2. Richert, D. A., and Westerfeld, W. W., *J. Biol. Chem.*, 1953, v203, 915.

3. De Renzo, E. C., Kaleita, E., Heytler, P. G., Oleson, J. J., Hutchings, B. L., and Williams, J. H., *Arch. Biochem. Biophysics*, 1953, v45, 247.

4. Reid, B. L., Kurnick, A. A., Svacha, R. L., and Couch, J. R., PROC. SOC. EXP. BIOL. AND MED., 1956, v93, 245.

5. Remy, C., and Westerfeld, W. W., *J. Biol. Chem.*, 1951, v193, 659.

6. Dannenburg, W. N., Reid, B. L., Rozacky, E. E., and Couch, J. R., *Poultry Sci.*, 1955, v34, 1023.

7. Morrison, A. B., Scott, M. L., and Norris, L. C., *ibid.*, 1955, v34, 738.

8. Camp, A. A., Reid, B. L., and Couch, J. R., *ibid.*, 1956, v35, 621.

9. Remy, C., Richert, D. A., and Westerfeld, W. W., *J. Biol. Chem.*, 1951, v193, 649.

10. Berttrand, D., *Compt. Rend.*, 1939, v208, 2024.

11. Marmoy, F. B., *J. Soc. Chem. Ind.*, 1939, v58, 275.

12. Morrison, A. B., Dam, R., Norris, L. C., and Scott, M. L., *J. Nutrition*, 1956, v60, 283.

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Isolation and Identification of Infectious Bovine Rhinotracheitis Virus in Tissue Culture. (23071)

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A disease of cattle characterized by severe inflammation of upper respiratory passages and trachea, accompanied by excessive nasal discharge, salivation, dyspnea and fever has been reported since 1950 in about 15 western states(1,2,3). This condition, observed in both dairy and beef cattle areas, has been described under various names such as acute upper respiratory infection, infectious necrotic rhinotracheitis, or more commonly, red nose disease. At a meeting of the U. S. Livestock Sanitary Assn. in Nov. 1955, it was generally agreed to call this infectious bovine rhinotracheitis (IBR). The disease could be reproduced regularly by intranasal inoculations of cattle with nasal washings from naturally infected cases, and protection tests in cattle, using infectious material from different parts of the country, indicated that the illness was caused by the same etiological agent(4,5). However, attempts to isolate this agent in chick embryos, weanling and suckling mice, guinea pigs and rabbits were unsuccessful(5). In a preliminary report, it was shown that with the use of bovine embryonic tissues in tissue culture, a virus producing a cytopathogenic effect on cells was isolated from infectious material of cattle(6). This paper presents additional information concerning this virus, and shows that it is the etiological agent of infectious bovine rhinotracheitis.

Material and methods. Tissue culture procedures. The cortex of bovine kidneys obtained from 8-9 months old feti was minced and trypsinized by a method similar to that described by Youngner(6). The prepared cells were suspended in nutrient medium consisting of 0.5% lactalbumin hydrolysate, and 5% to 10% horse serum made up in Earles basic salt solution. To this was added 200 units of penicillin and 200 μg of streptomycin/ml. This mixture was then dispensed in 2 ml amounts in tubes and allowed to stand in a stationary position for 5 days, or until good cellular growth was observed. At this time, the medium was changed and tubes placed in roller drums for use. Similar preparations were made with bovine testicular tissue. Tubes made with lung tissue, however, were prepared by a plasma-clot technic employing small pieces of minced tissue(7). *Sources of infectious material.* Various tissues, as well as nasal washings, were obtained during the early acute phase of illness from cattle naturally infected with IBR disease in Colorado, California, and Ohio. These specimens were placed in screw-cap vials and frozen immediately in an alcohol dry-ice bath, and stored at

TABLE I. Isolation of IBR Virus from Tissues of Naturally Infected Cattle from Different Areas.

Source	Specimen	Isolations/ Attempts	Year	Strain designation
Colorado beef	Turbinate	6/6	1955	Colorado I & II
	Larynx	2/2		
	Trachea	3/5		
	Nasal washing	3/3	1955–56	" III, IV, V
	Spleen	0/5	1955	
	Liver	0/4		
	Lung	0/7		
California beef	Nasal washing	1/1	1955	Blythe
" dairy	" "	1/1	1955	Los Angeles (L.A.)
Ohio beef	Turbinate	1/1	1956	Ohio I

–70°C until use.

