BOVINE "ENTEROTOXEMIA"
II. EXPERIMENTAL REPRODUCTION OF THE DISEASE*

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"CLOSTRIDIUM PERFRINGENS ENTEROTOXEMIA" receives wide acceptance when it refers to a disease condition in the ovine caused by the toxin of *Cl. perfringens* type D. It is perhaps presumptuous for some veterinary practitioners to have attributed sudden deaths in the bovine to the same etiological agent mainly because the latter species exhibited a somewhat similar clinical history and post-mortem picture to that seen in sheep known to have died of enterotoxemia.

This attitude on the part of veterinary practitioners and failure in previous studies (15) to isolate *Cl. perfringens* types other than A from clinically suspected cases of bovine enterotoxemia prompted an attempt to experimentally reproduce this condition in the bovine.

Considerable research has already been done on enterotoxemia in sheep. Bullen and co-workers (4, 3) experimentally reproduced it in over-fed sheep, by dripping type D organisms into the duodenum. The fate of such organisms when introduced into the rumen was also studied (5). Jansen (12) administered single doses of type D cultures mixed with dextrin by the intraduodenal route and was able to reproduce enterotoxemia. Griner (9) and Griner and Carlson (11) studied the effects of type D toxin on the brains of sheep and mice. Gordon *et al.* (8) injected culture filtrates of types A, C, and D into sheep by the intravenous route and observed the resultant pathology. The neurotoxic, and circulatory effects as well as the pharmacologic aspects of types D, B and C toxins injected intravenously into lambs, rabbits and cats were studied by Kellaway *et al.* (14, 13). Apparently the only reports on experimental reproduction of enterotoxemia in domestic animals other than sheep are those of Field and Goodwin (7) who used *Cl. perfringens* type C in piglets, and Griner and Bracken (10) who reproduced acute hemorrhagic enteritis in suckling calves with type C organisms.

MATERIALS AND METHODS

Cl. perfringens strains

Types A, C and D cultures were used. The type A strain (No. 398) was one of our own isolates whereas the type C (CN 3685) and the type D (CN 2068) strains were received from the Wellcome Research Laboratories, Beckenham, England. The same three strains were employed throughout the study.

Preparation of inocula

The cultures were grown in a meat medium (15) dispensed in 500 ml. Erlenmeyer flasks. Incubation was at 37° C. for six to eight hours under anaerobic conditions in MacIntosh-Fildes jars. Type A culture was usually grown slightly

*A part of this paper was presented at the 14th Annual C.V.M.A. Convention, Guelph, Ontario, July 15–18, 1962.

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longer than the other strains. Following incubation the cultures were strained through gauze to remove large meat particles and the liquid portions were used for inoculation. For bacteria-free filtrates these liquid portions were centrifuged and then filtered through Seitz "S" pads. Type D filtrate was trypsinized (0.025 per cent Difco trypsin) for one hour at 37° C.

Tests on filtrates

All batches of the bacteria-free filtrates were inoculated intravenously into mice to determine their toxicity and their minimum lethal dose (MLD, in 24 hours).

Hemolytic tests (16) were conducted using several batches of filtrates against bovine, sheep, horse, guinea pig and human washed red blood cells. In order to detect variability within a given species, red blood cells from 14 bovines and five ovines were used. Cells from the majority of the experimental animals were included in these tests.

Experimental animals

Beef type calves, which weighed between 300–500 lb. and which had not had access to grain, were obtained directly from the range. In most cases the animals were fed either one lb. of calf starter (17 per cent protein) or 1–2 lb. of a 50 per cent oats-barley mixture per day for four to six days prior to being used for experiment.

Six sheep, weighing between 55–75 lb. each and maintained on their regular diet were used for intravenous administration of filtrates. These intravenous inoculations were made to compare different filtrates as to toxicity and pathology (in cattle and sheep).

