Crystalline lysozyme (*3*) in the amount of 237 mg was exposed at room temperature to 1.8 c of tritium gas at a pressure of 0.39 atm for 72 hours (*4*). The specific enzymatic activity of the tritium-labeled protein was 90 percent of that of the nonlabeled material. Labile tritium was removed by dissolving the protein in water (5 mg/ml), then lyophilizing. This was repeated twice. A sample of the protein (22.5 mg) was then taken up in 5 ml of *p*H 6.5, 0.2*M* phosphate buffer and chromatographed on a 0.9 by 20-cm column of IRC-50, according to the method of Goncalves *et al.* (*5*).

The effluent fractions were examined for (i) optical density at 280 mμ; (ii) total protein, by the method of Lowry (*6*), with a reference curve prepared with nonlabeled lysozyme; (iii) enzymatic activity, by the method of Tallan and Stein (*7*); (iv) tritium radioactivity, by the method described in the accompanying report by Vaughan, Steinberg, and Logan (*8*) to permit direct assay in a liquid scintillation counter (*9*).

As is shown in Fig. 1, a single major protein peak was eluted, which emerged at the point where untreated lysozyme usually appears. The curves for enzymatic activity and radioactivity coincided exactly with the curve for total protein, as is indicated by the relatively uniform values for specific enzymatic activity and specific radioactivity in successive tubes.

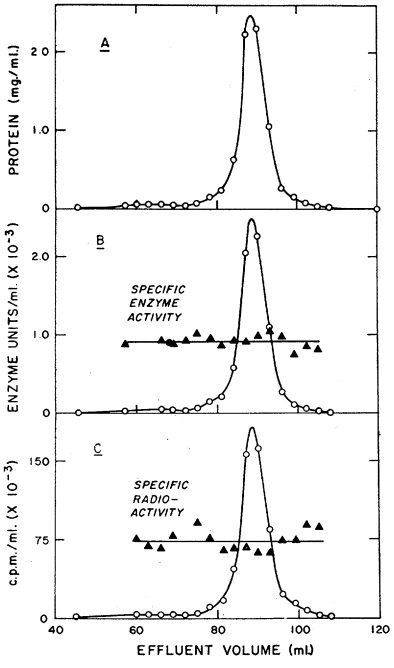

Fig. 1. Chromatography of tritiated lysozyme on IRC-50. (*A*) Total protein by method of Lowry (*6*). (*B*) Enzyme activity by method of Tallan and Stein (*7*). Solid triangles indicate specific enzyme activity in arbitrary units. (*C*) Tritium radioactivity by method of Vaughan *et al.* (*8*). Solid triangles indicate specific radioactivity in arbitrary units.

The specific enzymatic activity in the peak was 23 percent higher than that for the unfractionated labeled material. The specific radioactivity, on the other hand, was distinctly lower—only 1.87×10^5 count/min mg compared with 3.63×10^5 count/min mg before chromatography—and none of the other effluent fractions collected contained significant amounts of tritium. Only about one-half of the total nonlabile tritium was recovered in the peak, whereas the recoveries for total protein and enzymatic activity were 93 percent and 90 percent, respectively. This suggests the presence in the starting material of an enzymatically inactive component of high specific radioactivity, presumably formed by degradation during exposure to tritium, which remained tightly bound to the resin. A direct count on an aliquot of the resin confirmed the presence of tritium-containing material not eluted under the conditions used for chromatography.

In addition to the major peak, there was a very small but definite minor peak eluted just before it. The protein of this minor component was found to have the same specific enzymatic activity and specific radioactivity as that in the major peak.

