TABLE II. Incorporation of Acetate-1-C^{14} into Various Lipid Fractions of *T. foetus*.

Strains	Sterol esters	Sterols	Glycerides fatty acids	Phospho-lipids
		(% of C^{14} incorporation)		
BR	3.7	Traces	14.3	83.3
3P	3.3	"	17.1	79.6

The incorporation of radioactive acetate into various lipid factions is seen in Table II. As previously, there was no appreciable difference in incorporation between the 2 strains examined. Most of the radioactivity was incorporated into the phospholipids. Practically none was present in free sterols, and very little in sterol esters.

As appears from the results, the relative amount of cholesterol-like material is present in considerable amount in trichomonas, while no synthesis was demonstrated. As the medium contains an ample amount of cholesterol, it may serve as a source for this substance. This is in accord with the findings that trichomonads require sterol for their growth(8). It is of interest to note that while TF contain only "slow acting" sterol, several examined species of trypanosomidae such as *Leishmania tropica* and *Trypanosoma cruzi* contain considerable amounts of "fast acting" sterols(9).

The patterns of C^{14} distribution in lipids of TF incubated in the presence of radiocarbon labelled acetate was similar to that found in acetate injected chicks(3) and acetate fed rats(10).

Summary. Lipid content and metabolism of 2 strains of *Trichomonas foetus* were examined. The parasites contained cholesterol-like material in amounts of about one hundred-fold as much as glycerol and about half that of phospholipids. No synthesis of the sterol was detected.

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Binding of Fluorescent Insulin to Intracellular Antibodies in Guinea Pigs Immunized with Insulin.* (28273)

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The cellular aspects of antibody formation have been extensively studied(1-5) by means of the fluorescent antibody technique of Coons *et al.*(1), who found the indirect technique more sensitive than the direct method which employs fluorescent antigen. Nevertheless, demonstration of specific antibody in tissues by means of fluorochrome-labeled antigens under certain circumstances appears to offer a simple, direct approach to the study of antibody localization(6-9). The present report describes the preparation of fluorescein isothiocyanate conjugated insulin and the spe-

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cificity of its reaction with insulin antibodies, and also establishes the presence of intracellular antibodies in guinea pigs previously treated with large amounts of insulin.

While this work was in progress, other investigators reported the conjugation of beef insulin with fluorescein isothiocyanate(10, 11), the immunologic characteristics of such conjugates(10,12), and their use in staining tissues from diabetic patients(13).

Materials and methods. Immunization of animals. Of 14 hybrid guinea pigs weighing 350-400 g, 3 animals were immunized to bovine insulin (PZI, U-80) and 3 to porcine insulin (NPH, U-40) (Eli Lilly & Co.) as follows: One subcutaneous nuchal injection of 20 units of insulin in emulsion with 2 ml of Freund's complete adjuvant (Difco), followed by 10 units in one ml of adjuvant at weekly intervals for 3 weeks. Three other guinea pigs received only the Freund's adjuvant and 3 acted as noninjected controls. To study regional lymph node response specifically, 2 additional guinea pigs received 20 units of porcine insulin in Freund's adjuvant at weekly intervals for 4 weeks, followed at 4-day intervals by 2 injections of 10 units of insulin without adjuvant into the right hind foot pad. These 2 animals were sacrificed 4 days after the last injection. Surviving animals in the other group were sacrificed 5 days after the last injection. The animals were exsanguinated and tissues (injection sites; cervical, axillary, popliteal and inguinal lymph nodes; spleen; liver; kidney; and voluntary muscle) as well as plasma (from blood collected in 10% sodium versenate), were frozen and stored at –20°C. Titers of insulin binding antibodies were determined by the hydrodynamic flow technique(14,15).

