absorption of cholesterol.

Differences in lymph free cholesterol between control and experimental groups were insignificant except for those fed cholic acid, glycocholate, and commercial taurocholate. In these groups where cholesterol absorption occurred, values for free cholesterol were greater than in the control group; this relationship has been discussed(11). The data clearly demonstrate that certain bile salts have a direct effect on intestinal absorption of cholesterol in addition to the reported effect of these substances on hepatic metabolism of cholesterol(9,10).

Summary. 1. Effect of various bile acids on intestinal absorption of dietary cholesterol has been investigated in lymph-fistula rats. 2. Cholanic, lithocholic, and deoxycholic acids, with none, one and 2 hydroxyl radicals, respectively, produced no increase in lymph cholesterol over the control group. 3. Conjugated bile salts, glycocholate and taurocholate gave comparable significant elevations in total lymph cholesterol/24 hours. 4. Cholic acid with 3 free hydroxyl groups and an unconjugated carboxyl radical was the most effective bile acid in promoting cholesterol absorption from the intestine.

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Fluorescent-Antibody Technic for Serodiagnosis of *Trypanosoma cruzi* Infection.* (25009)

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Since Coons and his colleagues first reported the immuno-specific staining of tissue with fluorescein-conjugated globulin(1), numerous applications of the technic have been found. It was shown that fluorescein-labeled antibody not only provided a means of demonstrating localized antigen in tissues, but could be used to detect specific antibody developed during the course of infection with certain micro-organisms, *e.g.*, *Toxoplasma gondii*(2) and *Treponema pallidum*(3). The present report describes a fluorescent-antibody test developed for serodiagnosis of American trypanosomiasis (Chagas' disease), and gives an improved method for purifying antibody prior to conjugation with fluorescein. The procedure was evaluated in tests with homologous and heterologous sera and the results compared with those obtained in complement fixation tests.

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Materials and methods. The indirect technic, using a labeled antihuman antibody, was

employed. *Fluorescein conjugate*: Labeled anti-antibody was prepared by conjugating the euglobulin fraction(4) of anti-human gamma globulin horse serum with fluorescein isothiocyanate† according to the method of Marshall *et al.*(5). The euglobulin fraction was precipitated from whole serum by slowly adding, with constant stirring, 9 volumes of 0.0027 N HCl to 1 volume of chilled antiserum, and incubating the mixture at 1°C for 30 minutes to assure maximum precipitation. The euglobulin precipitate was sedimented by cold temperature (1°C) centrifugation for 10 minutes at 1000 g, and reconstituted to one-fourth of the original serum volume with 0.02 M phosphate-buffered salt solution, pH 6.8; trace amounts of insoluble residue were removed by centrifuging for 10 minutes at 1000 g. For conjugation, the euglobulin solution was diluted with sodium chloride solution (0.15 M) and carbonate-bicarbonate buffer (0.5 M, pH 9) so that the final solution contained 10 mg protein/ml and 10% buffer. The required quantity of fluorescein isothiocyanate (0.05 mg dye/mg protein) then was added to the alkaline euglobulin solution and the mixture stirred with magnetic stirrer for 18 hours at 3°C. Uncoupled fluorescein was removed by dialysis in the cold against successive changes of 0.01 M phosphate-buffered salt solution, pH 7.0 (PBS). Insoluble components of the dialysant were sedimented by centrifugation and the clear, labeled antibody solution stored at 3° or –20°C. Prior to use, each lot of labeled antibody was titrated with homologous and normal sera. The highest dilution of conjugate giving brilliant fluorescence in tests with *T. cruzi* antiserum and essentially no fluorescence with normal serum was used in the diagnostic tests; in most instances, a 1:10 or 1:20 dilution satisfied these conditions. *Sera*: Since the indirect technic using labeled anti-human antibody was employed, test sera necessarily were of human origin. Sera representing *T. cruzi* infection were from well documented cases, the diagnoses having been established either by demonstrating the organism in stained blood films, or by xenodiagnosis. The syphilitic sera were from patients with *T. pallidum* infection, whose diagnoses were confirmed by the *T. pallidum* immobilization (TPI) test. Normal sera were obtained from healthy donors undergoing physical examination as candidates for appointment to U.S. Military Academy; all were negative in the TPI test. The sera were stored at –20°C. Immediately before testing, *ca* 0.2 ml of thawed serum was heated for 30 minutes in water bath at 56°C. *Test procedure*: All reactions were carried out in test tubes to prevent drying of organisms. Cultures of *T. cruzi* (Brazil strain)‡ grown on a diphasic medium(6) for 8-12 days at 23°C served as source of antigen. The organisms were freed from debris by differential centrifugation and washed once with formalinized salt solution (0.15 M sodium chloride containing 0.1% formalin) and once with PBS. A PBS suspension containing 10^7 washed trypanosomes/ml was the standardized antigen. For each test, 1 ml of antigen suspension was transferred to 13 x 100 mm test tube and centrifuged 10 minutes at 1000 g. Following decantation of supernate, 0.1 ml of test serum was added to the sedimented trypanosomes, mixed, and incubated for 18 hours at 3°C. The organisms then were washed twice with PBS, sedimented, and treated with 0.1 ml of diluted fluorescein-labeled anti-human antibody. After incubation for 30 minutes at 3°C, the trypanosomes again were washed and finally suspended in 0.1 ml of PBS. A small drop (*ca* 0.02 ml) of test mixture was combined with equal volume of 90% buffered glycerine on a micro-slide, surmounted with a glass coverslip, and examined under fluorescent microscope. Degree of fluorescence was estimated according to criteria similar to those of Goldman(2), and results classified as: reaction (4+, 3+, 2+), weak reaction (1+), or no reaction (±, –). *Fluorescence microscope*: A standard monocular microscope fitted with first-surface mirror, reflecting dark field condenser, and a Corning #3496 yellow filter in the ocular was employed. The ultra-

