Reports

Isolation of the Infectious Bovine Rhinotracheitis Virus

During the fall of 1953 a disease characterized by sudden onset, pyrexia, abrupt cessation of milk flow, salivation, dyspnea, and severe inflammation of the upper respiratory passages and trachea was reported among dairy cattle in California (*1*). A clinically similar condition, although more severe, occurred among feedlot cattle in California in 1954 (*2*), and outbreaks have occurred intermittently since that time. Reports from Colorado indicated that a severe type of respiratory infection, clinically similar to that in California, had been prevalent there since 1951 among feedlot cattle and constituted a continuing problem for the cattle industry of that area (*3*).

The clinical syndrome could be reproduced regularly by the intranasal instillation of penicillin and streptomycin-treated nasal washings but not by the parenteral injection of blood, from early clinical cases. Calves that recovered from the experimental infection were immune to challenge with intranasal instillation of infectious nasal washings. Cross-protection tests showed that the clinical condition observed in California dairy and beef cattle and in Colorado beef cattle were the same disease. It was then recommended that the condition be designated as "infectious bovine rhinotracheitis" (IBR) (*4*).

Repeated attempts to recover an etiological agent in chick embryo, in weanling and suckling mice, and in guinea pigs were unsuccessful. These failures were followed by the successful isolation of the causative agent in tissue culture.

Tissue cultures of bovine embryonic kidney were prepared in tubes by a modification of the trypsin digest method of Youngner (*5*). Two nutrient media were used: (i) 0.5-percent solution of lactalbumin hydrolyzate in Hank's solution fortified with 6-percent lamb serum and (ii) mixture of 0.5-percent lactalbumin hydrolyzate in Earle's solution fortified with horse serum.

Nasal washings were obtained from calves that had been infected experimentally with nasal washings from naturally infected beef and dairy cattle in southern California and from beef cattle on Colorado feedlots. These washings, obtained in the acute phase of the disease, were treated with penicillin and streptomycin, diluted 1/10 with nutrient fluid, and inoculated into tubes of tissue culture. Cytopathogenic changes, characterized by rounding and shrinking of the cells and increased granularity and clumping, were seen in the tubes of beef embryo kidney 24 to 48 hours later. These changes continued through 96 hours until the majority of cells were affected, causing them to be released from the glass. Subpassages of infected tissue fluid regularly produced cytopathogenic changes in the tissue-culture cells of beef embryo kidney, testicle, and lung but never in HeLa cells, KB cells, L cells, or chick fibroblasts. A total of 37 serial transfers have been made in beef kidney with the Colorado isolate, and the cytopathogenic effect has occurred each time. Control material consisting of pooled nasal washings from normal animals produced no cytopathogenic effects, despite repeated serial passage. The virus has also been isolated from turbinate and tracheal scrapings and from the tissue of the larnyx but not from lung, liver, or spleen.

Titrations of the cytopathogenic agent have been made in tubes of embryonic bovine kidney cells by preparing decimal dilutions and inoculating 8 tubes per dilution. Replicate titrations show a $TCID_{50}$ of between $10^{5.5}$ and $10^{6.5}$/ml.

In two experiments cattle having no previous history of exposure to IBR were inoculated with infected tissue-culture material from the 4th, 7th, and 15th passages on bovine embryo kidney cells. The first experiment involved six calves inoculated intranasally with 4th-passage infectious or control tissue-culture fluid. Two animals were given California virus, two Colorado virus, and two normal nasal washings. The four animals that received the infected tissue-culture material developed pyrexia accompanied by nasal discharge, anorexia, dyspnea, and lassitude 3 to 5 days after inoculation—all characteristic signs of the illness described by McKercher *et al.* (*4*) and Jensen *et al.* (*6*) for the natural disease. The two animals given the control fluid showed no deviation from normal.

The second experiment involved two calves inoculated intranasally with infectious fluid from the 7th passage, and two with material from the 15th passage of the Colorado virus. Four additional calves were inoculated with the original nasal washings from which the tissue-culture virus was isolated. The clinical response of all of these animals was similar in every respect to that described for the animals in the first experiment.

