heterologous hapten-homologous protein is followed by a secondary dose of hapten-homologous protein, antibody response to the hapten is accelerated. A primary sensitizing dose of hapten, such as p-aminobenzoic acid (PABA) + protein such as hen egg albumin (HEA), followed by a secondary dose of the same hapten attached to heterologous protein, does not result in accelerated response to the hapten. These phenomena are explained if delayed hypersensitivity is considered an early step in antibody synthesis.

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Antigenicity of Connective Tissue Extracts II. Stimulation of Auto- and Iso-antibodies by Heterologous Antigen.* (25917)

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Previous studies in our laboratory(1) have shown that saline extracts from rabbit tendons combined with Freund's adjuvant are antigenic to guinea pigs. Circulating complement fixing (C.F.) and precipitating antibodies to these connective tissue extracts could be demonstrated(1). Growth of the majority of the guinea pigs injected subcutaneously with extracts of rabbit tendon was markedly diminished as compared to growth of control animals injected only with saline and adjuvant. This effect was not observed following injection of other tissue extracts (liver, leukocytes, kidney) and serum. It was considered possible that the stunted growth was related to development of antibodies against components of connective tissue. The present report deals with demonstration of fixed antibodies in various tissues of the sensitized animals.

Methods and materials. Preparation of extracts from rabbit tendon has been previously described(1). Since the previous study showed connective tissue extracts (C.T.) from old rabbits to be more antigenic than those from young animals, only tissue extracts from rabbits older than one year were used. Extracts from leg and foot tendons of guinea pigs were prepared in the same fashion and used as test antigen only. Guinea pigs of the Hartley Strain weighing approximately 300 g received weekly injections of C.T. suspended in Freund's adjuvant. The injection aliquots contained 150 μg of connective tissue protein

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TABLE I. Weight Gain of Guinea Pigs during First 35 Days* of Experiment.

	No. of animals	Initial wt range (g)	Wt gain (g) Range	Mean & S.D.
A. Inj. with C.T. in adjuvant	30	238-330	96-273	195 ± 47
B. " " adjuvant and saline	5	264-332	243-302	273 ± 22
C. Uninjected	7	266-320	180-310	245 ± 44
D. B and C combined	12	264-332	180-310	257 ± 37

Statistical comparison of wt gains of Groups B and C: t, 1.33; $p > 0.2$.
A and D: t, 4.04; $p < 0.001$.

* This is an arbitrarily chosen interval. The difference remained significant after longer intervals, but sample size diminished because of periodic killing of animals.

as determined with the technic of Lowry *et al.* (2).† After 4 such injections, monthly booster injections of half the dose were given. Guinea pigs were divided into 3 groups (Table I). Animals of group A were injected with C.T. in adjuvant. One group of control animals received injections of adjuvant and saline (group B), the other received no injections (group C). The guinea pigs were weighed each week. Twenty-three guinea pigs of group A and 8 control animals were bled by cardiac puncture on the 96th day of the sensitization regimen and their sera tested for C.F. antibodies to extracts of rabbit and guinea pig connective tissue (Table II). These animals were not sacrificed to permit long term observation. Nine guinea pigs of Group A and 7 guinea pigs of the control group were killed by exsanguination through the jugular vein in intervals from 13 to 68 days following start of injection regimen (Table II). Blood was collected and serum obtained in the usual manner for serologic, electrophoretic and hematologic studies. The animals were immediately dissected and tissue samples from the various organs were frozen in liquid nitrogen and sectioned in a cryostat. The sections, which were cut at a thickness of 5 μ, were placed on slides to which they adhered following thawing without addition of adhesive material. They were washed with buffered saline (pH 7.3-7.4) for three 10 minute periods, dried at room temperature, then overlaid with commercial fluorescein conjugated rabbit anti-guinea pig globulin(3). The latter had been previously mechanically agitated with powdered activated charcoal(4) at a concentration of 50 mg per ml of serum for one hour at room temperature and the charcoal removed at high speed centrifugation (18,000 rpm for 10 min.). Slides were kept overnight in a sealed moist chamber at 5°C, then washed with buffered saline (3x10 min.) and air dried. A drop of buffered glycerol (pH 7.4) was then added and cover slips applied. Microscopic examination was performed with fluorescent light illumination. A Zeiss fluorescent microscope was used with OSRAM HBO 200 light source, UG2 and