Fluorescein isothiocyanate conjugates. Fluorescein isothiocyanate-insulin conjugates (FII) were prepared as described by Halikis and Arquilla(10) with bovine and porcine zinc crystalline insulin‡ and fluorescein isothiocyanate (FIT) (Baltimore Biological Laboratories). Initial insulin : FIT ratios varied from 10:1 to 40:1. Conjugates, 2-4 mg/ml, were dialyzed for 4-5 days against frequent changes of 0.01 M phosphate (pH 7.2) in saline. FIT conjugates of mixtures of crystalline bovine insulin and bovine insulin-I^{131} (Abbott) were similarly prepared (FII-I^{131}). In some instances FII was adsorbed with a mixture of acetone powder of mouse liver and diatomite filter aid (Celite, Johns-Mansville Co.)(16), or passed through a column of dextran gel (Sephedex, G-25, Pharmacia A.B., Uppsala, Sweden) in an attempt to improve the purity of the preparation and reduce non-specific staining. FIT conjugates of bovine albumin and the serum globulin fractions (precipitated with half saturated ammonium sulfate) from 3 guinea pigs immunized to beef or porcine insulin were prepared by the method of Marshall(17), with initial globulin : FIT ratios of 50:1 by weight.

FII conjugates were characterized electrophoretically on cellulose acetate strips using a pH 8.6, 0.1 M barbital buffer. Wet strips were viewed under ultraviolet light and then stained with 0.1% nigrosin. The chromatographic mobilities of FII, FII-I^{131}, and crystalline insulin were compared on Whatman #3 paper using n-butanol : H_2O : acetic acid (12:5:2, v:v) in an ascending system(18) for 12-15 hours. The ability of the FII-I^{131} to bind to antibody was studied chromatographically by the hydrodynamic flow technique of Berson and Yalow(14) as used by Grodsky *et al.*(15). FII-I^{131} (0.02 ml containing 0.1 μg insulin-I^{131} and 0.08-0.16 mg insulin) was incubated with 0.2 ml plasma for 30 minutes and 0.1 ml of the mixture was subjected to hydrodynamic flow for one hour. In this system undegraded insulin remains at the origin unless bound to antibody. The chromatograms were viewed in ultraviolet light, stained with 0.01% bromphenol blue, and appropriate ones counted for radioactivity. A modified method of immunoelectrophoresis was performed on cellulose acetate strips in the pH 8.6 barbital buffer. Guinea pig plasma, 5 μl, was added on the anode side of the center of the strip. FII-I^{131}, 1 μl (0.7 μg), was loaded

‡ Dr. W. R. Kirtley of Eli Lilly & Co. kindly supplied the crystalline insulin (Lot No. 719106 and 723603) and glucagon preparations, (Lot No. 258-234B-167-1).

2 mm away from the antiserum towards the cathode, but still on the anode side of center. Electroosmotic flow caused a rapid movement of the globulins through the insulin, which remained almost stationary in the absence of antiserum during the 90 minute run. Albumin remained stationary or moved slightly toward the anode, and thus did not come in contact with the insulin. Strips were examined for I^{131}, distribution of fluorescence and nigrosin staining. To quantitate any loss in the ability of insulin to bind to antibody due to FIT conjugation, 4 preparations of FII conjugates of measured protein content(19) were assayed by the immunochemical technique of Grodsky and Forsham(20).

Preparation and staining of tissues from immunized and control guinea pigs. Frozen tissues were sectioned (5-6 μ) on a Harris International Cryostat and fixed immediately in 95% ethanol for 10-15 minutes at room temperature. Sections were stained for 30 minutes at room temperature with FII, diluted to approximately 4-8 μg/ml with 1% bovine albumin in pH 7.5-8 phosphate buffered saline, and then washed in 2 changes of buffered saline for 10 minutes. The slides were mounted in pH 7.5-8 buffered saline, and examined with a Zeiss fluorescence microscope utilizing a BG 12 primary filter and an 0G5 barrier filter. In some instances, adjacent frozen sections were stained with hematoxylin and eosin for comparison with fluorescent sections. Aliquots of all tissues frozen for fluorescence studies were fixed in 10% buffered formalin and processed for paraffin sectioning. *Specificity of fluorescent staining.* 1) Sections of lymphoid tissues were pretreated with solutions of insulin and also glucagon‡ (1 to 5 mg/ml in pH 8-9 phosphate buffered saline) for 30 minutes, washed in 3 changes of buffered saline for 10 minutes, and stained with FII for 10 minutes. Results were compared with sections stained for 10 minutes with FII alone. 2) Staining with FII and fluorescein-albumin conjugates was contrasted on comparative tissue sections. 3) FII (4 μg) was incubated 30 minutes *in vitro* with 0.2 cc antiinsulin guinea pig plasma and staining characteristics were compared with FII alone and FII incubated in control plasma. 4) Demonstration of intracellular antibody by the indirect technique was attempted. Sections were covered with insulin (1 to 5 mg/ml) for 30 minutes at room temperature, washed, and stained with the fluorescent antibody globulin preparations.

