A FILTERABLE VIRUS CAUSING ENTERITIS AND PNEUMONIA IN CALVES

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PLATE 15

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A disease of calves recognized by fever, diarrhea, and pneumonia has been observed among young animals in the dairy herds of the eastern United States, where calves less than one month of age are particularly susceptible and are severely affected. Death rarely follows if the animals are kept under good conditions, but they do not develop normally and are unprofitable to keep. The disease is seen throughout the year although it appears most frequently during the late fall, winter, and early spring. In certain years epidemics occur in which the mortality as well as the incidence is higher than in the inter-epidemic periods. There is a suggestion that these epidemics occur every 15 to 20 years, and almost certainly the years 1939 and 1940 were peak periods in the eastern United States. Reports from veterinarians indicate that this was true for the entire country, and that since that time there has been a gradual decrease in the amount of disease although sporadic outbreaks are still fairly numerous.

The literature shows that a similar condition affecting the respiratory and digestive tracts of calves is found in other countries. It has been called pneumonia, enteritis, or scours, probably depending on the signs that are prominent in the early cases. Sanders (1), in Florida, and Roberts (2) of Brazil, employ the term "pneumoenteritis" to include the total effects of the disease in one word. Udall (3) has reviewed the literature up to 1939, pointing out that it is sometimes difficult to decide whether the pulmonary infection is primary or secondary.

A study of the literature shows that bacteria, viruses, and vitamin deficiencies, singly or in combination, have been considered as the cause of enteritis and pneumonia in calves. The rôle of bacteria is discussed by Udall (3) who states that disease has never been produced by their inoculation, and that since cultures of lungs from some animals were negative, the bacteria found may be considered as secondary invaders. Thorp and his coworkers (4) isolated an influenza-like organism from pneumonic lungs and reported fairly successful results from its inoculation.

A virus cause was suspected by Nagel (5), working in Germany, because of his failure to demonstrate bacteria as the etiological agent and because cultures from lung lesions showed no growth. His attempts to demonstrate a virus were inconclusive. Only 1 of 4 calves which he inoculated with a Seitz filtrate of infective tissue developed any evidence of disease, and the autopsy of this animal 18 days after inoculation revealed

only pea-sized fibrous foci in the lungs. Lamont (6), in Ireland, observed an infectious disease of calves that he called influenza and considered the cause to be a combination of agents similar to that demonstrated by Shope (7) for swine influenza. He produced disease in calves with mixtures of a Berkefeld N filtrate of suspensions of infected lungs and a *Hemophilus*-like organism that had been isolated from the lungs of calves showing disease. He was unable to reisolate the bacterium or to establish its relation to the disease.

Vitamin A deficiency and lack of certain members of the B complex have been suggested by Phillips and his colleagues (8) as the cause of the diarrhea that is followed by pneumonia in calves. Their conclusions are based upon the reduction of the severity of the infection when these substances were administered in therapeutic doses.

In this laboratory, preliminary experiments showed that the typical disease could be transmitted to normal animals by contact. An investigation was therefore undertaken to procure the etiological agent and determine its nature.

Passage of the Etiological Agent to Mice

Since the intranasal route of inoculation in mice has been so helpful in the study of influenza and other virus diseases, it was used in attempting to demonstrate the agent causing enteritis and pneumonia in calves.

The animals used throughout the work came from a colony descended from 4 albino mice obtained by the Department in 1916. No mice from outside sources have been added to it.

The colony has been carefully checked for the presence of any latent viruses that produce pneumonia. Horsfall's (9) demonstration of such a virus in mice by passing lungs at 7 to 10 day intervals made imperative similar tests. During the course of the work reported here, 3 separate serial inoculations of lung extracts from apparently normal mice of the colony were made; 2 were carried through 6, and 1 through 12 passages, and in no instance did pneumonia appear.