Methods of administration

Surgical techniques were employed for the intraduodenal inoculations of whole cultures into 13 calves. Either the ventral or dorsal loop of the duodenum was exposed in the area on the right side, ventral to the third or fourth lumbar vertebral transverse process. In the first two trials the materials were injected directly into the lumen of the duodenum and the incision sutured. In subsequent trials, a polyethylene tube (6 mm. o.d.) was inserted in the duodenum, attached to the wall with intestinal silk, brought through the incision and an occluding clamp applied to the outside end before suturing the wound. In one case (type C culture) an injection through a low incision was made into the abomasum. Tranquilization with acepromazine[1] and paravertebral anaesthesia was employed. Aseptic surgical techniques were followed and in no case were antibiotics or other such chemotherapeutic agents administered during or following the surgery. Varying amounts of whole cultures along with a suspension of 40 per cent dextrin were given intraduodenally via the tube, depending on the clinical progress of the animal, for as many as five times over a three-day period. The animals that did not die in the experiments were used, along with new ones, for intravenous inoculation of filtrates (either the same or a different type). Animals being used for a second time were given their intravenous inoculations before possible immunity from the preceding experiments could develop.

Single doses of bacteria-free filtrates were given intravenously by the drip

[1]"Atravet", injectable. Ayerst, McKenna & Harrison, Montreal, Quebec.

method over a period of approximately 15–20 minutes. One calf, however, was given type A filtrate in divided doses over a two-hour period while another calf, as a control, received a single large dose of heat-inactivated (1 hr. at 70° C.) type C filtrate. A total of 21 animals (15 calves and 6 sheep) were employed in this experiment.

Oral administration of whole cultures was done in the form of a feeding experiment. Eight recently weaned range calves which had never been fed grain, were obtained. They were divided into four groups of two each and placed in separate enclosures. Adequate fresh water was provided. Twenty pounds of ground barley was made available to each calf. One half a litre of whole culture was mixed in with the feed each day. Three groups of animals received type A, C and D respectively and the fourth pair remained as controls, receiving no culture.

Necropsies were performed on all animals that died in these experiments. These were usually conducted about four hours after death, although on occasion this time interval varied from three hours to 15 hours.

Results

Properties of filtrates

Toxicity of the culture filtrates for mice remained constant from batch to batch of any one type. There was, however, a marked difference in the minimum lethal dose between the three types of filtrates. Type C filtrate was the most toxic for mice, being 20 times more potent than that of type D which had double the potency of type A. It was noted that about 70 per cent of the mice injected with type A filtrate showed blood from the mouth and nostrils at the time of death; this finding was not apparent following injection with type D and type C filtrates. The MLDs (ml./kg.) for mice are given at the bottom of Table I.

Hemolysis produced by the filtrates was most marked with type A. A considerable variation was observed, however, between the cells of individual animals. These results are shown graphically in Figure 1. The reactions with the type C filtrate paralleled those of type A, but were about one dilution lower. Type D filtrates showed hemolytic activity only when the culture was incubated more than six hours, and then only a slight reaction took place in the first few tubes.

Only five calves of the 15 bovines and 6 sheep survived the intravenous administration of culture filtrates. The results are given in Table I. The symptoms exhibited in these cases were of a peracute nature. With type D, nervous symptoms were predominant; animals showed disorientation, jumping up, circling, bellowing and frothing at the mouth, followed by convulsions and death. Symptoms with type A filtrates were characterized mainly by respiratory distress and the appearance of blood and bloody froth from the mouth and nostrils. Type C cases showed smooth progressive depression and terminal cyanosis. The differences in symptoms seen in calves were not evident in sheep; they merely collapsed and died.

Serum from one calf (No. 077) was used rather than saline as a diluent for type C filtrate when it was being titrated in mice. This was done to determine if bovine serum had a neutralizing effect on type C filtrate. No such effect was seen.

