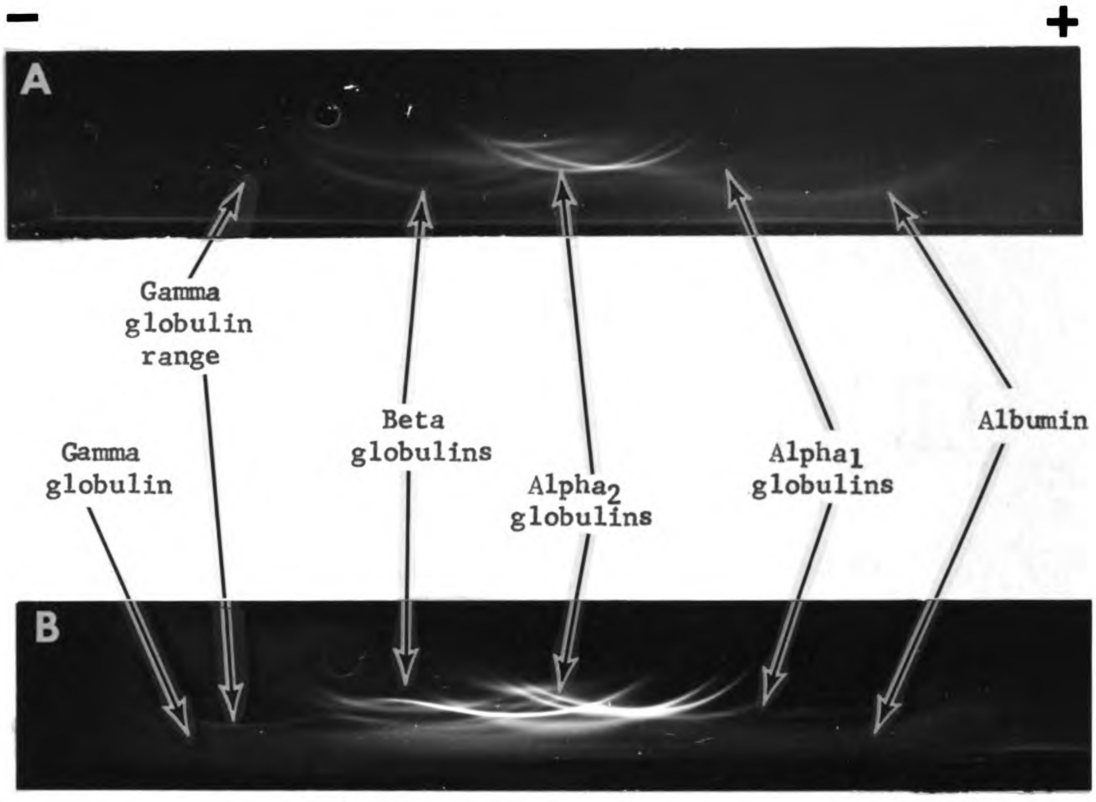

Figure 8. Immunoelectrophoretic identification of major groups of serum proteins in the serum of the (A) 24-hour-old germfree pig and (B) adult, conventionally reared pig.

