TABLE III. Results of Screening Sera for Antibody Against Parainfluenza Types 1 and 2 (HI Only).

| Species | Para 1 | Para 2 |
|---|---|---|
| Sheep | 0/5 | 0/9 |
| Swine | 0/5 | 0/9 |
| Dog | 0/15 | 4/10 (1:20) |
| Cat | N.T. | 1/9 (1:20) |

positives and the titers of the positives, some remained positive on HI.

Low titers to parainfluenza 2 were found in some dogs and cats (Table III) when tested by HI. All of these sera tested against parainfluenza 1 were negative.

*Conclusion.* Parainfluenza Type 3 neutralizing antibodies were found to be present in 81% of sheep sera tested. This points to this virus as probably being a common virus infection of sheep. The antibody appeared more closely related to the bovine strain of virus than to the human strain. Several sera from pigs, dogs and cats inhibited hemagglutination by parainfluenza 3, but failed to neutralize the virus in tissue cultures. In this series of sera examined, parainfluenza 1 and 2 did not appear to play a major role in sheep, swine, dog or cat infections.

NOTE ADDED IN PROOF: Since submission of this article, Woods *et al*(10) have confirmed the finding of HI antibodies to parainfluenza 3 in sheep and swine sera.

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## Amino Acids of Colon and Rectum. Possible Involvement of Diaminopimelic Acid of Intestinal Bacteria in Antigenicity of Ulcerative Colitis Colon.* (29952)

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Certain aspects of ulcerative colitis suggest the possibility of an immunologic process in the pathogenesis or course of the disease(1, 2). Evidence also has been adduced that ulcerative colitis colon may be more complex antigenically than normal bowel(3). The possible antigenicity of colon may be related to biologic or biochemical changes; as a consequence of an earlier, unrecognized infection (4). In ulcerative colitis, antigenicity of colon theoretically also might result secondarily from an alteration in its composition by the disease. Although infection has not been demonstrated as a primary mechanism in ulcerative colitis, the possible involvement of microorganisms, viruses or their constituents in the pathogenesis of the disease has not been excluded. It seemed desirable, therefore, to obtain information on (a) possible differences in the qualitative amino acid composition of normal and ulcerative colitis tissue, and (b) the possible role of some metabolic products of spherophorus necrophorus, an organism frequently present in the bowel of

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ulcerative colitis, in the antigenicity of colon mucosa.

*Amino acid survey. Methods and results.* Biopsies of the mucosa and submucosa of the rectum or of colon resected at operation were utilized from patients with ulcerative colitis, individuals without disease or patients with assorted gastrointestinal lesions. Standard aerobic and anaerobic cultures of the thoroughly washed specimens were negative. The tissues were subjected to acid hydrolysis with 20% hydrochloric acid (glass distilled). If not used immediately, the material was kept frozen at minus 10°C. The hydrolysis was performed in sealed evaculated glass tubes for 6 hours at 110°C (no proteins stainable with bromphenol blue remain after this treatment). After cooling, the hydrolysate was evaporated to dryness with a current of air over pellets of sodium hydroxide; the dry residue was dissolved in distilled water and evaporated; this procedure was repeated twice. The dry residue, free of hydrochloric acid, was dissolved in 10% isopropanol in distilled water (containing several drops of N/10 HCl per 100 ml), to yield 20-50 mg of tissue per ml. Chromatographic separation was performed with one- and two-dimensional ascending methods on paper(5-8). For the one-dimensional chromatographic separation, Munktell filter paper (#20/150) proved suitable. The solvent was: n-butanol:glacial acetic acid:distilled water (proportions 250:60:250)(9). The top butanol layer (N/1 acidity) was used for the chromatographic separation. The aqueous layer was placed in beakers in the chromatographic jar for saturation of the atmosphere.