Results. 1. *Isolation of virus.* Tissue cultures showing a continuous sheet of epithelial cells of bovine embryo kidney on the sides of tubes were selected for inoculation with infectious material. Portions of turbinate, larynx, trachea, lung, liver, and spleen from infected cattle were made into 10% suspensions in tissue culture medium, using a glass tissue grinder, and centrifuged at 1,000 rpm for 10 minutes. Supernatants of each suspension were inoculated in 0.2 ml amounts into each of several tubes. Similar tubes were inoculated with 0.2 ml of undiluted nasal washings. These tubes were observed daily for at least 7 days, following which transfers of 0.2 ml quantities of tissue culture fluid were made into new tubes of tissue culture cells. After a sub-culture was made and no demonstrable changes occurred, the tubes were discarded. A summary of various isolation attempts is presented in Table I.

Two to 3 days after inoculation with infectious material, a cytopathogenic effect (CPE) was observed. The cells became more granular, then rounded up and refractile in appearance. The affected cells grouped together to form clumps and strands. This effect was progressive until all cells became involved and were finally released from the wall of the tube. Similar cytological changes occurred when tissue cultures of testicular and lung tissues were inoculated with infectious material. The general appearance of this cytological change remained the same in all passages, although the rapidity with which all cells in the tube became affected depended on the titer of virus inoculated. A comparison of normal and infected bovine embryonic kidney epithelial tissue culture cells can be seen in Fig. 1.

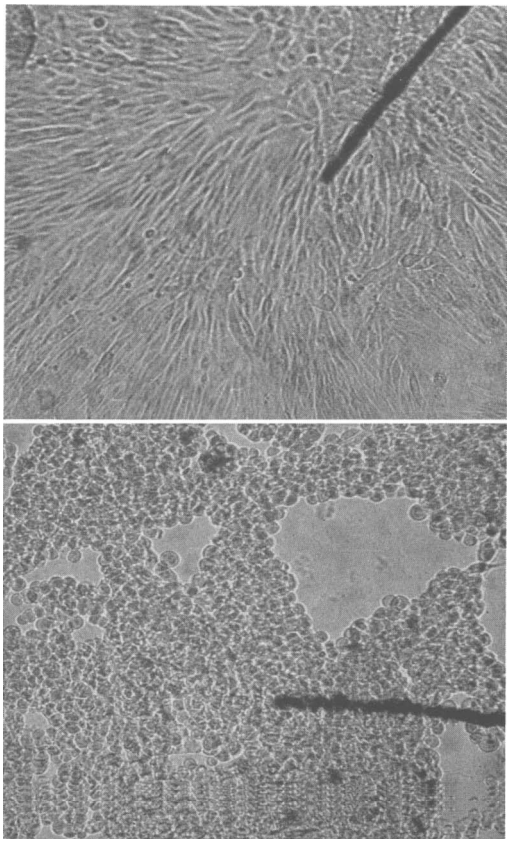

FIG. 1. Comparison of normal bovine kidney tissue (above) with a similar culture (below), showing complete cytopathogenic effect following inoculation with the virus of infectious bovine rhinotracheitis.

Titrations of infected kidney tissue culture fluids were made of a number of passages, using the CPE as an indicator of end point. By preparing 10-fold serial dilutions of the 8th tissue culture passage of 1 strain of virus (Colorado I) and inoculating 0.2 ml into each of 4 tubes for each dilution, a 50% end point ($TCID_{50}$) of infectivity was obtained at $10^{6.5}$. This would indicate that the virus had multiplied in the tissue culture, since the end point of titration for the 8th passage would represent a dilution of $10^{14.5}$ of the original tissue suspension. Subsequent titrations through 100 passages of this virus gave similar results with $TCID_{50}$ end points varying from $10^{5.5}$ to $10^{7.0}$.

2. *Studies with cattle.* (a) Reproduction of the Disease. All cattle used in these studies were either raised on our research farm or purchased from the area immediately surrounding the laboratory (a region where the disease has not yet been reported). The animals varied from 4-9 months of age, and were generally held in isolation for at least 2 weeks prior to inoculation. Daily temperature records, as well as other observations, were made to insure that animals were normal before use.