Crystalline ribonuclease (*10*) in the amount of 570 mg was exposed at 25°C to 6.73 c of tritium gas for 48 hours (*11*). The specific enzymatic activity of the tritium-labeled protein, determined by the method of Anfinsen *et al.*, was 82 percent of that for the unlabeled material (*12*). Labile tritium was removed by dissolving the protein in 0.1*N* NH_4OH and then lyophilizing. The protein was taken up in 0.2*M* *p*H 6.17 phosphate buffer and chromatographed on IRC-50, according to the method of Hirs, Moore, and Stein (*13*). A degraded fraction of very high specific radioactivity but without enzyme activity emerged with the front. This was incompletely resolved from the enzymatically active peak which followed. Because of the incomplete resolution, the degree of homogeneity of the enzymatically active peak could not be evaluated. However, the peaks of radioactivity, enzyme activity, and total protein (optical density at 280 mμ) coincided, and, in the descending limb at least, the enzyme specific activity and the specific radioactivity were reasonably constant in successive tubes.

These studies demonstrate the applicability of the Wilzbach method to the preparation of tritiated proteins. Although there is some degradation during the labeling procedure, good yields of chromatographically pure, enzymatically active protein are obtained, at least in case of lysozyme and ribonuclease. Since only a very small fraction of the enzyme molecules is actually labeled, it is impossible to be certain that the tritium-containing protein molecules are themselves enzymatically active, nor can subtle changes in structure be completely ruled out. Studies to characterize these labeled proteins further are in progress.

Many applications of this method suggest themselves. The fate of intact, internally labeled proteins in biological systems can be studied without the complications introduced when iodination or other "external" labels are used. The method should be particularly valuable for the preparation, in labeled form, of proteins present in the organism in very small quantity, such as the protein hormones, and for the preparation of labeled human proteins, since in these cases it is difficult or impossible to obtain satisfactory labeling by biosynthetic methods. Complex peptides of known structure can be prepared in labeled form by specific proteolytic degradation of tritium-labeled pure proteins or by tritiation of specific fragments derived from breakdown of unlabeled proteins. These labeled peptides, difficult to prepare by synthetic methods, should be valuable in studies of protein biosynthesis.

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12 June 1957

Cerebral Synaptic Inhibition by Serotonin and Iproniazid

In 1955 we (*1*) described the highly potent cerebral synaptic inhibitory action exercised by serotonin. On this basis, we suggested for this natural constituent of mammalian brain a highly important role as a neurohumoral synaptic inhibitor, overshadowing adrenaline and noradrenaline by virtue of its much greater potency.

The action was limited to the brain by the "close arterial" injection into the common carotid artery of the lightly anesthetized cat. Obviously, under these circumstances the serotonin penetrated the blood-brain barrier in order to inhibit the ipsilateral cortical synapses, as was indicated by the reduction in the cortically recorded action potential signaling the evoked response.

We were impressed by the high potency of this action. Serotonin proved effective in doses of as little as 1 μg/kg, and it was 20 times more potent than the next most active natural synaptic inhibitor that we had previously described—namely, adrenaline. The high potency appears to be characteristically cerebral, since the synaptic inhibition we had already recorded in the ciliary ganglion (*2*) required about 75 times the dose. Having dealt only with exogenous serotonin, one could properly ask for a demonstration of the action of endogenous or *in situ* serotonin. We believe that we have given this with the following data.

The action of serotonin is short-lived, because of its great susceptibility to monoamine oxidase (*3*). We reasoned that, if serotonin is naturally present at synapses, we should be able to make its action apparent by inhibiting cerebral monoamine oxidase (MAO), and thereby accumulating *in situ* serotonin until it reaches the threshold for synaptic inhibitory action. This we have done with iproniazid. On intracarotid injection, it reproduces the picture of serotonin action—namely, a reduction of both the evoked responses and the electrocorticogram recorded from the same electrode. As in previous work, we have evoked potentials in the optic cortex of the cat by initiating transcallosal impulses (*1, 4*). That the inhibition of evoked responses and of the electrocorticogram is the result of a poisoning of monoamine oxidase and consequent *in situ* accumulation of serotonin is borne out by the determination of cerebral monoamine oxidase at the time of maximum synaptic inhibition indicated electrically.