Samples of hydrolysates were placed with a platinum wire loop on the same spot 5 times, each after the previous drop had dried completely, on sheets 50 cm in length, at a distance of 7 cm from the lower edge of the paper. For some amino acids, present in very low concentrations in the tissue hydrolysate, 10 drops were superimposed on the same spot; or a more concentrated hydrolysate solution was used. After one run for 22 hours at room temperature, the dried (in hood, not heated) chromatogram was rechromatographed under the same conditions for an additional 22 hours, then dried, and sprayed with a 0.25% solution of ninhydrin in acetone. Sprayed sheets were left overnight in the dark; or after drying for 30 minutes in the dark, they were heated for 2 to 3 minutes in an oven, at approximately 100°C, over water vapor. The different color nuances of the amino acids are more definite on unheated sheets.

Amino acid standards, 1 mg/ml in 10% isopropanol (with hydrochloric acid), were employed as markers on the same paper, facilitating location of the positions of the amino acids in the hydrolysate by comparing the Rf. The amino acids were identified by the following procedure: to aliquots of the hydrolysate solutions, equal amounts of solutions of amino acid standards were added, one standard to each aliquot, and chromatographed at the same conditions. If the amino acid in question appeared on the chromatogram as one single spot with the standard amino acid, they were regarded as identical. By this procedure, the following amino acids could be identified in the hydrolysates of normal and of ulcerative colitis colon tissue: cystine, lysine, arginine, glycine, glutamic acid, alpha-alanine, proline, tyrosine, valine and leucine; histidine and serine unaccountably were not found by this procedure.

The 2-dimensional chromatographic separations were performed with 2 pairs of solvents on Whatman #1 paper. The solvent combinations recommended by Hausmann (10) could be utilized as follows: Solvent A) secondary butanol:3% $NH_4OH$ (proportions 25:10); Solvent B) secondary butanol:90% formic acid:water (proportions 30:6:4). The first ascending run in Solvent A continued for 70 hours at room temperature. The chromatograms were dried and chromatographed again in the same solvent until the same front was reached The separation in the second direction was made with Solvent B for approximately 50 hours. The chromatograms were dried and sprayed with ninhydrin. Three additional amino acids were identified thereby: aspartic acid, methionine and phenylalanine. Isoleucine could be separated from leucine and identified with the solvent of Wieland(11) on Whatman #1 paper:n-butanone:pyridine:$H_2O$ (proportions 70:15:15), with three consecutive runs for 19 to 22

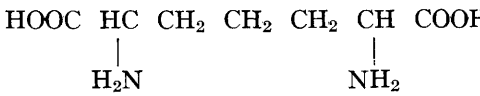
hours each. Leucine moved ahead of isoleucine for a distance of 2.5-3.0 cm, thus enabling differentiation of these amino acids.

Hydroxyproline could be identified on a chromatogram on Whatman #1 or #3 paper, with the solvent consisting of 80% phenol (previously purified), containing 0.1% cupron in the presence of 0.3% ammonia. The spots were stained by the dye isatin and Ehrlich's p-dimethyl-amino-benzaldehyde.

The identified 15 amino acids were found in hydrolysates of both normal and ulcerative colitis colon tissue. Tryptophane could not be detected in these tissues, hydrolyzed with 0.38 M barium hydroxide at 110°C for 6 hours. Chromatographic separations in the following solvents also were made: (1) aqueous phenol or phenol crystals equilibrated with an equal volume of phosphate buffer of pH 12.0, (resulting pH of the phenol phase, 8.0); this solvent was used with and without 0.3% ammonia; (2) s-collidine:$H_2O$ (proportions 125:44); (3) s-collidine:2-6 lutidine:$H_2O$:diethylamine (proportions 200:200:200:9); (4) 0.5% urea:acetone:glacial acetic acid (proportions 40:60:8:5); (5) Redfield(12) pair of solvents: (a) methanol:$H_2O$:pyridine (proportions 80:20:4), (b) tertiary butanol:n-butanone:$H_2O$:diethylamine (proportions 40:40:20:4).† None of these procedures demonstrated additional amino acids.