TABLE I

INTRAVENOUS ADMINISTRATION OF CULTURE FILTRATES

Calf No.	Type	ml./kg.	No. mouse MLD/kg.	Result
084*	D	1.7	7.1	Died in 1 hr.
080*	D	1.2	5.0	Died in 15 min.
081*	D	1.6	6.5	Died in 20 min.
B*	D	1.6	6.7	Died in 10 min.
001*	D	0.7	3.0	Died in 45 min.
090	A	0.8	1.6	Died in 5 min.
092	A	0.8	1.6	Died in 5 min.
079*	A	0.5	1.1	Survived
078*	A	0.4	0.8	Survived
280*	A	1.0 (2 hrs.)	2.0	Survived
077	C	3.0	252.5	Survived
343	C	4.3	355.8	Died in 10 min.
113	C	2.2	186.7	Died in 6 hrs.
371*	C	1.0	83.3	Died in 20 min.
085*	C (control)	3.0	equiv. 248.3	Survived
Sheep No.				
622	D	1.8	7.7	Died in 1 hr.
609	D	2.6	10.8	Died in 20 min.
623	A	1.3	2.6	Died in 5 min.
607	C	0.4	36.7	Died in 5 min.
626	C	1.2	98.3	Died in 2 min.
613	C	0.4	36.7	Died in 5 min.
Mice		MLD ml./kg.		
	A	0.5		
	C	0.01		
	D	0.2		

*These animals were first used for experiment with whole cultures, see Table II.

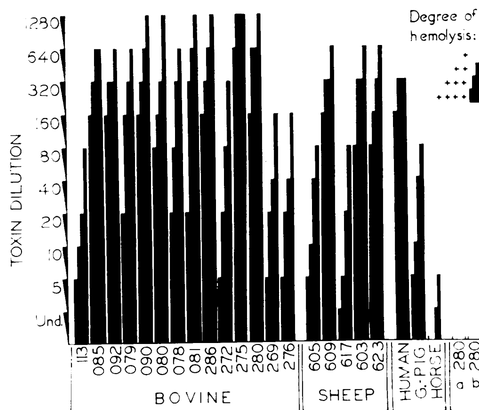

FIGURE 1. Hemolysis of various red cells produced by type A filtrate. Cells of No. 280 used for controls: (a) filtrate inactivated at 70° C. for one hour; (b) filtrate neutralized with type A antiserum. Complete hemolysis—++++.

Effect of whole cultures in calves

Two of the eight animals to which whole culture of type D was administered intraduodenally developed rapidly progressing muscular incoordination and nervous symptoms (increased excitability, dilated pupils, twitching) within two hours, leading to death in six–eight hours. One of these animals received a single dose of 140 ml. of culture plus dextrin, the other was given 300 ml. plus dextrin. The remaining six animals, even when given large repeated doses, showed no ill effects, other than on occasion slight transitory diarrhea.

Type A and C cultures were given to four and two calves, respectively, (including one type C injection into the abomasum) in large doses with no subsequent ill effects. Results of this experiment are summarized in Table II.

TABLE II

INTRADUODENAL ADMINISTRATION OF WHOLE CULTURES

Calf No.	Type	Amount of culture ml.	Amount of dextrin ml.	Result
085*	D	1465 (6 doses, 3 days)‡	1120 (divided doses)	Survived
078*	D	90	60	Survived
079*	D	90	120	Survived
083	D	140	160	*Died in 8 hrs.*
084†	D	450	—	Survived
086	D	300	300	*Died in 6 hrs.*
081*	D	500	250	Survived
272	D	2100 (2 doses, 24 hrs.)	250	Survived (killed)
084†	A	915 (4 doses, 3 days)	1080 (divided doses)	Survived
280*	A	3800 (5 doses, 3 days)	2500 (divided doses)	Survived
371*	A	1600 (3 doses, 2 days)	1400 (divided doses)	Survived
001*	A	1600 (3 doses, 2 days)	1400 (divided doses)	Survived
B*	C	2650 (4 doses, 3 days)	1170 (divided doses)	Survived
080*	C	130 (into abomasum)	200	Survived

*These animals were later used for intravenous work, see Table I.
†Type A first, changed to type D, then to intravenous method.
‡The total amount given in so many divided doses over a period indicated.

Whole cultures in the feed

Most of the eight calves, used for the feeding experiment, ate about 60 lb. of barley each over a four-day period. Their appetite, however, declined on the third day. The most severe symptoms were noticed in the control group which exhibited some evidence of posterior paresis as well as diarrhea. A tentative diagnosis of acidosis was made and when good alfalfa hay was made available, all animals recovered.