The data are displayed in Fig. 1. The monoamine oxidase titer of the injected hemisphere is expressed as percentage of the titer in the opposite or control hemisphere. The evoked potential and electrocorticogram findings are indicated by appropriate symbols at the base of each bar. The left-hand side of the diagram shows the influence of saline control injection. It is evident that there is no change in the electrocorticogram, which was the only electric record taken in this set of controls, and that the variation in monoamine oxidase content of the two hemispheres is relatively small. The extremes of this variation are extended by dotted lines to overlap the right-hand half of the figure, which indicates the data obtained with iproniazid. It is apparent that at the height of synaptic inhibition there is, on the whole, a reduction of monoamine oxidase on the injected side considerably exceeding the natural variations. Similar data were obtained by comparing subelectrode biopsies from the recording hemispheres with the symmetrical points on the opposite sides, but the results were not quite so consistent, probably because of the difficulty in obtaining subelectrode plugs containing identical proportions of the higher monoamine oxidase containing gray matter and the white matter on the two sides.

The direction of these preliminary findings is clear. However, substantiation by further numbers is required to determine whether the instances where electrically indicated inhibition is accompanied by a change in monoamine oxidase titer on the injected side that fails to exceed normal variations are, in fact, due to an initial asymmetrical monoamine oxidase distribution favoring the control side and therefore minimizing the apparent difference induced by iproniazid between the injected and control hemispheres.

This, and the influence of possible leakage across the brain of iproniazid in a few experiments, as well as the safety factor for monoamine oxidase, must be determined. We are now engaged in the final step of simultaneously measuring brain serotonin and monoamine oxidase activity at the time of synaptic inhibition brought about by intracarotid injection of iproniazid.

Since we have shown that many chemical psychotogens are synaptic inhibitors, serotonin or some similar substance seems a likely candidate for the role of an endogenous psychotogen acting by distorting synaptic equilibrium (*5*). That such a distortion could also sometimes be therapeutic is suggested by the recent report of improvement obtained by iproniazid administration in mental disturbance characterized by marked depression (*6*).

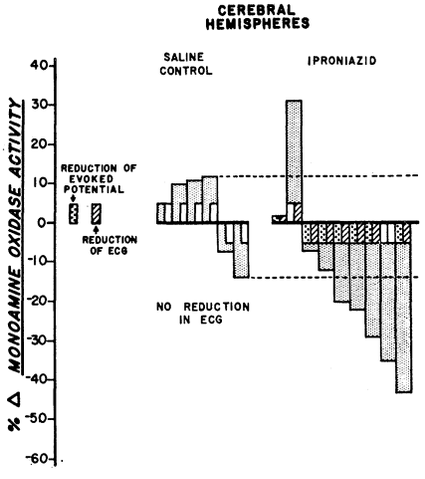

Fig. 1. Correspondence of differential iproniazid inhibition of monoamine oxidase and electric activity in brain. Iproniazid (10 mg/kg) injected into the common carotid artery of a cat anesthetized with sodium pentobarbital.

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2 July 1957

On the Nature of the Pigment in Retinal Pigment Epithelium

Although there have been no biochemical studies of the nature of pigment granules in the retinal pigment epithelium, it has been assumed that the pigment is melanin. This assumption is presumably based on the failure of pigment synthesis in the retinal pigment epithelium of the complete albino. Also, Miescher, in 1923, demonstrated "dihydroxyphenylalanine oxidase" by histochemical methods in the retinal pigment epithelium of the chick, rabbit, and guinea pig (*1*). In some recent manometric studies of homogenates and pigment granules from the retinal pigment epithelium of embryonic chicks, we have demonstrated the presence of the melanin-synthesizing enzyme, tyrosinase (*2*).

The retinal pigment epithelium, derived from neural ectoderm, is a cuboidal epithelium which lies immediately to the outer side of the layer of rods and cones. Pigment granules in human retinal pigment epithelium first appear in the fourth week (5 to 6-mm stage), and by the fifth week (10-mm stage) full pigmentation of the retinal pigment epithelium has occurred. Except for an increase in area, the retinal pigment epithelium remains essentially the same throughout life.

Although retinal pigment epithelium is intimately related to the rods and cones, it is easily separated as a delicate black membrane. Thus it is possible to utilize relatively pure retinal pigment epithelium in enzymic studies with substrates (tyrosine, cresol) that are known