However, the 2-dimensional chromatographic separation with an additional pair of solvents revealed, in hydrolysates of ulcerative colitis tissue, an amino acid spot, not present in the hydrolysates of normal colon tissue (concentration 500 mg tissue/ml). The first run was on Whatman #1 in 80% phenol containing 0.1% cupron, in the presence of 0.3% ammonia. The sheet was dried in a hood until complete evaporation of the phenol (2-3 days); then twice chromatographed (each for 17 hours) in the solvent:n-butanol:90% formic acid:$H_2O$ (proportions 360:55:415); the acidity of the butanol phase was 1.4 N. Dried sheets were sprayed with 0.25% ninhydrin in acetone, made 1.4 N with 90% formic acid (about 8 ml/100 of 90% formic acid). Ahead of the aspartic acid spot and in line with glutamic acid spot, a ninhydrin-stained spot (X) was located, corresponding to the position of alpha-epsilon diaminopimelic acid. This amino acid was observed in 6 of 22 samples of colon and rectal tissue from patients with ulcerative colitis. It was not demonstrated in similar studies of normal gastric tissue, the small intestine from a patient with regional enteritis, in uninvolved sections of the colon from an individual with ulcerative colitis, or in 25 samples of rectal biopsy tissue from normal persons. Apparently, DAP in the colon does not originate from a simple mechanical mixture of colon tissue with bacteria; whether or not the DAP in ulcerative colitis colon tissue is attributable to penetration, incorporation or adherence is not known at present.

Alpha-epsilon diaminopimelic acid (DAP) is 1.5 diaminopentane-1.5 dicarboxylic acid.



This amino acid has been found in the lipopolysaccharide and protein fractions of human virulent and nonvirulent and bovine strains of *mycobacterium tuberculosis*; in *B. proteus, Corynecbacterium diphtheria, Vibrio cholera, B. cereus,* in some strains of *E. coli,* and spherophorus necrophorus, among many microorganisms, in rumen contents from sheep, and in soil(13-20). The presence of alpha-epsilon diaminopimelic acid in the tissue hydrolysates suggests the possibility that it may contribute to the antigenic potential of the colon in ulcerative colitis; a similar role might be considered also for other constituents of intestinal microorganisms.

*Antigenicity of spherophorus necrophorus, diaminopimelic acid, and DAP containing bacterial or protein products.* To test this possibility, model experiments were conducted with spherophorus necrophorus and diaminopimelic acid. Spherophorus necrophorus (Syn. *Bacillus necrophorus*) is a necrosis-producing, pleomorphic, gram-negative microorganism that has been isolated from the colon and from regional lymph nodes of patients with ulcerative colitis(21). Suspensions of a human strain of spherophorus

† After use of the solvents 1, 3, 4 and 5, a suitable ninhydrin spray should be utilized.

necrophorus from cultures, fresh or frozen and thawed, or macerated for 7 days in distilled water, did not react antigenically with ulcerative colitis serum in agar gel diffusion tests. When the bacterial suspension was disintegrated by ultrasonic oscillation in water, the supernate, but not the precipitated cell debris, manifested antigenic properties *in vitro* (as denoted by agar gel precipitation with colitis serum). Apparently the intact bacterial cell is unable to react with ulcerative colitis "antibodies"; and only disruption of the cell or cell wall releases the antigenic component.

Since lysozyme has been found in increased amounts in the feces of patients with ulcerative colitis(22), the possible effect of lysozyme upon spherophorus necrophorus was investigated. Five-tenths ml of a thick, centrifuged suspension of spherophorus necrophorus culture was suspended in 1.5 ml of .9% sodium chloride; another 0.5 ml of the bacterial suspension was suspended in 1.5 ml of 0.03 M phosphate buffer solution of pH 7.0. Both samples (with toluene) were incubated with lysozyme overnight at 37°C; then heated for 15 minutes at 80°C to inactivate the enzyme, and centrifuged. The supernates were used as antigens in agar gel diffusion tests. The saline sample was inactive; but the buffered sample reacted as an antigen with ulcerative colitis serum. In the saline medium, the lysozyme had no mucolytic effect on the bacterial cell, but in the buffered medium at pH 7.0, the lysozyme digested the wall of the bacterial cell and presumably released the antigenic component. A lysozyme digest of a strain of *Bacillus proteus* isolated from human urine reacted in the same manner as the spherophorus necrophorus digest; but the digest of *E. coli* (isolated from human feces) did not react, or demonstrated nonspecific precipitation. *Bacillus proteus* contains DAP, whereas this amino acid is present in some, but not in all, strains of *E. coli*; presumably the *E. coli* strain employed did not contain DAP.