Groups of 3 or 6 mice, 5 to 7 weeks of age, were lightly etherized and inoculated intranasally with 0.05 cc. of bronchial washings or suspensions of lungs from naturally infected calves. The suspensions were prepared by grinding affected lung tissue in 25 per cent concentrations (based on wet weight) with sterile sand in buffered saline. The mice were autopsied at the end of 7 to 10 days and suspensions of their lungs were inoculated into other mice in a manner similar to the original passage. The lung suspensions from the mice were ground in a glass grinder with 0.5 cc. buffered saline added for each lung, to make approximately 25 per cent suspensions, but after transmissible disease was established the density of the suspensions was reduced to 10 per cent. This procedure, utilized in every attempt to isolate a virus, was continued for 5 passages and only if no visible lesions developed under such circumstances was it considered negative.

Agents which proved similar in cross-immunity tests were isolated from sick calves in two separate herds but the attempt to obtain them from the diseased animals of a

third herd failed. An agent which produced pneumonia in mice was also isolated from a sick calf at the New York State Veterinary College in Ithaca, where the work began, but it was lost before it could be thoroughly studied.

The establishment of a pathogenic agent in mice inoculated with the calf material became evident after 3 passages, when definite lesions in the form of small consolidations were observed in the lungs. Subsequent passages increased the extent of the pneumonia, and death occurred in the 8th passage. Continued passage increased the pathogenicity of the agent, as can be seen in Table I.

The agent was maintained easily by passage in mice, but in addition attempts were made to keep it by freezing and drying, storage at −4°C., and the use of 50

TABLE I

Increased Pathogenicity of the Virus for Mice As Result of Intranasal Inoculation in Serial Passage

Passage No.	Results				
	Dilution of virus				
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
2	0/6*				
12	30/6	12/6	0/6	0/6	
22	30/6	18/6	9/6	3/6	0/6
42	30/6	30/6	28/6	10/6	0/6

* Horsfall's infectivity score is used in the tabulation of the findings. The denominator represents the number of animals used; the numerator indicates the extent of disease (5, death; 4, consolidation of all lobes; 3, 3/4 of lung affected; etc.) multiplied by the denominator.

per cent glycerin. Frozen and dried specimens were active after a storage period of 4 months although passage was necessary to restore their pathogenicity completely. The reduction in it was probably due to the effects of the drying rather than the storage period, since it was found that suspensions carried through the drying procedure diminished in activity from a point at which 10^{-3} dilutions inoculated intranasally into mice caused death to one where 10^{-1} dilutions produced only small consolidations. Pathogenicity persisted in the frozen state at −4°C., although greatly diminished, for longer than 1 but less than 4 weeks. Storage in 50 per cent glycerin for a week resulted in complete loss of activity.

Tests were next made to learn whether the agent was filterable.

Lung suspensions from infected mice in which the agent had been serially transferred for 14, 16, and 35 passages were prepared as 10 per cent suspensions in broth. After centrifugation at 1500 R.P.M. for 30 minutes, the supernatants were removed and passed through Berkefeld N filters. The filters had previously been tested for defects

by observing passage of water (30 to 40 cc. in 5 minutes at 10 mm. Hg pressure passes through a sound filter). Immediately before filtration the filters were layered with 25 cc. of broth. Three separate tests were attempted with one new and two used filters, and in each experiment approximately 10 to 15 cc. of filtrate was obtained. Portions of each filtrate transferred to serum broth and cooked meat medium sealed with vaseline showed no growth after incubation. Mice inoculated intranasally with 0.05 cc. of the filtrates showed small consolidations in the lungs, but in the next passage they died in the usual manner. In all work that followed this strain of virus was used.

This experiment and the successful mouse passages were interpreted as together constituting proof of the filterability of the agent through a Berkefeld N filter.

Cultures of the lung suspensions from infected mice on blood agar plates showed either no growth or the presence of a few bacteria that could be classified as lung contaminants; and as just stated the agent producing the pneumonia proved capable of passing a Berkefeld N filter. It appeared therefore that a filterable virus was the cause of the pneumonia in mice; and since in similar passages the lungs of normal mice showed no pneumonia, it was considered probable that the virus came from the sick calves.