Results. Electrophoresis of FII and FII-I^{131} showed 2 fractions (I and II in order of decreasing mobility) in all instances and in several preparations a third slowest component (III). The number of fractions did not correlate with the initial insulin : FIT ratios used in the preparation. Fraction I contained 80-85% of the insulin as measured by fluorescence, nigrosin stain and radioactivity. Fraction III, when present, was less than 5%. Paper chromatograms confirmed the existence of 2 to 3 fractions. Fraction I, exhibiting the major bulk of material, had an Rf of approximately 0.40. The Rf of fraction II was 0.23. A faint third band had an Rf the same as non-fluorescent insulin (0.30). FII-I^{131} and unconjugated insulin-I^{131} behaved similarly in the hydrodynamic flow system; both fluorescence and radioactivity remained at the origin in control plasma or albumin, but moved with the plasma proteins when antibody was present (Fig. 1). Judging by parameters of both radioactivity and fluorescence, FII-I^{131} migrated in the immunoelectrophoretic system with the beta-gamma area. Migration was almost completely prevented by preincubation of the antiserum with unconjugated insulin. Immunoassay of FII preparations showed 70 to 80% retention of antibody binding activity as determined by comparison with unmodified crystalline insulin.

Antibody titers ranged from 1:250 to 1:1000 in the 5 surviving immunized animals, but showed no correlation with the species of insulin injected. Frozen sections of spleens from guinea pigs with the highest antibody titers contained numerous cells which were stained specifically by FII. These cells, showing a bright apple green cytoplasmic fluorescence and occasionally nucleolar fluorescence, were distributed primarily in clusters around splenic trabeculae and arterioles (Fig. 2). In hematoxylin and eosin sections most of these peritrabecular and perivascular cells