A further series of experiments utilized the globulins from pooled human sera or human gamma globulin; hydrolyzed partially (with pepsin at pH 1.5-2.0) to tetra or pentapeptides, and subsequently incubated with pepsin at pH 4.0; together with the amino acids: glutamic acid (another constituent of the cell wall of certain microorganisms), DAP, and gamma amino butyric acid; or with a lysozyme digest of spherophorus necrophorus. Solutions of the precipitated complexes were employed as antigens in agar gel diffusion studies with serum from patients with ulcerative colitis and with serum from normal persons. The glutamic acid preparation was inactive. The complex containing gamma-aminobutyric acid and the complex containing the lysozyme digest of spherophorus necrophorus occasionally demonstrated a one-line precipitation with ulcerative colitis serum; and did not react with normal serum. The preparation containing diaminopimelic acid reacted positively with all ulcerative colitis sera tested, and not with normal serum. Unhydrolyzed globulin solutions, incubated under the same conditions with diaminopilemic acid, did not react. These experiments suggest that, of the several constituents in the cell wall of some microorganisms, diaminopimelic acid may be more reactive antigenically; perhaps in common with ulcerative colitis colon mucosa. Gamma-aminobutyric acid was included in the experiments because of its presence in guinea pig intestine(23) and in some animal and bacterial cells, although this amino acid was not found in human colon tissue.

Lysozyme-treated colon tissue extracts were not active as antigens in either agar gel diffusion or hemagglutination tests; and the addition of diaminopimelic acid did not enhance their antigenic activity. Pepsin-hydrolized (pH 4.2) colon tissue from individuals with ulcerative colitis occasionally reacted as an antigen with ulcerative colitis serum. Incubation of diaminopimelic acid with pepsin hydrolysates of ulcerative colitis colon tissue, as described above, produced an antigen reacting with most ulcerative colitis sera. These observations thus suggest that alpha-epsilon diaminopimelic acid, and perhaps other components similar in antigenic properties, released by metabolic activity from certain intestinal bacteria, may contribute to the immunological potential of ulcerative colitis colon.

*Summary and conclusions.* In addition to

15 amino acids identified in normal and in ulcerative colitis colon mucosa, an amino acid, alpha-epsilon diaminopimelic acid, was demonstrated in some samples of ulcerative colitis rectal and colon tissue. This finding may be related to the presence of metabolic products of gram-negative intestinal bacteria. The supernates of disintegrated suspensions of spherophorus necrophorus and lysozyme digests of spherophorus necrophorus or *B. proteus* contain a component reacting similarly to colon mucosa with ulcerative colitis serum, as demonstrated by positive hemagglutination and agar gel diffusion reactions. Incubation of alpha-epsilon diaminopimelic acid and gamma-aminobutyric acid, with pepsin hydrolysates of globulin and colon tissue, yielded complexes also reacting as antigens with ulcerative colitis serum.

1. Fifteen amino acids were identified in hydrolysates of normal and ulcerative colitis rectal and colon mucosa and submucosa. 2. Rectal and colon tissue from patients with ulcerative colitis may contain an additional amino acid; possibly alpha-epsilon diaminopimelic acid, not yet demonstrated in normal colon tissue. 3. Alpha-epsilon diaminopimelic acid, a component of the cell wall of intestinal microorganisms, and perhaps other metabolic products of intestinal microorganisms, may contribute to the antigenic potential of ulcerative colitis colon.

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## Influence of Glucose Cycloacetoacetate and Its Hydrolysed Product on Capillary Resistance in Scorbutic Guinea Pigs. (29953)

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The characteristic increase in capillary fragility is one of the important manifestations of scurvy(1-5) which might implicate the internal haemorrhages in advanced cases of the disease. When ascorbic acid is administered to scorbutic animals capillary resistance is restored(4,6). Reppert *et al*(7) have suggested that ascorbic acid exerts this effect by inhibiting hyaluronidase thus causing increased accumulation of hyaluronic acid which is the cementing substance of endothelial lining. Glucose cycloacetoacetate (GCA), the glucose derivative obtained by condensation of glucose and ethyl acetoace-