sented.

Summary. The relation between activity of brain carbonic anhydrase and susceptibility to various grades of seizure patterns has been studied in young rats by observations of the effects of acetazolamide. The percentage inhibition of carbonic anhydrase and of the reaction catalyzed by the enzyme is related to the suppression of tonic and clonic seizure patterns. It is suggested that brain carbonic anhydrase and its specific catalytic activity may be essential for the propagation of the seizure discharge. Low levels of enzyme activity may be sufficient for focal discharge and the induction of minor seizure patterns while a relatively high degree of activity may be required for the propagation of a generalized seizure discharge and the induction of a major tonic seizure.

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Tetramethylrhodamine as Immunohistochemical Fluorescent Label in the Study of Chronic Thyroiditis.* (23822)

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Since development by Coons and his coworkers(1) of the fluorescent immunohistochemical technic using fluorescein groups as the fluorescent label, there has been interest in developing fluorescent labels of a color other than the fluorescein apple green. Thus, Clayton(2) reported labelling antibodies with 1-dimethylaminonaphthalene-5-sulfanyl chloride and nuclear fast red to produce yellow and red fluorescence, respectively. Silverstein(3) recently reported that tetraethylrhodamine can be coupled through ureido groups to protein to yield a label which fluoresces orange in ultraviolet light. We had been working on the fluorescent label problem also and had prepared the tetramethylrhodamine isocyanate which when coupled to protein fluoresces orange. We are reporting the use of this label in a study of the serum components associated specifically with chronic thyroiditis (Hashimoto's disease). Roitt, Doniach, Campbell and Hudson(4) reported that serum of patients with Hashimoto's disease contains material capable of precipitating with human thyroid extract. Witebsky, Rose, Terplan, Paine and Egan(5) have shown that serum of patients with chronic thyroiditis agglutinates tanned sheep red cells coated with human thyroid extract. Previously, Witebsky and Rose(6,7) had shown that thyroiditis similar to that in humans can be produced in rabbits by injecting animals with thyroid extract, either from same species, or from a portion of individual's own thyroid.† From these observations, it has been postulated that chronic thyroiditis in humans may be the result of an auto-immunization process in which an individual produces antibodies against tissue components in his own thyroid. The se-

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† Beutner, Witebsky, Rose and Gerbasi(8) have shown that rabbit thyroid is stained by fluorescein labelled sera from rabbits injected with rabbit thyroid.

rum components in a patient with chronic thyroiditis have been shown to be in the rapidly sedimenting globulin fraction(10). To determine which structures of the thyroid gland combine with precipitating chronic thyroiditis serum, we treated sections of normal human thyroid with such serum and determined where combination took place by staining subsequently with tetramethylrhodamine (and fluorescein) labelled rabbit anti-human gamma globulin antiserum. Similarly, we demonstrated that thyroid tissue from a patient with chronic thyroiditis is stained by anti-human globulin antibodies, indicating presence of globulin in that tissue.‡

Since thyroid sections used in this study fluoresced green in certain areas under ultraviolet light without any staining and since this green was difficult to distinguish from the green staining obtained with the fluorescein label developed by Coons, the newly developed tetramethylrhodamine label was particularly useful. The tetramethylrhodamine fluoresces orange-red rather than the green and can be easily distinguished from background fluorescence.

Materials and methods. The chronic thyroiditis serum used was described previously (10). The diseased thyroid was that obtained from the same patient. Normal serums of blood groups A, B and O were employed as controls. The normal thyroid tissues used as controls were obtained at autopsy. Rabbit antiserum to human globulin was prepared by injecting rabbits with commercial poliomyelitis immune globulin (human) in Freund's adjuvants(11). *Preparation of 3-amino-tetramethylrhodamine.* 4-Nitrophthalic acid was dissolved in excess ammonium hydroxide and reduced by heating at 80° with a solution of sulfur in excess ammonium sulfide. Sulfur was filtered off and aminophthalic acid precipitated with hydrochloric acid. The aminophthalic acid was dissolved in excess aqueous sodium carbonate and acetylated with acetic anhydride until the product was no longer diazotizable. The acetoaminophthalic acid was condensed with excess 3-dimethylaminophenol by heating at 160° to 170° in tetrahydronaphthalene for 5 hours. The solvent was decanted and the residue washed with benzene and dried. The residue was hydrolyzed by boiling for 10 min. with 5 N HCl. The product was diluted with water and neutralized with sodium carbonate, precipitating the dye. The amino dye so obtained was washed with water and dissolved in acetone. Dilution of the filtered acetone solution with water precipitated the dye, aminotetramethylrhodamine, which was then dried and used without further purification or separation into isomers.