The Experimental Disease in Calves

Production.—The choice of the experimental calves for the work to be described required considerable care because of the widespread distribution of the disease. Some of the animals came from a herd maintained for 11 years at The Rockefeller Institute in Princeton, New Jersey, and no evidence of the disease was noted in any of them. Unfortunately it was necessary to secure additional calves from other sources. Newborn calves, preferably obtained before receiving colostrum, were removed from the dams and placed in isolation units. Pasteurized colostrum was fed once and thereafter milk prepared from dried whole milk powder was given. They were observed for periods of at least a week for any evidence of disease. A few of the calves obtained in this manner developed maladies of one sort or another and were discarded.

Contact exposure consisted of placing a normal calf in the same isolation unit with one showing signs of disease. Intranasal inoculation was performed by placing a Luer syringe at the external nares and injecting 20 cc. of a 5 or 10 per cent suspension of lungs from infected mice. It should be noted that the number of mouse passages varied from 3 to 40 with the material used. Calves were inoculated intratracheally with suspensions and amounts similar to those employed for intranasal inoculation. The injection was accomplished by placing the point of an 18 gauge needle in the lumen of the trachea. The results of the contact experiments and the inoculations are given in Table II. In all, 17 calves were used.

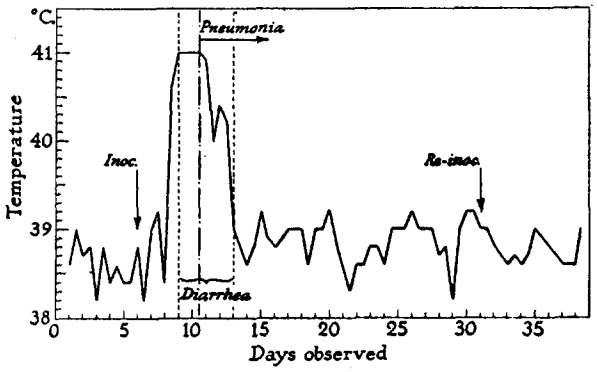
It can be seen in Table II that infection in calves regularly followed the intranasal inoculation of suspensions of lungs from infected mice, whereas intratracheal injection of such suspensions failed to infect 2 of 4 calves. In two ex-

periments, normal calves became sick which were in pen contact with calves that had fallen ill after inoculation with suspensions of lungs from infected mice. The disease was similar to field cases except that it was milder. Intranasal inoculations in which large doses of virus were administered produced a severe form of illness, and it was slightly milder in the calves infected by contact with experimental cases.

TABLE II

Production of Disease in Calves

Route of infection	No. of animals	No. of animals showing		
		Fever	Diarrhea and enteritis	Pneumonia
Contact with natural disease	3	3	3	2
Contact with experimental disease	2	2	1	1
Intranasal inoculation with suspensions of lungs from infected mice	8	8	5	7
Intratracheal inoculation with suspensions of lungs from infected mice	4	2	1	2



TEXT-FIG. 1. Reactions in a calf to intranasal inoculation of suspension of lungs from mice infected with the calf virus.

Features of the Illness.—The cardinal manifestations following contact exposure or intranasal inoculation were fever, diarrhea, and pneumonia in the sequence given. The onset of fever occurred 2 to 4 days after inoculation and the temperature rapidly increased to a peak of 40–41°C. The record in a typical experimental case is shown in Text-fig. 1. Diarrhea usually followed, the day after the fever began, the feces becoming soft, yellow, increased in amount, and possessed of a distinctive fetid odor. Blood-tinged mucus was found in fecal specimens from some of the calves, and in a few the evacuations reached a fluid stage. The diarrheal condition rarely persisted longer

than the febrile period, which usually lasted 3 to 5 days. Increase in the rate of the respirations, the first sign of pneumonia, appeared soon after the onset of fever and the respiratory rate not infrequently exceeded 100 per minute. Coughing was never spontaneous but later in the course of the disease it could be induced by pressure on the larynx or by occluding the air passages for a few seconds. This test does not usually induce coughing in animals with normal respiratory tracts.

During the active phase of the illness, the animals showed malaise and anorexia. They preferred the recumbent position and it was difficult to make them stand. Following the disappearance of fever, the animals began eating and soon appeared normal except for weakness and the respiratory symptoms of slightly increased respiratory rate and inducible cough.