Pathologic findings

Type D (culture). The two attempts which were successful in reproducing enterotoxemia by intraduodenal administration of whole cultures, animals No. 083 and 086, showed identical pathological findings. A considerable amount of straw-colored fluid was present in the peritoneal and pleural cavities and in the pericardial sac. Petechial hemorrhages were found on the serosal surfaces of the intestines and diaphragm and in the endo- and peri-cardium. The omentum showed patchy, orange-colored, slightly raised serosanguinous deposits on the

surface (Fig. 2). Perhaps the most striking change was found in the lungs, an extreme degree of edema. The interlobular spaces were greatly dilated and filled with clear yellowish gelatinous (coagulated) exudate. These spaces had a thickness of up to three-eighths of an inch and were visible through the pleural lining

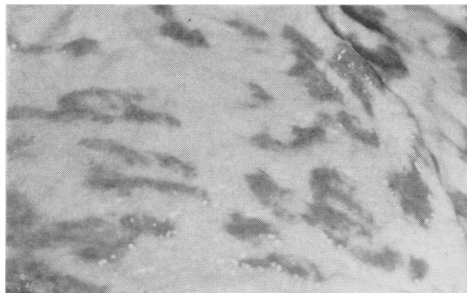

FIGURE 2. Serosanguinous deposits on omentum. Calf No. 083. Inoculum: type D whole culture. Route: intraduodenal.

FIGURE 3. Edematous lung. Note large interlobular spaces filled with exudate, on the cut surface (lower left) and visible through the pleura (upper right). Calf No. 083. Inoculum: type D whole culture. Route: intraduodenal.

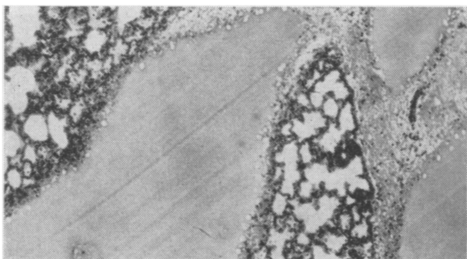

FIGURE 4. Photomicrograph of edematous lung. Proteinaceous material in interlobular spaces; alveoli in this section clear. (× 128). Calf No. 083. Inoculum: type D whole culture. Route: intraduodenal.

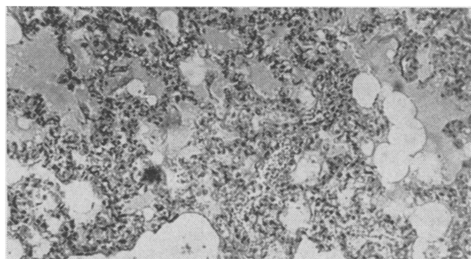

FIGURE 5. Photomicrograph of edematous lung (× 200). Calf 086. Inoculum: type D whole culture. Route: Intraduodenal.

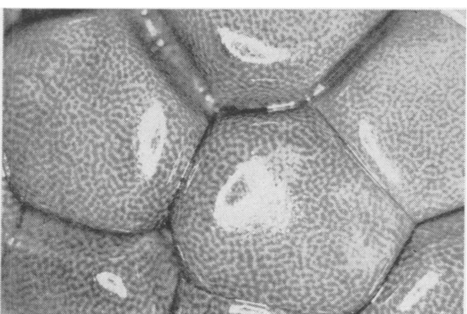

FIGURE 6. Hemorrhagic mottling of kidney. Calf No. 086. Inoculum: type D whole culture. Route: intraduodenal.

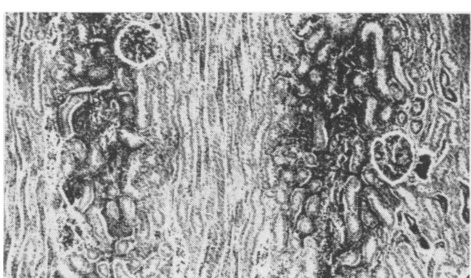

FIGURE 7. Photomicrograph of kidney. Radial section; hemorrhagic cortical rays visible (X 128). Calf No. 086. Inoculum: type D whole culture. Route: intraduodenal.