Tetramethylrhodamine and fluorescein labelled proteins. Essentially the same procedure described by Coons and Kaplan(12) for conversion of aminofluorescein to the isocyanate by treatment with phosgene and coupling the isocyanate to protein was used for fluorescein and tetramethylrhodamine coupling. The coupled protein solution was dialyzed and was stored in the frozen state. Prior to use, the labelled protein was absorbed with acetone rat liver powder(13). *Staining procedure.* Sections of normal thyroid tissue were cut and dried under a lamp for 10 min. In some cases, the tissue was fixed in acetone, methanol or ethanol for 15 min. at room temperature before drying. A drop of the serum to be tested was placed on the section and incubated for 1 hr at room temperature. The sections were then washed in saline for 10 min. with agitation and incubated similarly with tetramethylrhodamine or fluorescein labelled rabbit antibody to human globulin for 1 hr and washed again. Observations were made with a Reichert fluorescence microscope; the section was mounted in glycerol. Sections of Hashimoto's disease thyroid and lymph nodes were fixed as above or in formalin for 30 min. and stained directly by the rhodamine labelled anti-human globulin antibody.

Results. When normal thyroid obtained at necropsy was employed in the staining procedure, there was specific staining only when the chronic thyroiditis serum was employed. Staining occurred in the colloid region and in the glandular epithelium of the thyroid as

‡ Dr. R. G. White has made somewhat similar observations with fluorescein labelled serum from a Hashimoto's disease patient. Beutner *et al.*(8) also mention similar observations.

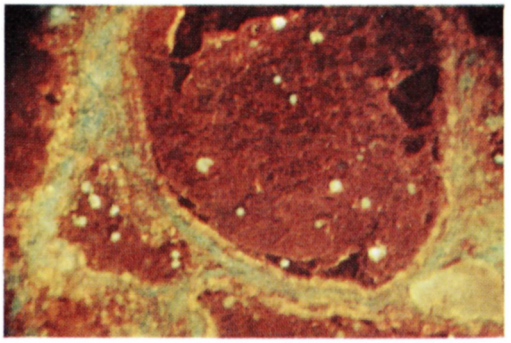

FIG. 1. Normal thyroid treated with chronic thyroiditis serum and subsequently with tetramethylrhodamine labeled rabbit anti-human globulin antibody. Orange fluorescence due to staining was seen in the colloid region of the thyroid and also in the glandular epithelium of the thyroid. Strong green autofluorescence of the stroma was present.

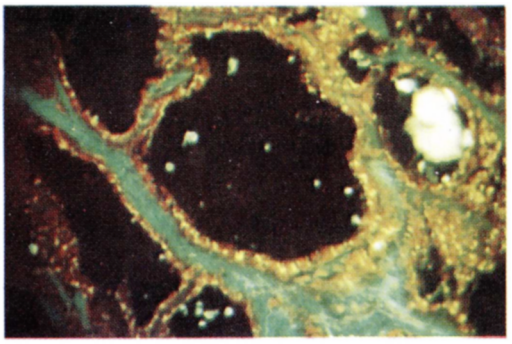

FIG. 2. Normal thyroid treated with normal human serum and subsequently with tetramethylrhodamine labeled rabbit anti-human globulin antibody. No staining was seen in the colloid region—much of the colloid appeared to be washed away. Nonspecific staining occurred in the glandular epithelium about equivalent to controls not treated with human serum prior to staining.