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TABLE I. Relative Sensitivity and Specificity of Fluorescent-Antibody and Complement Fixation Tests for *T. cruzi* Infection.

	Persons exhibiting the given serologic result* in							
	Fluorescent-antibody tests				Complement fixation tests†			
Diagnostic status	No. tested	Reaction	Weak reaction	No reaction	No. tested	Reaction	Weak reaction	No reaction
Chagas' disease	40	39	1		40	28	9	3
Syphilis	20		2	18	49			49
Healthy	70		3	67	100			100

* Classified as reaction (4+, 3+, 2+), weak reaction (1+), or no reaction (±, —).

† Tests with purified protein antigen fraction derived from *T. cruzi*.

violet light was supplied by AO-Spencer fluorescence illuminator #633 using Osram HBO-200 mercury arc lamp; a Corning #5850 blue filter was used for transmitting light in the near ultraviolet wavelength.

Results. Sensitivity and specificity of the fluorescent-antibody reaction was evaluated in tests with homologous, syphilitic, and normal sera, and the results compared with those obtained in complement fixation tests using a purified protein antigen derived from *T. cruzi* (7). The findings are summarized in Table I. Tests with 40 sera obtained from well documented cases of *T. cruzi* infection illustrate the relative sensitivity of 2 procedures. All sera reacted serologically in the fluorescent-antibody test, whereas only 37 of 40 specimens gave reactions in the complement fixation test. Results obtained with syphilitic and normal sera, on the other hand, provided an index of relative specificity of test procedures. Two of 20 syphilitic and 3 of 70 normal sera gave weak reactions in the fluorescent-antibody tests. In contrast, the complement fixation reactions were uniformly negative.