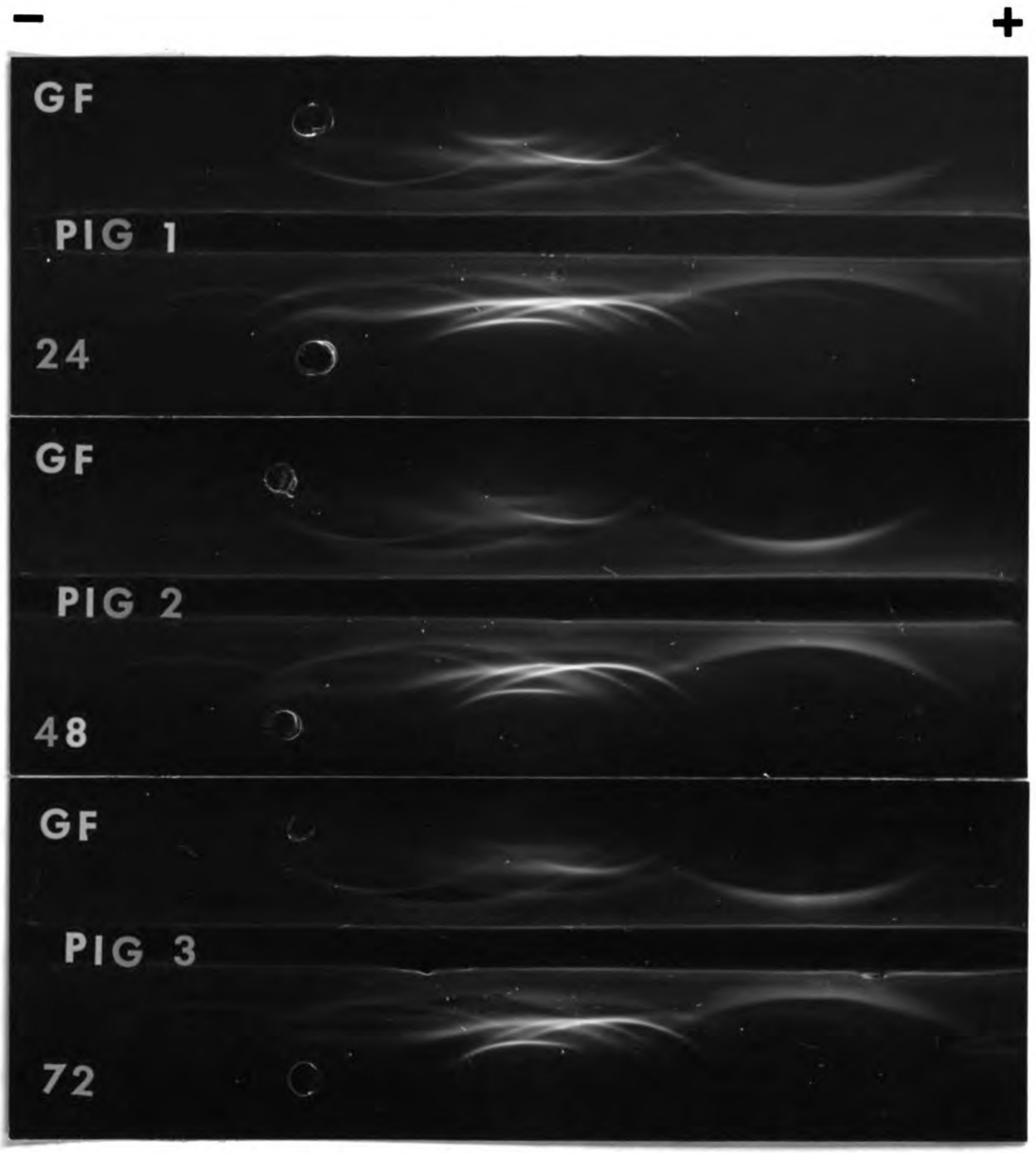

Figure 9. Serum immunoelectrophoretic patterns of 3 pigs from litter 3. (GF) denotes initial samples prior to inoculation at 24 hours of age. Numbers indicate hours after subcutaneous inoculation with E. coli.

TABLE 1. Sampling intervals and total serum protein values for litter 1[a]

Pig No.	Terminal bleeding (hours)[c]	Initial total protein[b] (Gm./100 ml.)	Terminal total protein (Gm./100 ml.)
J 5726	4	3.0	3.7
J 5712	8	---	2.8
J 5713	12	2.3	2.6
J 5714	17	---	2.3
J 5715	20	2.6	2.6
J 5716	25	---	2.4
J 5717	32	2.4	2.3
J 5718	40	2.4	2.8
J 5719	48	2.4	2.5
J 5720	72	2.6	3.0
J 5721	121	2.6	2.7
J 5722	168	---	3.3
J 5723[d]	36	2.4	3.0
J 5724[d]	146	2.6	2.9
J 5725[d]	168	2.3	3.2

[a]Inoculated subcutaneously with 1.35×10^6 organisms per pig.
[b]Twenty-four hours after birth.
[c]Hours from time of inoculation.
[d]Control, not inoculated.