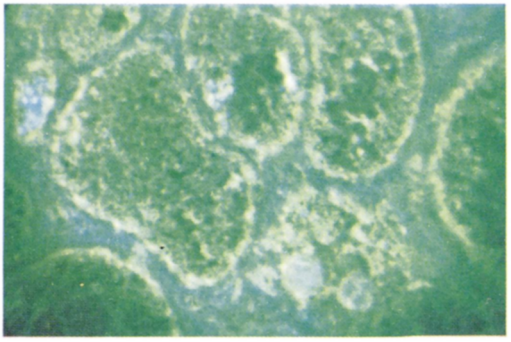

FIG. 3. Normal thyroid treated as in Fig. 1 except that fluorescein rather than tetramethylrhodamine was used as label. It is apparent that the colloid is stained by the fluorescent antibody but the green fluorescence of the unstained stroma does not allow observation of any specific staining in or close to the stroma.

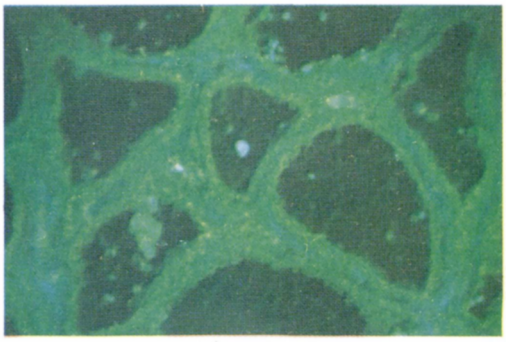

FIG. 4. Same as Fig. 2 except that fluorescein rather than tetramethylrhodamine was used as label. Nonspecific staining of the glandular epithelium is also seen.

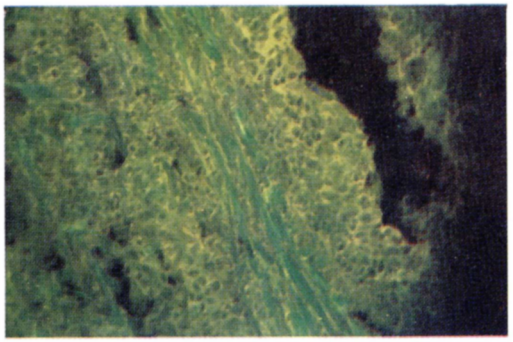

FIG. 5. Chronic thyroiditis thyroid treated with rhodamine labeled rabbit anti-human globulin antibody. Staining was present in the areas of lymphoid cell infiltration, showing the presence of globulin in or around these cells. Strong green-blue autofluorescence was present in the stroma.

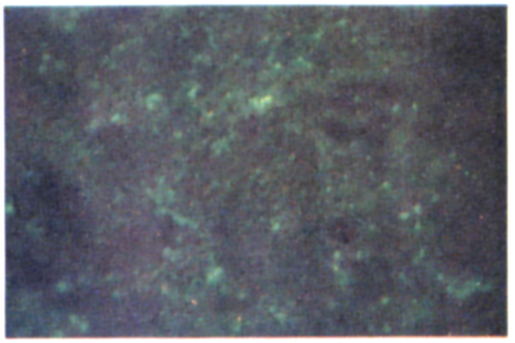

FIG. 6. Normal lymph node treated with tetramethylrhodamine labeled rabbit anti-human globulin antibody. The lymphoid tissue except for occasional weak areas of orange fluorescence was negative.

shown in Fig. 1 where staining was done with the tetramethylrhodamine labelled serum. The staining in the glandular epithelium may have been largely nonspecific since control sections treated with serum of individuals of the several blood groups showed no fluorescence in the colloid but there was staining of the glandular epithelium about equivalent to the staining of control sections which had not been treated with any human serum prior to staining with rabbit anti-human globulin antibody. The colloid appeared to have been leached out (Fig. 2).

The results obtained with the tetramethylrhodamine label (Fig. 1 and 2) were compared with results obtained with the fluorescein label (Fig. 3 and 4) where similar sections treated with chronic thyroiditis serum (Fig. 3) and normal human serum (Fig. 4) prior to staining with fluorescein labelled anti-human globulin antibody are shown. Here, it can be seen that the colloid was stained when chronic thyroiditis serum was used, but much of the other detail was lost because the fluorescence due to fluorescein label was indistinguishable from the background fluorescence of the tissue itself. Differentiation between fluorescent stain and adjacent background fluorescence is only possible when the tetramethylrhodamine stain is used.