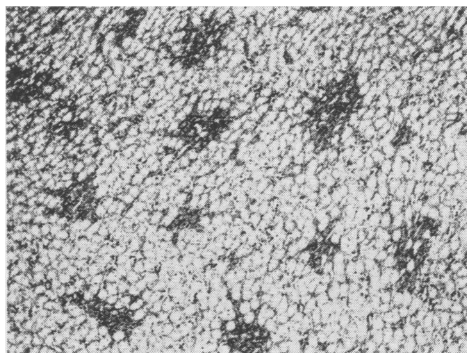

FIGURE 8. Kidney as in Fig. 7; tangential section (× 128). Hemorrhagic "islands" visible.

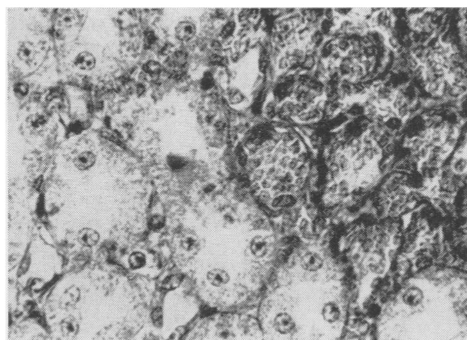

FIGURE 9. Kidney as in Fig. 8, (× 1280). Blood filled area ("island") seen at upper right.

which itself had a diffuse milky appearance (Fig. 3). Microscopic sections of these lungs revealed faintly-staining proteinaceous material in the interlobular spaces. The alveoli were normal in some sections (Fig. 4), whereas in others intra-alveolar edema, accompanied by congestion, was observed (Fig. 5). Edema was also present in the lymph nodes; the fluid, however, was not coagulated, and when cut, remained dripping.

The kidneys had an intense hemorrhagic mottling on the surface (Fig. 6) although the organs appeared firm and normal in shape. On sections, radial hemorrhagic streaks were found, coinciding with the cortical rays (Fig. 7). A tangential section of such an area is shown in Figure 8, and in higher magnification in Figure 9. General tubular damage was further demonstrated by urinalysis which showed the following results: ketones–negative; sugar–++ (¾ per cent); albumin–++++; microscopic examination revealed an abundance of degenerated epithelial cells (apparently cuboidal type) and a few lymphocytes; no red blood cells were seen.

A heavy concentration of gram-positive rods was seen in smears made from the intestinal contents of these two animals. A few of these same bacterial types were also found in blood smears. Anaerobic cultures of liver, spleen, muscle and pleural fluid resulted in no growth, whereas *Cl. perfringens* type D was isolated from the heart blood. Type D toxin was demonstrated by neutralization tests in filtrates of material taken from the duodenum, ileum, jejunum and cecum. Filtrate toxicity was greatest in the cecal contents and lowest in the duodenal contents.

Type D (filtrate). In the intravenous trials with type filtrates, animals No. 084 and 001 exhibited essentially similar pathological changes to those already described, including identical pathology in the lungs, and, to some extent, in the kidneys. As the bladder of No. 084 was empty, urinalysis could not be done but that of No. 001 showed a "trace" of sugar in the urine. Cultural procedures were not attempted. Of the remaining three calves and two sheep that died, only calf No. 081 revealed slight pulmonary edema, the others were normal in this respect. Kidney damage and other lesions were much less marked than those found in Nos. 084 and 001; apparently death occurred too suddenly for such lesions to develop.

Type C (filtrate). Some petechial hemorrhages were observed in the endocardium, particularly in the left ventricle. Edema and fluid in the body cavities were absent. More outstanding, however, was the dark, intensely injected vascular system of the digestive tract in both calves and sheep. The blood vessels appeared engorged with blood and were clearly visible at a distance (Fig. 10). All other organs were somewhat congested. In addition to general congestion calf No. 113 also displayed an intensely hemorrhagic abomasal mucosa. In calf No. 371 the mucosa of the small and large intestine was markedly congested.

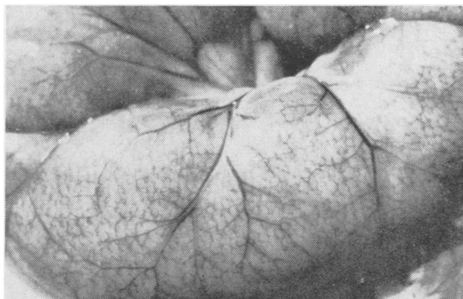

FIGURE 10. Injected intestinal blood vessels. Sheep No. 626. Inoculum: type C filtrate. Route: intravenous.