TABLE 2. Sampling intervals and total serum protein values for litter 2[a]

Pig No.	Terminal bleeding (hours)[b]	Terminal total protein (Gm./100 ml.)
J 6208	1.5	2.7
J 6209	2.5	2.4
J 6200	4.0	2.1
J 6201	6.5	2.5
J 6202	8.0	2.2
J 6203	12.0	2.5
J 6204	16.0	2.7
J 6205	24.0	2.6
J 6206	48.0	3.1
J 6207	72.0	4.4
J 6210[c]	36.0	2.9
J 6211[c]	72.0	2.8

[a]Inoculated subcutaneously with 6.3×10^7 organisms per pig.
[b]Hours from time of inoculation.
[c]Control, not inoculated.

TABLE 3. Sampling intervals and total serum protein values for litter 3[a]

Pig No.	Terminal bleeding (hours)[c]	Initial total protein[b] (Gm./100 ml.)	Terminal total protein (Gm./100 ml.)
J 6785	1.5	2.8	2.6
J 6784	2.5	2.6	2.6
J 6787	4.0	2.9	2.9
J 6786	7.5	2.9	2.8
J 6782	9.0	2.6	2.5
J 6788	12.0	3.5	2.6
J 6783	24.0	2.9	3.2
J 6789	32.0	2.7	2.7
J 6790	48.0	2.7	2.6
J 6791	72.0	3.0	3.2
J 6792[d]	3.0	3.3	2.8
J 6793[d]	24.0	3.0	2.9

[a]Inoculated subcutaneously with 8×10^5 organisms per pig.
[b]Twenty-four hours after birth.
[c]Hours from time of inoculation.
[d]Control, not inoculated.

TABLE 4. Sampling intervals and total serum protein values for litter 4[a]

Pig No.	Terminal bleeding (hours)[c]	Initial total protein[b] (Gm./100 ml.)	Terminal total protein (Gm./100 ml.)
K 52	1.5	3.6	3.3
K 53	1.5	3.2	2.5
K 57	4.0	3.5	3.5
K 58	4.0	3.7	2.8
K 62	8.0	3.2	3.2
K 54	16.0	3.4	3.2
K 55	22.0	3.2	2.8
K 56	24.0	3.4	3.1
K 59	32.0	3.8	3.4
K 60	40.0	3.6	2.7
K 61	123.0	3.6	3.8
K 63[d]	8.0	3.8	3.0
K 64[d]	24.0	3.3	2.8

[a]Inoculated orally with 4.8×10^6 organisms per pig.
[b]Forty-eight hours after birth.
[c]Hours from time of inoculation.
[d]Control, not inoculated.

Effect of hemolysis and absorbed nutrients

Electrophoresis. Hemoglobin migrated between the alpha and beta globulins (Figure 10). Absorbed nutrients did not have any apparent effect on serum electrophoresis, although the serum was grossly chylous as evidenced by the cloudy appearance (Figure 11).

Immunoelectrophoresis. Hemoglobin distorted the precipitin arcs, especially in the alpha and beta globulin fractions (Figures 12 and 13). The presence of absorbed nutrients had no apparent effect on immunoelectrophoretic patterns.

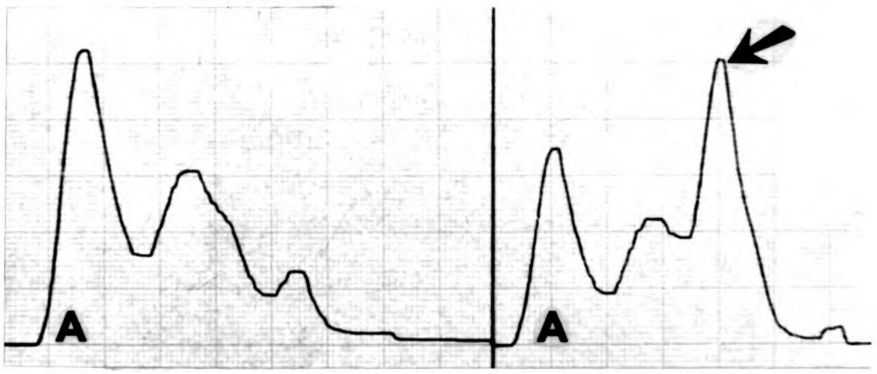

Figure 10. Electrophoresis of germfree pig serum. Left, normal, unhemolyzed serum. Right, serum with 1 Gm. of hemoglobin per 100 ml. Arrow indicates peak due to hemoglobin and (A) indicates albumin peak.