Pathology.—At various times some of the sick calves were stunned by a blow on the head and bled to death by transection of the jugular vein. The trachea was closed with artery forceps in order to prevent inhalation of blood. The lesions found were consistent with the signs observed during the course of the disease.

The small intestine, when diarrhea existed, was diffusely reddened, especially in the lower ileum, and the mucosa was covered with a sticky mucus. The mesenteric lymph nodes were enlarged and contained much fluid.

Well defined pneumonic lesions were not present in animals killed during the early phase of the disease. Changes in the lungs at this time consisted of patchy, raised, and slightly reddened areas in the cardiac lobes. Animals killed at the end of the febrile period when signs of pneumonia were present usually showed consolidation of more than half of the lung tissue of the anterior lobes and a few scattered areas around the hilus in the diaphragmatic region. The pneumonic areas were of a purplish red color and a small amount of fluid exuded when they were sectioned. Lungs from one animal autopsied several weeks after recovery showed small fibrotic areas, while those from another presented no visible evidence of previous disease. Animals autopsied during the acute disease often showed a slight reddening of the nasal mucosa, small pin-point hemorrhages in the larynx, a viscid mucoid exudate in the lower trachea and bronchi, and slight enlargement of the bronchial lymph nodes.

Other organs presented slight changes. The liver was pale in color and often had a greenish yellow tint. There was a slight enlargement of the spleen, while no visible changes were observed in the kidneys.

Histopathology.—The ileal region of the small intestine presented a picture of catarrhal enteritis. Mixtures of desquamated epithelium and leucocytes were observed on the mucosal surface in a few calves. Desquamation of the epithelium was also noted in the crypts, and localized denuded areas were surrounded by leucocytic accumulations. Cellular infiltration into the tunica propria was observed, consisting of mixtures of polynuclear, large mononuclear, and plasma cells (Fig. 4). Capillaries in this layer were filled with blood. The submucosa was edematous and contained numbers of leucocytes. Similar changes were noted in the serosal layer.

Examination of the lungs of a calf killed at the onset of fever revealed an exudative bronchitis, the exudate consisting of more fluid than cells. The exudate from a lung in which pneumonia was well established was rich in cells that were mainly polynuclear. Little or no peribronchial reaction was observed in the early stage, but in the later one cellular accumulations were noted. These accumulations consisted of round and

polynuclear cells, many of which had infiltrated an apparently undamaged bronchial epithelium.

Sections taken from the center of pneumonic areas showed involvement of entire lobules. Vacuolated cells in the bronchial epithelium were noted. The alveolar architecture was indistinct, due to the presence of leucocytes, serous exudate, and red blood cells. Occasional areas of necrosis were found. A milder disease process was observed at the periphery of the pneumonic area. The epithelium of small bronchi was well preserved. Some of the alveoli adjacent to small bronchioles contained serous exudate and a few leucocytes, but most of them were collapsed with their walls

TABLE III

Immunity Tests

Calf No.	Initial infection produced by	Test for immunity to intranasal injection of infected mouse lung suspension	
		Days after infection	Results
1	Infected mouse lung suspension intranasally	23	No evidence of disease
2	Control		Fever, enteritis, pneumonia
3	Contact with experimental case that had received mouse passaged virus	16	No evidence of disease
4	Control		Fever, enteritis, pneumonia
5	Infected mouse lung suspension intranasally	50	No evidence of disease
6	Control		Fever, enteritis, pneumonia
7	Berkefeld N filtrate of mouse lung intranasally	14	No evidence of disease
8	Control		Fever, enteritis, pneumonia
9	Infected mouse lung suspension intranasally	17	No evidence of disease
10	Control		Fever, enteritis, pneumonia

thickened by congested capillaries and infiltrations of mononuclear cells. The surrounding alveoli were usually unchanged, but many immediately adjacent contained some exudate, a few leucocytes, and red cells (Figs. 1 and 2).

Other organs showed only slight changes, with the most constant lesions noted in the liver in which small focal accumulations of leucocytes and occasional necrotic liver cells were present (Fig. 3). Nothing was found in the spleen and mesenteric lymph nodes except a possible hyperplasia. The kidneys showed no consistent changes.

No inclusion bodies were found in examinations of any tissue.