Both preinfection and convalescent serums from experimentally and naturally infected cattle were tested for specific neutralizing antibodies against the isolates from both California beef and dairy cattle and Colorado beef cattle. Serial twofold dilutions of serum were mixed with approximately 1000 TC_{50} units of virus and held at room temperature for 2 hours. The mixtures were then inoculated into beef embryo kidney cell cultures, 8 tubes per dilution. In all cases a 1–10 dilution of convalescent serums from both naturally and experimentally infected cattle neutralized 1000 TC_{50} units of virus as indicated by the absence of cytopathogenic changes. The preinfection serums failed to prevent these changes.

There was no evidence to indicate that the virus isolates, of either source, were different antigenically. Convalescent California serum neutralized Colorado virus to the same degree as it did the homologous virus. These results are in keeping with those of cross-neutralization tests in cattle by McKercher *et al.* (*4, 7*).

Attempts to incriminate other microorganisms as the potential etiological agent from both infected nasal washings and infected tissue-culture fluids have proved uniformly unsuccessful. The infected tissue-culture fluids used in these studies have been cultured and found to be bacteriologically sterile. In addition, a careful search of infected tissue-culture fluids has failed to reveal pleuropneumonialike organisms (PPLO) when cultured on standard PPLO media by the method of Adler *et al.* (*8*).

The etiological agent of IBR will pass through a fine sintered glass filter and will survive storage at –70°C for at least 7 months and at 37°C for at least 96 hours (*9*).

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Identification of Protein Disulfide Reductase as a Cellular Division Enzyme in Yeasts

Previous work in this laboratory (*1*) has shown that divisionless mutant 806 of *Candida albicans* is genetically blocked at a reductive reaction in such a manner that metabolically generated hydrogen is "spilled over" in quantity, during growth, for nonspecific reductions. (Added dyes, and tetrazolium compounds, with redox potentials as low as $E_0' = -0.150$ v are readily reduced by growing cells of the mutant but not by the parent strain). This "waste" of reductive capacity by the mutant is not at the expense of demands of hydrogen acceptors participating in syntheses essential for growth, since mutant and normal strains synthesize cell mass at approximately the same rate; nor is it at the expense of respiration, since the mutant reduces oxygen even more rapidly than does the normal strain (*2*).

It is possible environmentally to induce filamentation and dye reduction in the normal strain by the incorporation of a powerful metal-chelating agent in the culture medium. Based on these facts, a biochemical lesion underlying the morphological alteration has been postulated to involve a flavoprotein locus, which has been converted to a diaphorase (causing nonspecific dye reductions) in the filamentous form, but which is presumably a metallo-flavoprotein in the normal strain and catalyzes the reduction of some hydrogen acceptor participating in cellular division.

The existence of a protein (containing 2.1 percent sulfur) bound to the mannan component of the cell wall of baker's yeast has recently been demonstrated (*3*). Enzymatic reduction of disulfide linkages in this protein was achieved by the use of cell-free particulate preparations from baker's yeast (*4*). Polysaccharide-protein complexes (containing about 2 percent sulfur) have also been found in the clean cell walls of both normal and filamentous strains of the yeast *C. albicans* (*5*). We wish to report that mitochondrial particulates obtained from the normal strain of *C. albicans* show powerful protein disulfide reductase activity on cell-wall protein, whereas similar preparations from the divisionless (filamentous) mutant possess such activity only to a very slight extent. The data presented in this paper permit identification of the "hydrogen acceptor participating in division" as the disulfide bond of the cell-wall mannan-protein component. Furthermore, the protein disulfide reductase that catalyzes this reduction may be termed a "division enzyme" and is the first such catalyst to be identified.