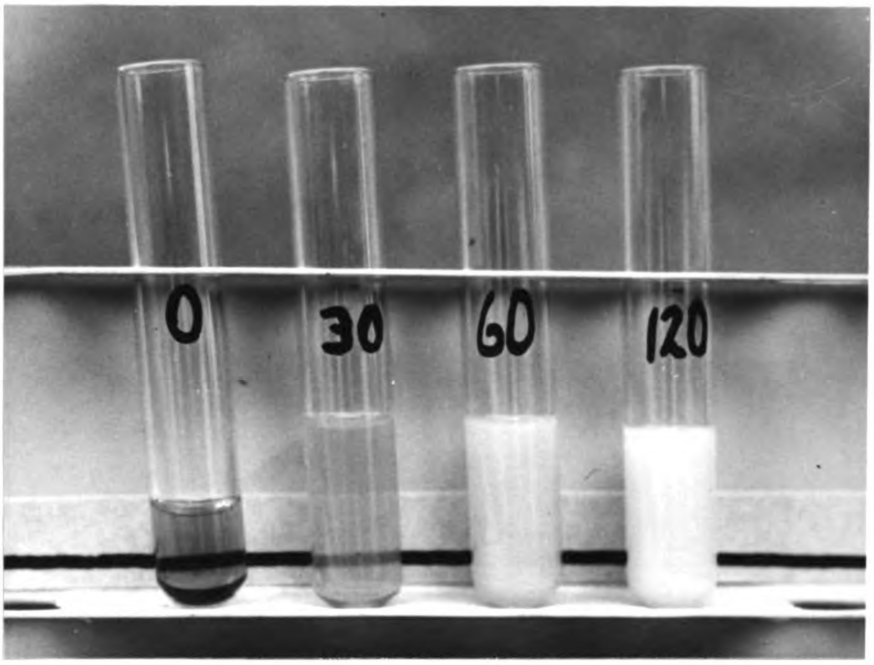

Figure 11. Serum from germfree pig showing increased opacity resulting from absorption of food from the intestine. Numbers indicate minutes after ingestion of milk.

Figure 12. Effects of hemolysis and feeding on immunoelectrophoresis of germfree pig serum. (A) unfed newborn, (B) same serum as A but containing hemoglobin, (C) 72-hour-old pig 30 minutes after eating, (D) same serum as C but containing hemoglobin. Note distortion in samples B and D, which contained 1 Gm. of hemoglobin per 100 ml.

Figure 13. Effects of hemolysis and feeding on immunoelectrophoresis of germfree pig serum. (A) 72-hour-old pig 60 minutes after eating, (B) same serum as A but containing hemoglobin, (C) 72-hour-old pig 120 minutes after eating, (D) same serum as C but containing hemoglobin. Note distortion in samples B and D, which contained 1.8 Gm. of hemoglobin per 100 ml.

DISCUSSION

The serum proteins of young pigs maintained under gnotobiotic conditions underwent changes which were, in part, influenced by environment. Changes appeared to be a normal "maturation" or development of serum proteins to types and levels found in adult pigs' serum. Exposure to a pathogenic strain of E. coli (serotype O 138:K 81 NM) resulted in an acceleration of the changes in the serum proteins during the first hours of life as compared to the changes in those pigs maintained under germfree conditions. It should be noted that the changes which occurred in the serums of inoculated pigs eventually occurred in the serums of pigs which were maintained under germfree conditions.