† Other determined constituents of C.T. included hydroxyproline, uronic acid, hexose, hexosamine and sialic acid. We are grateful to Dr. L. Robert, Dept. of Biochemistry, Univ. of Illinois College of Medicine and Dr. R. D. Coleman, Senior Research Biochemist, VA West Side Hospital, for performing these studies which will be reported later in detail.

TABLE II. Guinea Pigs Sensitized with Rabbit Connective Tissue in Adjuvant.*

No. of animals	Day after start of sensitization	Reciprocal of titer of complement fixing antibodies to Rabbit C.T.	Guinea pig C.T.	Fluorescent antibodies
2	13	neg.	neg.	neg.
		4	"	pos.
2	26	8	8	"
		8	neg.	"
1	34	8	"	"
1	40	neg.	"	"
1	47	4	"	"
1	60	32	4	"
1	68	128	8	"
23	96	32 (2)	neg.(10)	n.d.†
		64(10)	4 (9)	
		128 (7)	8 (3)	
		256 (4)	32 (1)	

* 16 animals served as control. Neither complement fixing antibodies nor fluorescence could be demonstrated in these animals.

† Not done.

UG5 exciter filters, and BG23 and GG4 barrier filters. An attempt was also made to demonstrate fixation of anticonnective tissue antibodies to organ sections from normal