In the staining procedures, there was some leaching out of the soluble antigen which gave precipitate with the added chronic thyroiditis serum. No such precipitate was obtained with control serum. Although acetone fixation did not give completely satisfactory results, it appeared better than ethanol, methanol or formalin fixation. The precipitate formed in solution and was removed on subsequent washing. Staining was obtained even when the precipitate formed. This indicates that there was an excess of staining antibody present or that perhaps the components which are being stained in the tissue are of a different antigenic nature than the material which leached out and formed a precipitate. The possibility does exist that in chronic thyroiditis there may first be formed antibodies directed against components other than thyroglobulin and these are the originally cytotoxic antibodies. Antibodies to thyroglobulin may be formed only subsequent to the initial insult by these hypothetical antibodies directed against other components.

It appears from the staining results that antibody prepared in rabbits against the polio globulin contains antibody against the component of chronic thyroiditis serum responsible for precipitation with thyroid extract. It has been shown that this component is associated with the rapidly sedimenting globulin(10). Similar rapidly sedimenting components are known to be constituents of normal serum. Therefore, the chronic thyroiditis serum component must be similar antigenically to normal serum components.

To determine whether human globulin was present in the chronic thyroiditis thyroid, formalin fixed§ diseased thyroid was treated with tetramethylrhodamine labelled rabbit anti-human globulin antiserum for 1 hr. Formalin fixed sections of lymph node were treated simultaneously as control since chronic thyroiditis thyroid is primarily a mass of lymphocytic infiltration. The sections were washed and observed. Good staining was present in the case of the thyroid from the patient with chronic thyroiditis with orange fluorescence being present primarily in the infiltrating lymphocytic cells, showing the presence of globulin in or perhaps around the cells while the stromal elements showed strong green and blue auto-fluorescence (Fig. 5). The lymphoid tissue except for occasional weak areas of orange fluorescence was negative (Fig. 6). This indicates that the infiltrating cells contained globulin and may have been important in the destruction of the thyroid during the course of the disease.

To demonstrate specificity of the staining, thyroid sections from chronic thyroiditis were first incubated with unlabelled anti-human globulin (2 hr), washed briefly and then incubated for 10 min. with tetramethylrhodamine labelled rabbit anti-human globulin. The sections were washed for 5 min. before observation. There was definite inhibition of staining. As controls, similar sections were treated with normal rabbit serum in place of the unlabelled anti-human globulin serum and

§ Similar results were obtained with acetone fixed sections.

showed no inhibition in subsequent staining with the labelled anti-human globulin. This proves that the staining observed originally was due to the reaction of anti-human globulin with human globulins in the section.

Whether the slides were fixed in formalin‖ or acetone or not fixed at all, there was a definite precipitate formation when the unlabelled antihuman gamma globulin was added. The precipitate formed in solution and was removed on subsequent washing. The fact that staining was obtained in the presence of the precipitate forming in the suspension would indicate here also that there was excess antibody present and perhaps that the components which are being stained in the tissue may be of a different nature than the material precipitating.

Summary. 1) A new fluorescent label, tetramethylrhodamine isocyanate, is described and its use demonstrated in the immunohistochemical fluorescence technic. The label fluoresces orange and is of particular value for staining tissues which show a green autofluorescence similar to fluorescein fluorescence even when unstained. 2) Serum from a patient with chronic thyroiditis combined with normal thyroid tissue in the colloid region and perhaps in the glandular epithelium, while normal human serum showed no such staining. Thyroid from a patient with chronic thyroiditis, infiltrated with lymphocytic cells, was stained by rabbit antibody to human globulin in the region occupied by the infiltrating cells. Normal lymph node showed no such staining. This indicates that the infiltrating cells contained human globulin and may have been important in the destruction of the thyroid during the course of the disease.

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‖ The fixation process did not render the antigens completely insoluble.

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Role of 11β-Hydroxyprogesterone as Intermediary in Biosynthesis of Cortisol and Corticosterone.* (23823)

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There is considerable evidence that production of 17α-hydroxycorticosterone (cortisol) and corticosterone from cholesterol in the adrenal cortex proceeds *via* the reaction scheme illustrated in Fig. 1(1,2). The indicated sequence of hydroxylations of progesterone, shown by solid arrows, has been re-

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