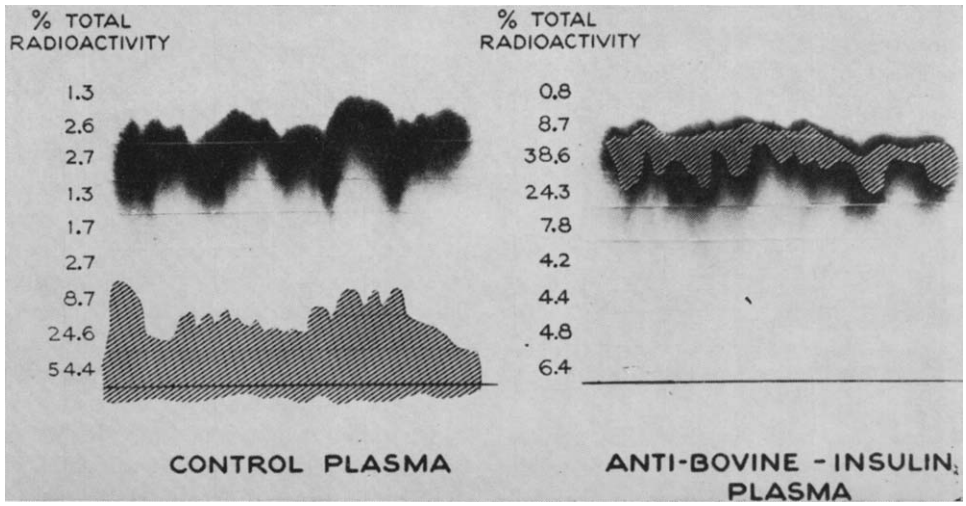

FIG. 1. Photograph of hydrodynamic flow chromatograms. Fluorescein isothiocyanate-insulin-I^{131} incubated with control plasma (guinea pig injected with Freund's adjuvant alone) and with anti-bovine-insulin plasma (guinea pig injected with bovine insulin-Freund's adjuvant emulsion). Black areas represent plasma proteins stained with bromphenol blue, lined areas the yellow-green fluorescence of fluorescent insulin.

appeared to be immature plasma cells (type B)(5). Mature plasma cells were present in smaller numbers, but relatively few of these showed affinity for the FII (Fig. 3). Nonspecific staining by FII was reduced to a minimum by dilution of conjugates to 4-8 μg insulin/ml. Passage through a Sephedex column and adsorption with mouse liver powder —Celite produced no further reduction. Sections of the spleens of control animals showed no cells that stained specifically with FII, although a plasmacytosis was present in the spleens from animals that received adjuvant only. When spleens from animals immunized with bovine insulin were stained with porcine FII and vice versa, no differences in intensity of specific fluorescence or numbers of cells taking the stain could be detected.

Splenic sections, from immunized animals, layered with FII preincubated with antiserum developed no fluorescence, whereas, staining by FII similarly exposed to control plasma

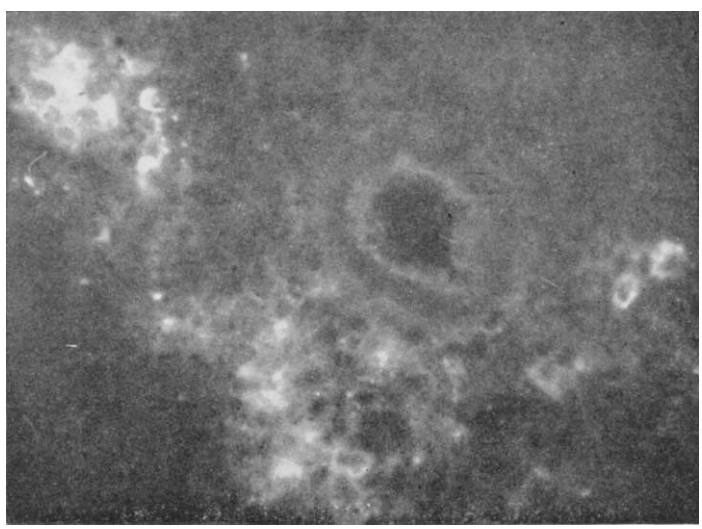

FIG. 2. Section of spleen from guinea pig immunized with bovine insulin, stained with bovine FII. ×400.

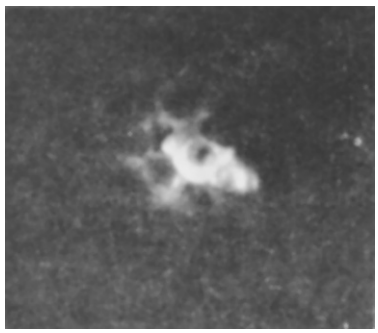

FIG. 3. Section of spleen from guinea pig immunized with porcine insulin, stained with porcine FII. ×640.

was unaltered. A significant but less marked blocking effect was produced by pretreatment of sections with nonfluorescent insulin followed by staining with FII for 10 minutes. Prolonged staining by the latter resulted in a reversal of this effect. When sections were pretreated with glucagon in the same fashion, no inhibition of staining was noted, and staining with bovine albumin-FII alone produced only diffuse nonspecific fluorescence. Staining of sections of spleen and lymph nodes from immunized animals by the indirect technique of Coons produced only diffuse nonspecific fluorescence of relatively low intensity.