Normal strain 582 of *C. albicans* (ATCC No. 10261) and filamentous mutant strain 806 (ATCC No. 10259) were grown in a glucose, ammonium sulfate, biotin, salts medium with continuous agitation at 28°C for 48 hours. Batches of 20 lit were grown in 2-lit flasks (0.5 lit per flask) on a large reciprocating shaker to obtain sufficient cells for preparation of isolated clean cells walls by the methods previously described (*3*). Batches of 4 lit were grown in 250-ml flasks (100 ml per flask) on a rotatory shaker to obtain cells from which mitochondrial particulates were isolated, as was previously described (*4*). Mannan-protein was solubilized from isolated clean cell walls (*3*), and sulfhydryl groups of the protein were oxidized with ferricyanide. The resulting oxidized cell-wall protein was incubated with active or boiled mitochondria isolated from both strains of yeast (*4*). After incubation, the reaction mixture was centrifuged at high speed to remove particulates. The —SH content of the protein in solution was assayed spectrophotometrically by mercaptide formation with *p*-chloromercuribenzoate by the method of Boyer (*6*).

As is shown in Table 1, mitochondrial particulates isolated from the normal strain of *C. albicans* exhibit vigorous protein disulfide reductase activity against the cell-wall protein obtained from this strain and from the mutant. In contrast, the mutant exhibits only slight enzymatic reduction of —S—S— linkages of its own cell-wall protein. The relative activities, per unit density of mitochondrial suspension, for normal versus mutant strain were 24.4 to 1.0. Whereas mitochondria from the normal strain vigorously reduced disulfide of the mutant cell wall (7.3 times faster than the mutant itself), mitochondria from the mutant strain were completely lacking in ability to reduce —S—S— of the normal cell wall. Enzymatic reduction of disulfide covalent bonds in mannan-pseudokeratin, a major structural component of the cell wall of yeasts, is thus seen to be a reaction essential for cellular division of yeasts.

It is not possible here to consider the physical consequences of formation and breakage of covalent bonds between the molecular fibrils that make up the fabrics that constitute the cell wall of yeasts. However, it seems important to mention two aspects of this attempt to analyze the molecular bases of this area of cellular morphology. Examination of individual frames of time-lapse photographs (darkfield microscope) of budding yeast (*7*) reveals, in a most striking manner, that a bud-initial arises as a "blowout" from the mother cell. Localized enzymatic reduction of protein disulfide may operate at this stage of the division process to convert a portion of

Table 1. Protein disulfide reductase activity in normal and divisionless strains of *Candida albicans*.

Reaction system*	Mercaptide formation† (optical density at 255 mμ)	
	Enzyme system from normal yeast	*Enzyme system from divisionless mutant*
Oxidized cell-wall protein from normal yeast (1.156) + Mitochondrial particulates (0.520)	1.676	1.946
Cell-wall protein + particulates‡	3.800	1.880
Oxidized cell-wall protein from filamentous mutant (0.766) + Mitochondrial particulates (0.520)	1.286	1.556
Cell-wall protein + particulates‡	1.920	1.730

* The components indicated were added to the following basal mixture and incubated at 37°C for 2 hours: sodium succinate, 10 mg; ethanol, 4.5 mg; liver coenzyme concentrate (Armour) 50 μg; and 0.02*M* phosphate buffer, *p*H 7.0; reaction volume 3.5 ml. Where indicated, mannan-protein from 12 mg cell wall, solubilized as described (*3*), and 0.5 ml of mitochondrial particulate suspension (in 8.5 percent sucrose) were added.

† For determination of sulfhydryl content of protein, 2.0-ml samples were taken from the incubated mixtures and added to 1.0 ml of 0.3M acetate buffer, *p*H 4.6, and 0.5 ml 1.2×10^{-4}M *p*-chloromercuribenzoate (assayed spectrophotometrically at 234 mμ in 0.1M acetate buffer, *p*H 4.6, according to the method of Boyer, *6*). Mercaptide formation was allowed to proceed for 90 minutes at 37°C and then determined at 255 mμ.

‡ Values for controls using heated mitochondria were not greater than the sum of the constituents.