Bacteriology.—No significant bacteria were obtained on blood agar plates from the organs of experimentally infected calves or those showing the natural disease. Cultures from some lungs showed a few cocci while others had no growth. One naturally infected animal which did not recover as ordinarily but died 3 weeks after the onset of disease was found to harbor *Pasteurella*. This organism may have been a secondary

invader, for it could not be recovered from another calf that had been in immediate contact with the sick one and was autopsied when pneumonia first became evident. This is not to say that the bacteria may not have had an important rôle in causing death.

TABLE IV

Effect of Intranasal Inoculation into Mice of a Mixture of Equal Parts of Undiluted Serum and a 1 Per Cent Suspension of Infected Mouse Lungs

Sera tested		Results after 14 days		
Source	Animal No.	Mouse No.		
		1	2	3
Non-immune calves	C-26	++++*	++++	++++
	C-35	+++++	+++++	++++
	C-37	++++	++++	++++
	C-38	++++	+++	+++
	C-39	++++	+++	+++
	C-44	++++	++++	++++
	S-206	+++++	++++	++++
Calves recovered from experimental infection with the mouse virus	C-26	+	+	0
	C-35	++	+	0
	C-37	++	+	+
	C-44	+	+	+
Calves recovered from natural infection	S-166	+++++	++++	++++
	S-182	+++	+++	0
	S-200	+++++	+++	0
	S-206	+	+	+
	D-1	+++	++	0
	D-2	+++++	+++++	+++++
	D-4	+	+	0
	D-5	+	+	+
	VC1	++	++	++
	VC2	++	+	+

* +++++ = death. ++++, +++, ++, + = extent of consolidation of lung in animals killed while sick. 0 = no pneumonia.

Cultures from organs other than the lungs showed no growth, except those from the kidneys in which coliform organisms were noted in an occasional calf, and some taken from the liver, these containing a few bacteria of the coliform type and other forms whose identity was undetermined. Specialized media, for example cooked meat sealed with vaseline, broth, and agar plates containing 10 to 30 per cent horse serum, failed to yield any anaerobic or pleuropneumonia-like organisms.

Immunity Phenomena.—Tests for acquired immunity were made with 5 calves which had been rendered ill by the intranasal inoculation of suspensions of lung from infected

mice or Berkefeld N filtrates of similar suspensions, or by contact with calves made ill by the mouse passaged virus. The illness ran the ordinary course of the enteritis with pneumonia, and from 14 to 50 days after infection the animals were inoculated intranasally with 20 cc. of 10 per cent lung suspensions from mice infected with the filterable agent. Since these immunity tests were made at different times, a fresh control animal was utilized for each recovered one. The results are given in Table III.

It can be seen that all of the recovered animals were immune to infection whereas the controls all developed the typical disease. One of the recovered calves when tested again in the same way 3 months later remained well.

TABLE V

Distribution of the Virus in Various Organs of Infected Calves As Determined by Mouse Inoculation Tests

Animal No.	Mode of infection	Stage of disease	Days from onset of fever	Organ tested						
				Lungs	Intestines	Serum	Liver	Spleen	Kidney	Mesenteric lymph nodes
1	Intratracheal inoculation	Onset	1	+	−	−	−	−	−	−
2	Intranasal inoculation	Onset	1	+	+	−	−	−	−	−
3	Contact with natural disease	Midcourse	3	+	+	+	+	+	+	+
4	Intranasal inoculation	Midcourse	4	+	+	+	+	+	+	+
5	Contact with natural disease	End	6	+						
6	Intranasal inoculation	Recovered	23	−						

Neutralization of the virus was tested for by inoculating mice intranasally with mixtures of equal parts of calf serum and a 1 per cent suspension of the lungs from infected mice. Each specimen was allowed to stand in the refrigerator for 1 to 2 hours before inoculation. The results are shown in Table IV.

It can be seen that in many instances the serum of recovered calves exerted a pronounced neutralizing effect but that complete neutralization was rarely obtained. The sera of 4 normal calves failed to neutralize the virus but after they had recovered from the experimentally produced disease their sera did so. The sera of 6 animals recovered from the natural disease neutralized the virus, but there was little or no neutralization with sera from 4 other recovered cases.

