tion of 3-HOA by patients with tumors of the bladder.

Summary. A method is presented for the stabilization of urinary 3-hydroxyanthranilic acid (3-HOA) to enable the acquisition of more reliable data concerning the urinary excretion of 3-HOA by patients with tumors of the urinary bladder. The data show that significant amounts of 3-HOA can decompose in urine samples of some tumor patients if such samples are allowed to incubate at 37°C for 6 hours, and that the presence of high levels of L-ascorbate in such samples inhibits this decomposition.

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Differences in Colonic Staining with Technical Variations in Fluorescent Antibody Conjugation.* (32548)

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Immunologic mechanisms have been implicated in the course of ulcerative colitis(1-3). However, there have been conflicting reports regarding the demonstration of circulating antibodies to colonic mucosa in patients with this disease(4,5). Application of immunohistochemical techniques has led to emphasis on the cytoplasm of the colonic mucosal epithelial cell as the site of an antigen to which serum globulins from certain ulcerative colitis patients bind(6-9). On the other hand, some investigators have been unable to demonstrate a specific colonic mucosal epithelial antigen, using sera from ulcerative colitis patients (2,4,5). To understand better the possible reasons for these discrepancies, the present studies were designed to evaluate several variables involved in the direct fluorescent antibody technique and to demonstrate factors which may result in nonimmunospecific staining of the colonic mucosa. Careful attention was directed to the uniformity of labeling, fluorescein:protein ratios and final protein concentrations of globulin conjugates.

Materials and methods. The fluorescent antibody conjugation and staining methods used were essentially those of Goldstein *et al* (10,11), as previously utilized in this laboratory(5,12). The experimental design is shown in Fig. 1. Globulin fractions of whole serum were obtained by precipitation with cold saturated ammonium sulfate and gel filtration with a Sephadex G-50 column. Pressure dialysis was employed to adjust globulin fractions to a concentration of 1.25% before the conjugation procedure, and two 8 ml (100 mg) globulin preparations of a given serum separately were conjugated with fluorescein. One 100 mg globulin preparation was con-

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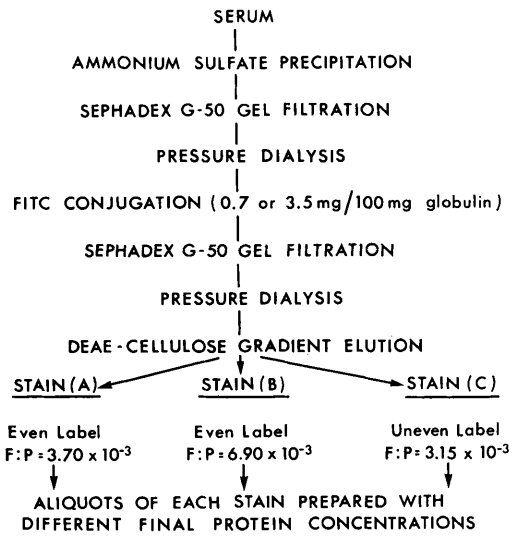

FIG. 1. Experimental design. Globulin conjugates from a given serum were prepared to differ as to uniformity of labeling, F:P ratios or final protein concentrations.

jugated using 0.7 mg crystalline fluorescein isothiocyanate (Baltimore Biological Laboratories); while the second 100 mg globulin aliquot was conjugated in the same way, except that 3.5 mg crystalline fluorescein isothiocyanate was employed. Sephadex G-50 column chromatography then was utilized to remove excess fluorescein from the conjugated proteins. After reconcentration the conjugates were subjected to DEAE-cellulose chromatography with gradient elution. The final protein concentrations and fluorescein: protein (F:P) ratios of the conjugated protein fractions were determined(13). As described below, globulin conjugates from a given serum were prepared to differ as to: a) uniformity of labeling; b) F:P ratios; or c) final protein concentrations.

Three direct immunohistochemical stains were prepared from these conjugated protein fractions. One stain (A) was made by pooling fractions which had a F:P ratio of approximately 3.5×10^{-3} and resulted in a final F:P ratio of 3.70×10^{-3}. Stain (B) was made by pooling the conjugated protein fractions with F:P ratios in excess of 3.5 and had a final F:P ratio of 6.90×10^{-3}. The third stain (C) consisted of pooled heterogeneous conjugated fractions which had F:P ratios outside the $2.0\text{-}3.5 \times 10^{-3}$ range, but with a composite final F:P ratio of 3.15×10^{-3}.

Three different colonic tissues were employed. The first tissue was obtained from the colon of an ulcerative colitis patient (M.R.) at the time of total colectomy; the same patient provided the serum to make up the above 3 fluorescein-conjugated globulin stains. Another tissue was obtained at the colectomy of a different ulcerative colitis patient (H.H.). The third colonic tissue was a grossly normal section from a patient (D.M.) who underwent subtotal colectomy for adenocarcinoma and diverticulitis coli.