To determine whether the virus causing the CPE in tissue culture produced IBR disease in experimental cattle, groups of animals were inoculated intranasally with 1 ml of infected tissue culture fluid, using different tissue culture passage levels of the Colorado I strain. The calves were inoculated as follows: 4 with T.C. passage 2; 9 with T.C. passage 4; and 6 with T.C. passage 15. Within 2-3 days after inoculation, a febrile response occurred in each inoculated animal varying from 103.6° to 106.6°F and lasting from 1-9 days, with average duration of 3½ days. Other signs of illness, such as nasal discharge, depression, inappetence, dyspnea, and coughing were also noted, although not all of these were observed in each experimental animal. A high percentage of animals showed marked hyperemia of the nasal mucosa as well as small, white areas of serofibrinous exudate which adhered tightly to the anterior portion of mucosal surfaces. This condition generally persisted a few days after disappearance of the febrile response. In more severe cases, this serofibrinous exudate became extensive and covered not only anterior areas of the nasal mucosa, but also extended posteriorly over the surfaces of turbinates. There appeared to be no discernible difference in response of the cattle to different passage levels of tissue culture material tested. Several animals, inoculated with normal tissue culture fluid, remained healthy and susceptible to challenge with virulent virus 2-3 weeks later.

TABLE II. Cross-Protection Tests in Cattle with Several Strains of Infectious Bovine Rhinotracheitis Virus.

Immunizing strain	Challenging strain: Col. I T.C.*	Col. V N.W.†	Col. V T.C.	Blythe T.C.
Colorado IV N.W.	0/ 1‡	N.T.§	N.T.	N.T.
" I T.C.	0/20	1/11	0/3	0/3
Blythe T.C.	0/ 1	N.T.	N.T.	N.T.

* T.C. = Tissue culture-propagated virus.
† N.W. = Nasal washings from naturally infected cattle.
‡ Numerator = No. of animals reacting to challenge; denominator = No. of animals challenged.
§ N.T. = Not tested.

(b) Identification by protection tests. To determine whether the experimentally produced disease was actually infectious bovine rhinotracheitis, 39 calves were immunized with one or another virus strain. They were challenged intranasally 2-3 weeks later with homologous or heterologous virus shown to have disease-producing properties by inoculation of one or more susceptible control animals. The results are summarized in Table II.

3. *Identification by serum neutralization test.* As further proof that the cytopathogenic agent isolated from infectious material was the cause of IBR, serum neutralization tests were undertaken. In these tests, equal amounts of serum, or serum dilutions, were mixed with equal amounts of infected tissue culture fluid in a final concentration between 100 to 1,000 $TCID_{50}$ of virus. This mixture was incubated for 2 hours at 37°C and 0.2 ml then inoculated into each of 3-4 kidney tissue culture tubes and observed for several days. The serum neutralization effect was

TABLE III. Reciprocal Cross-Neutralization Tests with 3 Strains of Infectious Bovine Rhinotracheitis Virus, Using Homologous and Heterologous Immune Sera against 32-200 $TCID_{50}$ of Virus.

Sera tested		Strains of virus used		
		Colorado 1	Blythe	L.A.
Colorado	1	1: 10	1: 9	1: 9
	2	1:180	1:100	1:180
	3	1: 6	1: 3	1: 3
Blythe	1	1: 10	1: 19	1: 10
	2	1: 45	1: 45	1: 10
	3	1: 27	1: 27	1: 23
	4	1: 13	1: 10	1: 10
L.A.	1	1:180	1:180	1:180
Normal serum*		0	0	0

* Normal serum represents 3 tests with pre-inoculation sera from cattle inoculated with Colorado 1 virus.

recorded when approximately 100 $TCID_{50}$ units of virus were observed in the virus control titration carried out simultaneously.

By this method, it was demonstrated that the animals inoculated with Colorado I developed antibodies against this strain in a titer ranging from 1:2 to 1:64. Uninoculated control animals, or animals inoculated with normal tissue culture fluid, were negative for neutralizing antibodies against this virus. Serum samples collected from 71 animals which had recovered from IBR during natural outbreaks in California, Colorado, Illinois, Nebraska, and Ohio had neutralizing antibodies against Colorado I. Colorado V, Blythe, L. A., and Ohio tissue culture isolates were also neutralized by Colorado I antiserum.

To examine more completely the antigenic relationship between these viruses, 3 strains obtained from widely separated areas (Colorado I, Blythe, and L.A.) were used in reciprocal cross-neutralization tests employing antisera against each of these viruses. The results of this work, summarized in Table III, indicate a close, if not identical, antigenic relationship between these strains of virus.