Distribution of the Virus in the Animal Body

The presence of virus in the various organs at different stages of the disease in naturally and experimentally infected calves was determined by inoculating

suspensions of the tissue intranasally into mice. All were made in the same way as the lung extract ordinarily used. The distribution of the virus in the animal body as thus ascertained is shown in Table V. When the inoculated material came from calves experimentally infected with the virus from mice, pneumonia often appeared in the first passage, showing that active virus was present, whereas serial passage in mice was necessary to demonstrate it in material from calves that showed the natural disease. Animals which had fallen ill as result of pen contact with calves that had been inoculated intranasally with suspensions of lungs from infected mice yielded pathogenic virus,—a fact which can be taken as added proof of the experimental transmission of the disease.

It can be seen from Table V that immediately following the onset of fever, the virus was demonstrable only in the lungs and intestines, but that later in the course of the disease it was generally present in the blood stream and all the organs that were tested. In the exceptional instance of calf 1 in which virus could not be demonstrated in the intestines, it may not yet have been distributed from the lungs after the intratracheal inoculation.

DISCUSSION

Enteritis with pneumonia is a prevalent disease in calves and, as shown above, it has been possible to procure from sick animals by passage through mice a virus that produces the characteristic disease when it is taken back to calves. The transmission has been successful with material from two herds, yet it does not follow that all cases of infection are due to the same virus. In this relation the fact may be important that there are outbreaks in calves in which the pneumonic signs predominate and others in which diarrhea is the outstanding characteristic. If both are due to the virus it seems probable that secondary invaders have sometimes greatly modified the picture.

It is desirable that more cases of this disease be examined and hence mention will be made of certain requirements that are essential in experimenting with it.

A stock of mice free from viruses capable of producing pneumonia on passage is essential to the work. We were fortunate in this respect. If we had been compelled to use any of the mice supplied by the dealers known to us the work might have been impossible, for most of the stocks we and others have tested carry virus that will produce pneumonia in mice when lung material is passaged. Whether these viruses are capable of producing pneumonia in calves has not been determined, but in any case the results obtained would be questionable.

The calves used for the experimental work also present a great problem. They should either come from a disease-free herd or be obtained at birth and fed as described in the text or in other well controlled ways. They must be kept under observation from birth to the time they are used and must have shown no temperature or signs of disease. In addition they must be strictly isolated for a week before they are used in an experiment.

SUMMARY

An infectious disease of calves has been described which is characterized by fever, diarrhea, and pneumonia, followed soon by recovery. On autopsy of animals killed at the height of the disease, there is found a catarrhal enteritis and a bronchopneumonia that is usually confined to the anterior lobes of the lungs. From this disease an agent has been secured by the serial inoculation of lung extracts that produces a pneumonia in white mice. Attempts to demonstrate by the same means a similar agent in uninoculated mice from the same stock have yielded negative results. Suspensions of the lungs of the mice with pneumonia, when inoculated intranasally or intratracheally into calves, cause a disease like the natural infection, characterized by fever, diarrhea, and pneumonia. In two experiments pen contact of normal calves with calves inoculated with the passed material resulted in the typical disease. Early in its course the causative agent is found only in the lungs and intestines, but at its height is generally distributed throughout the body. Calves that have recovered from the induced disease are resistant to subsequent infection and their blood serum will neutralize the causative agent as not previously. Sera from calves that have recovered from the natural disease also neutralize the agent. Cultures from the infected lungs of calves and mice as a rule show no growth, and material that has been passed through Berkefeld N filters produces the characteristic disease. It is therefore concluded that this disease of calves is caused by a filterable virus.

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EXPLANATION OF PLATE 15

All sections stained with hematoxylin and eosin.