Immunoelectrophoresis revealed a protein fraction which migrated toward the cathode. This could have been either a beta globulin or a gamma globulin. Specific anti-globulin antiserum was not available to make the differentiation. If it were gamma globulin it did not appear to be serologically active against E. coli antigens to which the pigs were exposed, and it is not the same gamma globulin most readily absorbed from colostrum by suckling pigs. Gamma globulin acquired from colostrum forms a broader precipitin arc which is located closer to the antibody slot. It may be possible that it is physiologically active as an opsonin or some other nonspecific, immunologically active globulin. This speculation is raised since most of the infected pigs were able to cope with the organism and confine its growth to the lumen of the intestine (Christie, 1966).

Aiken and Blore (1964) presented information which led them to conclude that persistence of an antigen would stimulate production of antibodies in young pigs. Since the serotype of E. coli used in the present work was found by Christie to persist within the pigs throughout the experiment, it seems likely that some other factors, such as age of the animal, type of antigen and route of inoculation, may be involved.

The antigenic and pathogenic properties of E. coli 0 138:K 81 have been described in detail by Johnston (1964). An immunologic response in the inoculated pigs may not have been detected because the experiments were terminated no later than 7 days after inoculation. It is possible that serum taken 3 to 4 weeks after inoculation could have contained detectable amounts of antibodies against E. coli. It can only be said that 7 days after inoculation with E. coli, antibodies were not detected by the precipitation or agglutination techniques described.

Since most of the pigs eliminated the bacteria from all organs except the lumen of the intestine, it would appear that some mechanism was present to aid in the removal. It seems unlikely that the bacteria could not survive because of inadequate nutrients or growing conditions in organ tissues other than the intestinal lumen, in light of its pathogenic character. If a pre-existing antibody of nonspecific nature were involved in the elimination of the bacteria, some qualifications must be made to the natural selection theory of antibody production described by Jerne (1955, 1960) to make it acceptable. The ability of an animal to produce specific immune antibodies must be based on the physiologic and biochemical maturity of its antibody producing cells as well as on pre-existing antibody. It is also possible that an antibacterial substance

such as a lysozyme, which is not an antibody, was present within the pigs and accounted for the elimination of the bacteria.

Jerne's theory of pre-existing antibody's being required for the production of immune antibodies could be better evaluated by using older germfree pigs and/or simultaneous injection of dilute, specific antiserum with the antigen as described by Segre and Kaeberle (1962). The information from the present research provides no basis to evaluate Jerne's theory.

Although Brummerstedt-Hansen and Hirschfeld (1961) reported that immunoelectrophoretic patterns were not influenced by the addition of hemoglobin to pig sera prior to immunoelectrophoresis they did not mention the amounts of hemoglobin which they used. Our results show that when hemoglobin was added to pig serum in sufficient quantity it distorted immunoelectrophoretic patterns (Figures 12 and 13). Smaller amounts of hemoglobin had no noticeable effect. Laurent (1964) noted the occurrence of nonspecific binding of hemoglobin by human globulins which was detected by immunoelectrophoresis on cellulose acetate and the benzidine staining reaction. This phenomenon was not detected in these experiments with immunoelectrophoresis of germfree pigs' serum in agar gel. The stains used in the attempt to detect hemoglobin were Lepehne-Pickworth's benzidine and Okajama's alizarin as described by McManus and Mowry (1960). Hemoglobin binding by 2 $alpha_2$ globulins and 1 beta globulin of pig serum was detected by Brummerstedt-Hansen and Hirschfeld (1961) using immunoelectrophoresis in agar followed by a benzidine stain.

Difficulties in obtaining blood from germfree pigs predisposes to slight hemolysis. Although this presented no problem in the immunoelectro-

phoretic analysis of serum proteins the fractions separated by electrophoresis on agar gel could not be quantitated accurately, since the hemoglobin migrated with the alpha and beta globulins. In addition, small amounts of cellular debris in the serum remained at the site of application to the agar and interfered with quantitation of the electrophoresis slides.