Discussion. The evidence presented seems to leave little doubt that the tissue culture virus is the etiological agent of IBR disease. A significant fact is that this virus produced illness in experimental cattle characteristic of that observed in naturally occurring cases. It is possible, however, that similar upper respiratory infections might be caused by a number of agents. Therefore, additional support for the relationship of this virus to the disease is provided by protection tests in cattle. If the tissue culture virus was not the etiological agent, then animals immunized with this virus should have responded to a challenge inoculum of IBR infectious material obtained from field outbreaks of the disease. As has been shown, this did not occur. Final proof as to the identity of the virus is provided by the fact that antibodies produced by the first tissue culture isolate neutralized equally well its homologous virus and viruses isolated from infectious material from different IBR outbreaks. Conversely, sera from these outbreaks readily neutralized the tissue culture virus.

Production of a cytopathogenic effect in tissue culture cells by this virus provides an easy and accurate means of measuring the titer of infectious material. It also furnishes a method for additional isolations of the virus from various outbreaks of this disease. The neutralization test in tissue culture makes possible a serological test that can be used to study the epidemiology of the disease in individual herds or areas, as well as to identify new outbreaks of the infection that may occur in different parts of the country.

Since bovine embryonic tissues are readily available, growth of this cytopathogenic virus in tissue culture is a major step forward in propagating the virus in quantity for possible use in development of a practical vaccine. Furthermore, the fact that all strains of virus so far isolated appear to be antigenically the same greatly simplifies the task of developing an effective control program.

Summary. An agent producing a cytopathogenic effect has been isolated in bovine embryo tissue culture cells from upper respiratory tissues of cattle having the disease of infectious bovine rhinotracheitis. Lack of growth in bacteriological culture media, resistance to antibiotics such as penicillin and streptomycin, and ability to pass through fine porosity filters demonstrates that this agent is a virus. Reproduction of a disease in experimental cattle identical to that which occurs naturally, protection tests in cattle using

tissue culture virus and infectious material from naturally occurring cases, as well as neutralization tests with serum obtained from naturally and experimentally infected cattle, show that this virus is the etiological agent of infectious bovine rhinotracheitis. Serum neutralization tests also indicate that viruses isolated from different parts of the country are closely related antigenically.

1. Schroeder, R. J. and Moys, M. D., *J.A.V.M.A.*, 1954, v125, 471.

2. McKercher, D. G., Moulton, J. E., and Jasper, D. E., *Proc. U. S. Livestock Sanitary Assn. 58th Meeting*, 1954, 260.

3. Miller, N. J., *J.A.V.M.A.*, 1955, v126, 463.

4. McKercher, D. G., Moulton, J. E., Kendrick, J. W., and Saito, J., *Proc. U. S. Livestock Sanitary Assn. 59th Meeting*, 1955, 151.

5. Chow, T. L., Deem, A. W., and Jensen, R., *ibid.*, 1955, 168.

6. Youngner, J., PROC. SOC. EXP. BIOL. AND MED., 1954, v85, 202.

7. Weller, T. H., Robbins, F. C., and Enders, J. F., *ibid.*, 1949, v72, 153.

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Increased Production and Utilization of Circulating Glucose During Growth Hormone Regimen.*† (23072)

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Hypophysectomized dogs exhibit extensive abnormalities in carbohydrate metabolism (1), many of which are reversed by growth hormone(2). Large doses of the hormone may produce diabetes in the normal dog(3). Recently, by application of a technic whereby body glucose pool was tagged with C^{14}(4), it was shown that the unanesthetized hypophysectomized dog in postabsorptive state, in comparison with the normal dog, had a smaller body glucose pool and lower rate of glucose turnover, *i.e.*, glucose inflow from liver into plasma, and glucose outflow from plasma into tissues(5).

The present study is concerned with effect of a growth hormone regimen on turnover rate of the glucose pool in the unanesthetized hypophysectomized dog, studied with the aid of C^{14} glucose.

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Materials and methods. Adult hypophysectomized male and female dogs, maintained on standard diet(2), were used. The animals were 57-850 days postoperative. Growth hormone (Lot C_5I_5; prepared and kindly supplied by Dr. Robert Bates) was administered intramuscularly in daily doses of 1 mg/kg for 4-5 days, last injection given 18 hours prior to experiment. The animals were in good physical and nutritional state before and during injection period. The experiments were performed after 17-18 hours fast and without anesthesia. A complete description of experimental material and procedure has been given previously(4). Uniformly labeled C^{14} glucose was administered intravenously by initial priming injection, followed immediately by constant continuous infusion. Total amount of glucose administered did not exceed 4 mg. Samples of blood were drawn at intervals for determination of concentration of plasma glucose and its C^{14} content. Plasma glucose content was determined on aliquots of Somogyi zinc-barium filtrates(6), by the method of Hagedorn-Jensen(7). Unlabeled carrier glucose was then added to other aliquots of the filtrate and glucose was