Results. The first staining procedure consisted of exposing tissue sections from all 3 patients with each of the stains following excessive concentration by pressure dialysis. Thus, stains (A), (B) and (C) had final protein concentrations of 5.18, 3.49, and 8.75 mg/ml, respectively. As shown in Table I, the application of these 3 concentrated stains to the 3 tissue specimens resulted in diffuse nonspecific mucosal staining in all instances.

TABLE I. Direct Immunohistochemical Studies of Colonic Tissues with Stains Prepared from the Serum of an Ulcerative Colitis Patient.

		Evenly labeled stain (A)			Heavily labeled stain (B)			Unevenly labeled stain (C)		
		Protein concentration (mg/ml)								
Colonic tissue	Patient	5.18	1.50	0.50	3.49	1.50	0.50	8.75	1.50	0.50
1. Autologous ulcerative colitis	M.R.	NS*	+†	+	NS	NS	NS	NS	NS	NS
2. Homologous ulcerative colitis	H.H.	NS	+	+	NS	NS	NS	NS	NS	NS
3. Grossly normal (diverticulitis)	D.M.	NS	0‡	0	NS	NS	NS	NS	NS	NS

* Nonspecific fluorescence. See text.
† Immunospecific fluorescence of the cytoplasm of leukocytes. See text.
‡ No immunofluorescence.

The mucosal epithelial cells, connective tissue and leukocytes of the inflammatory cell infiltrate showed a characteristic apple-green fluorescence. This staining was not inhibited by prior application of an unlabeled globulin aliquot from the same serum (M.R.).

In the next staining procedure all 3 stains were diluted to an optimal protein concentration of 1.50 mg/ml. Stain (B) gave diffuse nonspecific staining of mucosal epithelium, connective tissue and leukocytes (Table I). The nonimmunospecific staining was not quite as prominent as in the previous experiment. Stain (C) also gave nonimmunospecific staining. On the other hand, the tissue staining with the homogeneously labeled stain (A) with a F:P ratio of 3.70×10^{-3} and a final protein concentration of 1.50 mg/ml resulted in virtually no nonspecific staining. The autologous ulcerative colitis tissue (M.R.) showed staining of the cytoplasm of some of the lymphocytes and plasma cells of the mucosa and submucosa which could be inhibited by pre-incubation with the unlabeled globulin aliquot. The cytoplasmic fluorescence was homogeneous and sometimes extended beyond the borders of these round cells. The polymorphonuclear leukocytes and eosinophils showed no immunospecific fluorescence. In no instance was there staining of the epithelial cells of colonic mucosal glands. Stain (A) gave similar results with the homologous ulcerative colitis tissue (H.H.).

The third staining procedure utilized the 3 stains each adjusted to a protein concentration of 0.50 mg/ml. Using stain (B), even at this lower protein concentration, the mucosal epithelial cells and the leukocytes of the infiltrate of the autologous and homologous ulcerative colitis tissue, as well as the mucosal glands of the normal colonic specimen, all demonstrated nonimmunospecific staining (Table I). Stain (A) gave similar results at a protein concentration of 0.50 mg/ml, as had been obtained at a concentration of 1.50 mg/ml, although the reduced protein concentration did cause a decrease in the intensity of the fluorescence. The same immunospecific staining of the mononuclear cells was seen in both the autologous and homologous ulcerative colitis tissues. Again no specific staining of the mucosal epithelial cells was observed. The unevenly labeled stain (C) caused nonimmunospecific staining in all 3 tissues.

A supplementary staining procedure employed the same 3 stains at the 0.50 mg/ml concentration, but the tissues were prepared with the paraffin embedding technique described by Sainte-Marie(14). In addition to the 3 previously used tissues, a section of normal colon was employed which was obtained from a patient (D.C.) who had a resection for cancer of the colon. In this technique, cold ethanol was used as a fixative, the tissue was dehydrated in absolute alcohol, passed through 3 xylene baths, and embedded in paraffin. Following sectioning and deparaffinization, the tissues were stained with the standard immunofluorescent procedure. As shown in Table II, the results were the same as those obtained with the cryostat-cut sections. Both of the normal colonic tissues showed the same staining pattern when the 3 stains were applied. There was no problem in differentiating the apple-green color of fluorescein isothiocyanate from the bluish-white autofluorescence of colonic mucosa. The evenly labeled stain (A) gave no staining of the epithelial cells or the leukocytes of the normal colonic tissues. Stains (B) and (C) gave nonimmunospecific staining of the epi-

TABLE II. Direct Immunohistochemical Studies of Colonic Tissues Using the Paraffin Embedding Technique of Sainte-Marie and Stains with Final Protein Concentrations of 0.50 mg/ml.

Colonic tissue	Patient	Evenly labeled stain (A)	Heavily labeled stain (B)	Unevenly labeled stain (C)
1. Autologous ulcerative colitis	M.R.	+*	NS†	NS
2. Homologous ulcerative colitis	H.H.	+	NS	NS
3. Grossly normal (diverticulitis)	D.M.	0‡	NS	NS
4. Normal	D.C.	0	NS	NS

* Immunospecific fluorescence of the cytoplasm of leukocytes. See text.
† Nonspecific fluorescence. See text.
‡ No immunofluorescence.

thelium, connective tissue and occasionally of mononuclear cells.