Discussion. The present report is comprised of studies on 2 discrete but related problems. One deals with development of a serologically specific fluorescent-antibody procedure, the other with an improved method for purifying antibody to be labeled. It was found early in the present studies that dried smears of *T. cruzi* were unsatisfactory for fluorescent-antibody tests, although other investigators successfully have used fixed smears of micro-organisms for a variety of fluorescent-antibody procedures. With *T. cruzi,* dried unfixed smears as well as those treated with various concentrations of methanol, ethanol, formalin, or with heat regularly gave nonspecific reactions with normal sera. The untoward reactions, moreover, were not affected by preliminary treatment of smears with crystalline bovine serum albumin solution. Efforts to improve the specificity of staining by using the "combined" or "sequence" inhibition (blocking) technics employed by Goldman in studies on toxoplasmosis(2) likewise were unsuccessful. Continued investigations revealed that non-specific staining was associated with desiccation of the trypanosomes. Solution of the problem therefore was found in tests conducted in test tubes wherein drying of the organisms was prevented. With the indirect test procedure that evolved from these studies, *T. cruzi* antisera readily could be differentiated from syphilitic or normal sera. In tests with homologous antisera, the organisms fluoresced brilliantly and exhibited the typical yellow-green color of fluorescein. Conversely, the trypanosomes were only faintly visible in tests with heterologous sera, and the color was a nondescript yellow-orange rather than the yellow-green characteristic of the specific reaction.

Serologic evaluation with homologous, syphilitic, and normal sera showed the fluorescent-antibody test to be superior in sensitivity, but slightly less specific than the complement fixation test using a purified *T. cruzi* antigen (Table I). On the other hand, specificity of the fluorescent-antibody procedure was greatly superior to that exhibited in complement fixation tests using the commonly employed unpurified *T. cruzi* antigens; *e.g.,* in earlier studies(8) a buffered-saline extract of *T. cruzi* reacted with 7 of 75 normal sera and 9 of 21 specimens from syphilitic donors. The findings suggest that the fluorescent-antibody test

may provide a relatively simple, reliable procedure for diagnosis of *T. cruzi* infection and be of particular value to laboratories not having access to purified complement fixing antigen. Furthermore, the fluorescent-antibody test, in view of its high degree of sensitivity, appears especially well suited for use as a screening procedure and could reduce the number of sera requiring study with the slightly more specific but intricate complement fixation test.

The need for further evaluation of the fluorescent-antibody procedure is evident since heterologous sera representing parasitic diseases other than American trypanosomiasis were not available during the present experiments. Moreover, any further studies should consider the possible improvement of specificity by arbitrarily reducing the sensitivity level of the test procedure. Investigations to determine whether the same antibodies are responsible for the complement fixation and fluorescent-antibody reactions likewise seems a worthwhile subject for future study.

With regard to preparation of fluorescein-labeled antibody, many investigators contend that the quality of conjugates can be improved by removing non-antibody serum components before labeling. Usually this is accomplished by repeated precipitation of the antiserum with half-saturated ammonium sulfate. The resultant antibody-containing globulin fraction then is tagged with fluorescein. Certain inherent problems, however, are encountered with this procedure. It is not only time-consuming, but requires extensive dialysis for complete removal of residual ammonium sulfate. In addition, the globulin fraction undergoes considerable dilution during dialysis, and frequently must be concentrated before it can be labeled. These difficulties were obviated in the present studies by isoelectric precipitation of the serum euglobulin fraction with dilute HCl(4). Specific staining capacity of euglobulin-fluorescein conjugate repeatedly was observed to be significantly greater than that exhibited by labeled ammonium sulfate-precipitated globulin or whole serum. The superiority of the euglobulin fraction was further indicated by the relatively small amount of precipitate that developed during conjugation, suggesting little if any gross alteration of antibody components.

Summary. A fluorescent-antibody technic for serodiagnosis of American trypanosomiasis is presented. While nonspecific staining regularly occurs in tests using dried smears of trypanosomes, specificity is excellent when reactions are conducted in test tubes and drying of organisms is prevented. Serologic findings indicate the fluorescent-antibody procedure, used either independently or in conjunction with the complement fixation reaction, can play an important role in serodiagnosis of *T. cruzi* infection. An improved method for purifying antibody prior to conjugation with fluorescein also is given. Advantages of purification by isoelectric precipitation with dilute HCl over ammonium sulfate as a precipitant are noted.

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