Type A (filtrate). A hemorrhagic picture predominated in these cases. Petechial hemorrhages were found on the serosal surfaces, in the endo- and peri-cardium and particularly in the laryngopharynx (Fig. 11). The lungs were intensely congested and hemorrhagic; when cut, blood and bloody froth oozed out of bronchioles and interlobular spaces (Fig. 12). Microscopic sections showed most of the alveoli filled with blood. The lungs of sheep No. 623 were not hemorrhagic, although some petechia were found on serous membranes. All carcasses revealed a relatively rapid production of gas in the tissues.

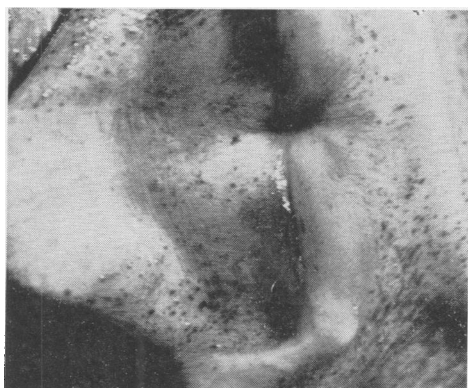

FIGURE 11. Petechial hemorrhages on laryngopharynx and epiglottis. (× 1.4). Calf No. 090. Inoculum: type A filtrate. Route: intravenous.

FIGURE 12. Hemorrhagic lung. Congestion and bloody froth visible on cut surface (× 1.0). Calf No. 090. Inoculum: type A filtrate Route: intravenous.

Discussion

As can be seen from the results, only limited success was achieved in reproducing enterotoxemia in susceptible bovines with *Cl. perfringens* type D culture, and it was difficult to assess the conditions necessary for its development. It seemed that the mere presence of type D organisms in the digestive tract could not be solely responsible for the development of the disease, because in some cases administration of large amounts of whole culture failed to reproduce this condition (Table II). Such findings were in keeping with work done previously on the presence of type D organisms in the alimentary tract of normal sheep (1).

No success was obtained with types A and C cultures, although large amounts had been administered. The feeding experiment which was meant to simulate many a feed-lot operation, induced only an acidosis-like condition in the control animals but failed to reproduce enterotoxemia.

The results of the intravenous trials with culture filtrates pointed to the relative resistance of these calves to type C toxin, although the MLD's could not be calculated. The MLD's for sheep, as shown by Gordon *et al.*, (8) as expressed in terms of number of mouse MLD/kg were: A–11.0, C–45.0, and D–25.0. Whether or not similar ratios would hold true for bovine susceptibility to these toxins was not established; were this so then the calves should be least susceptible to type C filtrates and most so to type A toxin. It should be noted however, that in mice, type A filtrate is only a fraction as toxic as that of C.

It appears that *Cl. perfringens* type D can, under certain conditions, be responsible for the production of a true enterotoxemia in susceptible bovines. If type D organisms happen to be present in the digestive tract and sufficient epsilon toxin is produced therein at a rapid rate, it is activated by proteolytic digestive enzymes and then absorbed into the blood stream. The presence of this toxin apparently increases the permeability of the intestine, which in turn facilitates the absorption of more toxin (2). Whether or not types A and C toxins (if formed and not destroyed in the intestine) could be absorbed through the intestinal mucosa is not clear.

The lethal potential of type A toxin in the blood stream would not be considered great if the studies on the metabolism of this toxin by Ellner (6) are taken into account. Using a radio-chemical method he discovered that *Cl. perfringens* type A toxin was rapidly metabolized and eliminated from the blood stream of mice and rabbits. This, perhaps, applied to our test animal No. 280 (Table I) which withstood a higher intravenous dose of toxin when it was given over a period of two hours than did other animals receiving a smaller dose during a 15–20-minute period.