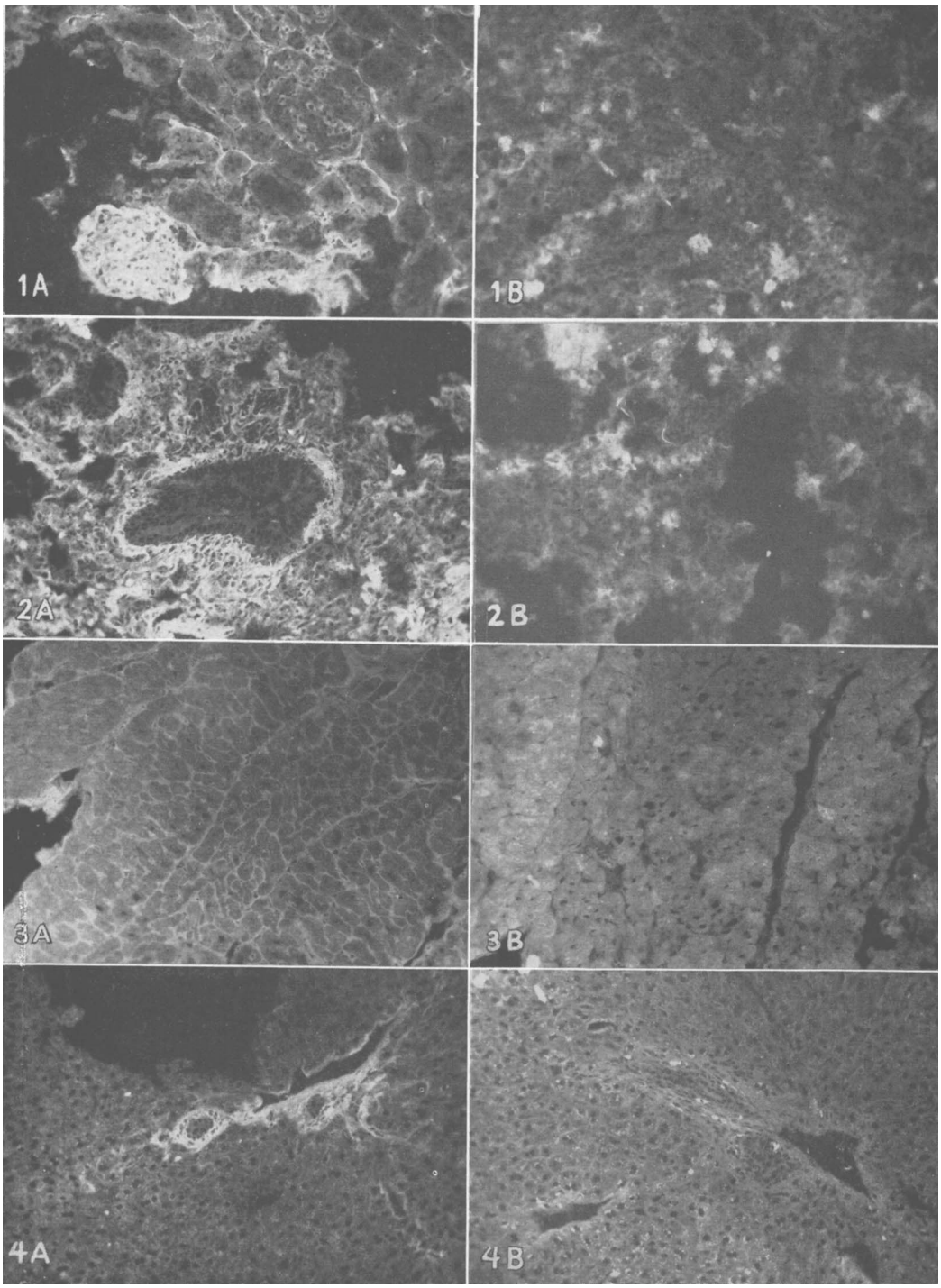

FIG. 1-4. Tissue sections from sensitized (a) and control (b) guinea pigs incubated with fluorescent antiserum. (1) Kidney, (2) Lung, (3) Heart, (4) Liver.

guinea pigs and rabbits. For this purpose these sections were overlaid with antiserum to rabbit C.T. and others with normal guinea pig serum. The slides were then placed in a moist sealed container which was mechanically rotated for one hour at room temperature. They were washed in buffered saline for 3 changes of 10 minutes each, air dried and overlaid with conjugated antiguinea pig globulin rabbit serum as described above. The test for C.F. antibodies was performed according to the standard technic of Kolmer.

Results. The weight gain of the group of animals injected with C.T. was markedly diminished as compared to control animals (Table I). In the examined tissue sections from 8 of the 9 guinea pigs injected with C.T., fluorescence was observed following incubation with the fluorescein conjugated antiguinea pig globulin (Fig. 1a-4a). The sections which did not show fluorescence came from an animal sacrificed 13 days following first injection. Fluorescence appeared to be localized in interstitial spaces, basal membranes and vascular walls of all examined organs, which included kidney (Fig. 1a), lung (Fig. 2a), heart (Fig. 3a), adrenals, spleen and liver (Fig. 4a). In kidney, fluorescence was most marked in the glomerular basement membrane; in lung, in the interalveolar septa and peribronchial tissue. In no organ was intracellular fluorescence demonstrated.

Tissue sections from normal guinea pigs overlaid with normal guinea pig serum followed by incubation with fluorescein conjugated antiserum did not reveal specific staining (Fig. 1b-4b). If, however, serum from guinea pigs which had been injected with C.T. were used, specific fluorescence was observed following incubation with the conjugated antiserum. As a control the same procedure was performed on tissue sections from 2 normal rabbits with similar results.

Complement fixing antibodies were observed in serum of 30 of 32 sensitized guinea pigs with rabbit C.T. as test antigen. Titers increased during sensitization and ranged from 1:4 to 1:256 (Table II). Sixteen guinea pigs also developed C.F. antibodies to guinea pig C.T. at titers of 1:4 to 1:32. Anti-connective tissue antibodies could not be demonstrated in control animals. In all animals injected with rabbit C.T., the electrophoretically determinable gammaglobulin fraction was higher than in non-injected animals. The Coombs test remained negative and the hematological examination did not reveal abnormalities.