The presence of absorbed nutrients in the serum (Figure 11) had no apparent effect on either the electrophoresis or immunoelectrophoresis. This would not be true, of course, if the pigs had received colostrum from the sow, since colostral proteins are absorbed, unaltered, by newborn pigs. Furthermore, any change in diet is likely to change the development of serum proteins, according to Lecce et al. (1962).

Despite technical problems the germfree pig appears to be an excellent animal with which to test various theories of immunity and to study the variables which control or influence the development of serum proteins.

SUMMARY

Serum proteins of germfree and gnotobiotic pigs changed during the first 8 days of life. Electrophoretic separation revealed a decrease in relative amounts of alpha globulin and an increase in albumin and beta globulin. These changes occurred in both germfree and Escherichia coli infected pigs. Immunoelectrophoretic analysis revealed that the serum proteins of neonatal germfree pigs gradually increase in their ability to form sharply delineated precipitin arcs with rabbit anti-pig serum antiserum. This ability was detected at a younger age and to a greater degree in animals exposed to E. coli by oral or subcutaneous inoculation. An immunoprecipitin arc in the gamma globulin range was detected in the serum from germfree and gnotobiotic pigs.

Antibodies to E. coli could not be detected by agglutination or immunodiffusion techniques in 8-day-old pigs which were inoculated at 1 day of age.

The effects of hemoglobin and nutrients absorbed from a milk diet on electrophoresis and immunoelectrophoresis of neonatal germfree pig serum were determined. Hemoglobin in concentration of 1 Gm./100 ml. resulted in a distortion of immunoelectrophoretic precipitin arcs and migrated with alpha and beta globulins during electrophoresis. Absorbed nutrients had no detectable effects on either electrophoresis or immunoelectrophoresis of the serum.

APPENDIX

Phosphate buffered saline, ionic strength 0.7, pH 7.2

Na_2HPO_4	28.0 Gm.
KH_2PO_4	6.5 Gm.
NaCl	51.0 Gm.
H_2O	qs 6000 ml.

Veronal buffer, ionic strength 0.0375, pH 8.5

Dilute 1 part buffer B-2[a] with 1 part distilled water.

Trichrome stain (Crowle, 1961)

Thiazine red R	0.1 Gm.
Amidoswarz 10B	0.1 Gm.
Fast green	0.1 Gm.
Mercuric chloride	0.1 Gm.
Glacial acetic acid	2.0 Gm.
H_2O	100 ml.

Stain as required (15 minutes for micro-immunodiffusion).

Differentiate with 2% acetic acid.

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[a]Beckman Instruments, Inc., Fullerton, California.

Brom phenol blue solution

Brom phenol blue	1.0 Gm.
Methyl alcohol, absolute	1000 ml.

Stain agar coated slides for 20 minutes. Remove excess dye with 2 or 3 rinses in 5% acetic acid, approximately 5 minutes per rinse.

Modified Folin phenol reagents

Copper sulphate solution

$CuSO_4 \cdot 5H_2O$	1.5 Gm.
$KNaC_4H_4O_6 \cdot 4H_2O$	6.0 Gm.
KI	1.0 Gm.

Dissolve in 500 ml. H_2O and add 300 ml. 10% NaOH. Dilute to 1000 ml. with H_2O.

Phenol solution

Harleco phenol reagent #2690[a]

Dilute 1:10 for working solution.

[a]Hartman Leddon Co., Philadelphia, Pa.

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VITA

The author was born in Bay City, Michigan, on December 1, 1940. He lived there and graduated from Thomas Lincoln Handy High School in 1958. After attending Bay City Junior College for 1 year he enrolled at Michigan State University. He received his B.S. degree in Medical Technology from Michigan State University in 1963, after completing a 12-month internship at St. Mary's Hospital, School of Medical Technology, Grand Rapids, Michigan. Later that same year he became certified with the Registry of Medical Technologists of the American Society of Clinical Pathologists.

In July 1963 the author accepted the position of Senior Pathologist and enrolled in a program of graduate study in the Department of Pathology at Michigan State University. There he worked in experimental pathology until his appointment as Instructor in the same department in July 1966.

He is a member of the Lansing Area, the Michigan, and the American Societies of Medical Technologists.