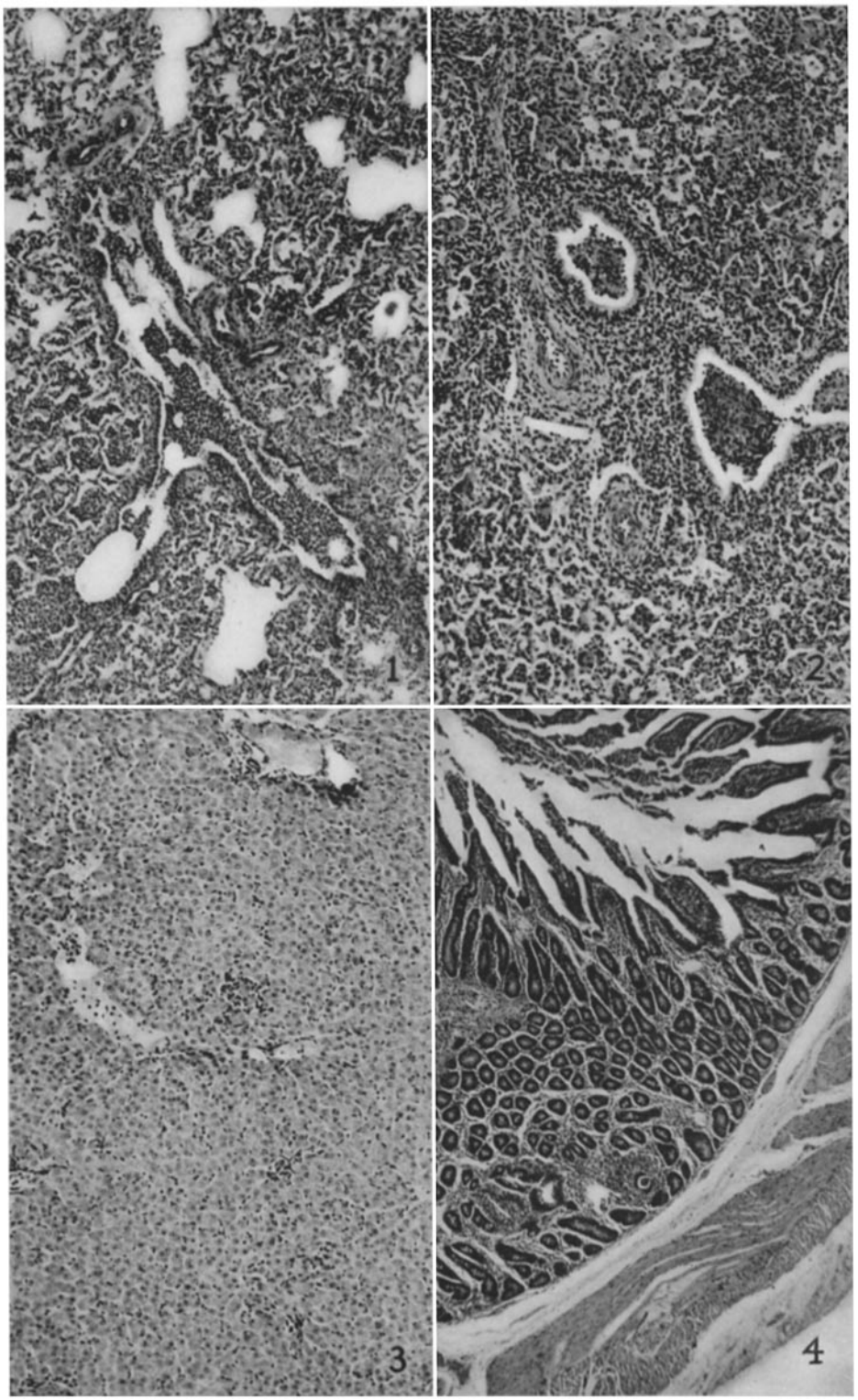
The photographs were made by Mr. J. A. Carlile.

FIG. 1. Pneumonia in lung of naturally infected calf. Note bronchial reaction. × 103.

FIG. 2. Pneumonia in lung of calf infected experimentally by giving lung suspensions from infected mice intranasally. The similarity to Fig. 1 is evident. × 103.

FIG. 3. Liver from experimentally infected calf, showing small focus of necrotic cells and infiltration with leucocytes. × 103.

FIG. 4. Small intestine of calf infected by contact, showing inflammatory reaction. × 46.



(Baker: Virus causing enteritis and pneumonia in calves)