The spleens from the 2 animals that received additional insulin in the foot pads (and had the lowest titers, *i.e.*, 1:250) showed a striking plasmacytosis with mature forms predominating. However, the percentage of plasma cells specifically binding FII was less than was seen in spleens from the guinea pigs with higher titers. Except for the foot pads and their draining lymph nodes in the above 2 animals, injection sites and lymph nodes generally contained few or no cells with insulin antibodies despite variable degrees of plasmacytosis. The histological changes produced by Freund's adjuvant were prominent in these tissues. Frozen sections of nonlymphoid tissues which included liver, kidney and voluntary muscle from the high titer animals showed no specifically staining elements except for a very few isolated plasma cells. In particular, the renal glomeruli contained no demonstrable insulin binding antibody.

Discussion. Both Halikis and Arquilla(10) and Tietze *et al.*(11) have demonstrated 2 electrophoretic fractions in their preparations of FII conjugates. The latter showed that the fast component is a monoconjugated insulin whereas the slow fraction is diconjugated and, further, that the terminal phenylalanine on the B chain of insulin is the site of conjugation with FIT. Berns *et al.*(12) using a recrystallized fluorescent insulin preparation, found only one electrophoretic fraction. Our preparations, in which initial insulin : FIT ratios of from 10:1 to 40:1 were used, always contained at least 2 electrophoretic and chromatographic components and frequently a faint third component, but the number of components did not appear to correlate with insulin : FIT ratios. Despite the presence of these 2 to 3 different components, the fluorescent insulin preparations appeared to be immunologically homogeneous, as far as could be ascertained with the form of immunoelectrophoresis used. Unlike Berns *et al.*(13), we found a 20-30% reduction in the ability of FII to bind insulin antibody, as compared with unmodified crystalline insulin, even in preparations in which the initial I : FIT ratio was the same as that employed by these authors. Whether this comparatively small loss of activity is due to handling or to changes in the antigenic structure produced by conjugation with FIT is not known although the latter has been suggested(10).

FII staining of lymphoid tissues from guinea pigs immunized to insulin produced specific cytoplasmic fluorescence in plasma cells and the correlation between the age of plasma cells and antibody titers appeared, in general, to agree with the findings of Vasquez (5). Any differences that might have been present between the two species of fluorescent insulin in degree of binding to antibody containing cells were not of great enough magnitude to be detected by fluorescence microscopy and, in fact, these species differences have been shown to be quite small by refined techniques(22). Apparently because of the low final titers of the fluorescent globulin preparations, we were unable to show specific staining of these same cells by the indirect method of Coons. However, it is also possible

that with insulin the indirect technique may be less sensitive than with other antigens since insulin appears to be relatively poor in immunologically reactive groups(21). The success obtained by Berns *et al.* in staining lesions in kidneys of diabetic patients by the indirect technique may be related to the quantity or nature of antibody material in the lesions or to higher antibody titers in their fluorescent globulin preparations, or both. When stained with FII the kidneys of the hyperimmune guinea pigs in our study showed no antibody material in glomeruli, tubules or vessels, demonstrating that circulating insulin binding antibodies do not accumulate in these areas, at least in short-term exposure.

Summary. Fluorescein isothiocyanate-insulin (bovine and porcine) conjugates, containing 2 to 3 chromatographic and electrophoretic fractions, showed a 70-80% retention of capacity to bind insulin antibodies, produced in guinea pigs, as compared to unmodified crystalline insulin. The conjugates and unconjugated insulin-I^{131} behaved similarly, electrophoretically and chromatographically, in the presence of antibody. Direct staining, with fluorescent insulin, of frozen sections of lymphoid tissues from guinea pigs immunized with insulin produced specific cytoplasmic fluorescence in plasma cells which were present in increased numbers. Non-lymphoid tissues from the same animals contained no specifically staining elements beyond occasional scattered plasma cells. No differences in staining were observed when lymphoid tissues from animals immunized with bovine insulin were stained with porcine fluorescent insulin and vice versa.

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