Discussion. Immunologically nonspecific staining of colonic mucosal epithelial cells and other colonic components has been observed with uneven labeling, F:P ratios exceeding 3.7×10^{-3}, or with final protein concentrations greater than 1.5 mg/ml. Using optimum conjugation conditions, no immunospecific staining of autologous or normal colonic mucosal glands was demonstrated. Since nonimmunospecific staining of colonic glands *was* induced, technical factors are considered critical to the reproducibility and interpretation of immunofluorescent results in studies of colonic tissues.

In the past the use of a coupling ratio of 50 mg fluorescein isothiocyanate per gram of protein was advocated(15,16) but, as a result of the work of Goldstein and coworkers (10,11), such a technique was found to produce conjugates which were capable of giving nonimmunospecific staining. They found that DEAE-cellulose gradient elution after using a coupling ratio of 6-8 mg fluorescein isothiocyanate per gram of protein would eliminate uncoupled molecules and resultant nonspecific fluorescence. They recommended that the final protein concentration of the stain be kept under 2.0 mg/ml and that the F:P ratio be maintained between 2.0 and 3.5×10^{-3}. Others have reported that an F:P ratio of $3.8\text{-}5.5 \times 10^{-3}$ could be used(16), and in the original study of Coons and Kaplan a F:P ratio of $2.2\text{-}5.1 \times 10^{-3}$ was considered optimal(15). Our data emphasize the need to carefully control the F:P ratios and final protein concentrations, as well as to insure uniformity of labeling, in order to prevent nonspecific staining. These technical factors in the preparation of the conjugates may be among the causes for the conflicting results of various immunohistochemical studies of ulcerative colitis.

The differing reports on the demonstration of antibodies to antigens derived from colonic tissue are difficult to evaluate because often the techniques used are not described fully, or a technique has been employed which others have shown to be capable of resulting in nonimmunospecific fluorescence. Additional factors which may result in nonspecific staining include temperature, pH and/or ionic environment(17-21). It is apparent that the techniques used in preparing the stain and the tissues are important if a meaningful interpretation of immunofluorescent data is to be obtained.

It is of interest that there appeared to be immunospecific fluorescence associated with the cytoplasm of mononuclear cells, as in some of our earlier immunofluorescent experiments(5). The immunospecificity of this staining was established when inhibition occurred upon pretreatment with unlabeled autologous globulin aliquots. The source and nature of this apparent antigen were not identified. More exacting controls of the immunologic specificity of these preliminary data must await isolation and purification of the involved antigen(s)(3,22).

Summary. Several different parameters involved in preparing direct fluorescent antibody stains were studied using autologous and homologous ulcerative colitis and normal colonic tissues. Diffuse nonimmunospecific staining was observed when conjugates were labeled unevenly, had high fluorescein:protein (F:P) ratios or when final protein concentrations exceeded 1.5 mg/ml. Some apparently nonimmunospecific staining of the mucosal epithelial glands was observed, but there was no specific fluorescence at these sites. Using an evenly labeled stain with an F:P ratio of 3.70×10^{-3} and final protein concentrations of either 0.50 or 1.50 mg/ml, specific fluorescence of the cytoplasm of some mononuclear cells was observed in autologous and homologous ulcerative colitis tissues, but not in normal colonic specimens.

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Effect of Nicotinic Acid Administration on Plasma Growth Hormone Concentrations.* (32549)

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In recent years, it has been demonstrated that the secretion of human growth hormone (HGH) is markedly influenced by hypoglycemia(1), glucose(2,3) or amino acid(4) administration. In addition, other stimuli such as exercise, prolonged fasting or stress have been shown to augment plasma HGH(2,5). Similar to its various physiological effects, the secretion of HGH appears to be controlled by a variety of factors.

In this laboratory, an attempt was made in normal subjects, to induce plasma non-esterified fatty acids (NEFA) change by administration of nicotinic acid, with or without heparin injection, and to determine serial plasma HGH concentrations. This report describes our finding that marked increase of plasma HGH has occurred following the administration of nicotinic acid alone, and this increase was abolished when nicotinic acid was given in combination with heparin injection.

Materials and methods. All test subjects were healthy male students, their ages ranging from 21 to 24 years old. They were non-obese, and had no family history of diabetes mellitus. All had fasted overnight for 13-15 hours. Upon arrival at the hospital next morning, the subjects had bed rest for at least 30 minutes before starting the test. They were on bed rest and told to relax but not to sleep during the study.

Indwelling needle was inserted into the antecubital vein and slow infusion (approximately 10 drops per minute) of physiological saline was performed to prevent blood coagulation throughout the test. All injections and blood samplings were done through the indwelling needle.

Tests were divided into 3 groups as follows: (a) Nine cases received 20 ml saline at 0 and 20 minutes. (b) Five cases received 100 mg nicotinic acid in 20 ml saline at 0 and 20

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