If the necropsy findings in these experimental cases can be considered pathognomonic for the natural disease, then the bovine enterotoxemia caused by type D could be characterized by edema, particularly in the lungs, and kidney damage. Formation of pulmonary edema is one of the characteristics most commonly associated with type D intoxication according to experimental work conducted in lambs, rabbits and cats by Kellaway *et al.* (14). The term "pulpy kidney", however, could not be applied to any of the animals dying from exposure to type D materials in these experiments. The pathology produced with the intravenous

administration of type A filtrate (acute hemorrhagic syndrome) and type C filtrate (general congestion and, perhaps, hemorrhagic enteritis) may or may not be pathognomonic for the natural disease.

All of the experiments conducted and results described were obtained using only three single strains of *Cl. perfringens*. Because of variability in the make-up of the toxin components within a type somewhat different results might have ensued had different strains been used in this work. The inter-action of toxins of different types of *Cl. perfringens* acting simultaneously in the one animal could lead to confusion.

Summary

Experimental reproduction of enterotoxemia with *Cl. perfringens* types A, C, and D was attempted in 300–500 lb. calves. Success was obtained only when type D culture was administered intraduodenally, along with some dextrin. The animals that died showed extensive edema, particularly in the lungs, and kidney damage. "Pulpy kidney" was not apparent.

Administration of types A and C cultures, even in large amounts, and the feeding of all three cultures failed to reproduce enterotoxemia.

When bacteria-free culture filtrates were administered intravenously, type A was as toxic as type D. The animals used in this work appeared relatively resistant to type C filtrate.

On the basis of these experiments, it appears that enterotoxemia due to *Cl. perfringens* type D may occur under certain conditions in susceptible bovines. The significance of *Cl. perfringens* types A and C in bovine enterotoxemia could not be ascertained.

Résumé

Des essais de reproduction expérimentale de l'"entérotoxémie bovine" furent accomplis sur des veaux de 400 à 500 lb. en employant les types A, C et D de *Cl. perfringens*. On obtint des résultats positifs avec des cultures de type D administrées par voie intraduodénale et accompagnées d'une certaine quantité de dextrine. A l'autopsie on observa de l'oedème généralisé, surtout des poumons, et des lésions rénales, cependant les reins n'étaient pas "pulpeux".

Des cultures de types A et C, même s'ils furent administrées en grandes quantités, ne reproduisirent pas la maladie.

Lorsque des filtrats de cultures furent données par voie intraveineuse, le type A s'avéra aussi toxique que le type D. Les animaux employés dans ces expériences semblaient être résistants au type C.

Comme conclusion à ces expériences, il semble que l'entérotoxémie à *Cl. perfringens* type D peut survenir chez des bovins, dans certaines conditions définies. Le rôle des types A et C de *Cl. perfringens* dans l'entérotoxémie des bovins n'a pu être démontré.

Acknowledgements

The authors gratefully wish to acknowledge the co-operation and advice of Dr. M. Sterne and his co-workers of the Wellcome Research Laboratories, Beckenham, England. The technical help of Miss G. S. MacDonell is acknowledged. Our thanks are also due to Dr. P. J. G. Plummer and Mr. J. L. Byrne for reading the manuscript.

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BOOK REVIEW

Veterinary Preventive Medicine. E. G. White and F. T. W. Jordan. Published by Baillière, Tindall, and Cox, London, England. 1963. 334 pages, illustrated. $5.40.

The purpose of this book, as outlined in its preface, is to provide an introduction to the principles of disease prevention for veterinary students, practising veterinarians and students of animal husbandry. These aims have been realized. The text is divided into four parts: first, an introduction to the principles of epidemiology; second, examples of diseases grouped according to etiology and chosen to illustrate varied problems and control measures; thirdly, a discussion of the sociologic, climatic, and geographic factors which either hinder or facilitate disease control; and finally, a discussion of the effects of diseases of animals upon man, as they affect his economy or directly, his health.

This book emphasizes how broad in scope is the field of preventive medicine. It requires a knowledge of epidemiology, all types of disease causation, diagnosis and therapeutics; even law enactment and enforcement. Attempting to cover this broad field has made this book shallow, of necessity, in its treatment of many subjects. It cannot substitute for textbooks on epidemiology, microbiology, parasitology, and genetics, although each of these disciplines and many more are drawn upon for example of disease control and prevention. *Lars Karstad.*