In view of fixation of guinea pig globulin in tissue sections of parenchymatous organs of sensitized guinea pig, it was considered likely that serum of these animals would also react with saline extracts from such guinea pig organs in the complement fixation test. This was tested with tissue extracts from guinea pigs (liver, kidney, heart, lung). Titers were of same magnitude as titers of isologous antibodies to tendon extract.

Discussion. The presence of gammaglobulin in tissue sections as visualized by its specific reaction with a fluorescein labelled antigammaglobulin is generally interpreted to be the result of a specific antigen-antibody reaction in the tissue with fixation of the antibody to the antigenic tissue component. Our findings, therefore, appear to indicate fixation of anti-connective tissue antibodies in interstitial spaces and basement membranes and vascular walls of several organs. Since rabbit tissue was used for sensitization of guinea pigs, the presence of circulating antibodies in guinea pigs to the rabbit connective tissue is not surprising; however, induction of tissue fixed antibodies in guinea pigs appears to be consistent with the possibility that autologous antibodies had been induced by heterologous antigen. This appears to apply also to circulating C.F. antibodies although the reactions were not as strong as with the heterologous antigen (Table II). The reaction of guinea pig tissue with anti-rabbit tissue antibodies could also be demonstrated *in vitro* following incubation of tissue sections from organs of normal uninjected guinea pigs with anti-rabbit connective tissue serum. Sera of sensitized guinea pigs also reacted with isologous organ extracts. These phenomena suggest the possibility that the occasionally reported cross reactions(5,6,7) of antisera to various organs with other organs are caused by the

antigenicity of ubiquitous connective tissue.

Goodman(8) has recently reported that rabbits injected with human liver nucleoprotein developed antibodies not only to human nucleoprotein extracts, but also to such extracts obtained from rabbit liver. The results of the present study suggest that auto- and iso-antibodies to connective tissue may also be induced by foreign material. It is uncertain if the stunted growth of the guinea pigs is related to development of these anti-connective tissue antibodies.

Identification of the antigenic component in connective tissue is under study.

Summary. Tissue fixed globulin has been demonstrated in interstitial tissue and basement membranes of various guinea pig organs following inoculation of guinea pigs with saline extracts of rabbit tendon. These animals had a significantly diminished growth. It is likely that the fixed globulin represents "auto-antibody" induced by heterologous antigen.

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Effect of Thyroxine and Propylthiouracil on Magnesium Metabolism in the Rabbit. Study with Mg^{28}.* (25918)

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The factors regulating the metabolism of magnesium are obscure. Previous studies have shown that insulin and glucose(1), cortisone(2), pyridoxine and desoxypyridoxine (3), and alloxan (unpublished) may affect distribution of Mg^{28} in rabbits. Very little is known about the relationship between magnesium metabolism and the thyroid gland, although it has been suggested that either hyperthyroidism or hypothyroidism may alter the partition of magnesium in serum(4). The present study was designed to investigate the effects of experimentally induced hyper- and hypo-thyroid states on magnesium metabolism in the rabbit. Before and after administration of thyroxine or propylthiouracil to 2 groups of animals, the external balance of magnesium was determined and exchangeable magnesium content was measured with Mg^{28}. Mg^{28} was also used to determine relative tissue radioactivity in rabbits which had been on larger doses of thyroxine or propylthiouracil.

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Material and methods. Thirty-two normal domestic adult male rabbits were kept in individual stainless steel metabolism cages and were fed a stock diet of compressed pellets. Except in exp. 2 and 4, tap water was given without restriction. *Thyroxine sodium* was dissolved in physiologic saline solution made slightly alkaline with a few drops of 1 N NaOH, and was made up to a final concentration of 0.3 mg/ml. In preliminary observations, 7 rabbits were given 2 intravenous injections of thyroxine, 0.3 mg per kg of body weight, within 48 hours. A striking loss of body weight ensued, and 3 of the 7 rabbits died within 7 days. In the definitive experiment, therefore, only one dose of 0.3 mg/kg was administered initially. When it appeared