individuals and laboratories referred to in Table I for furnishing us with the samples which made this investigation possible.

Summary

A series of protein, enzyme, hormone, and miscellaneous preparations have been assayed for B vitamins. Only traces have been found in most; however, inositol appears to be a constituent of purified amylase and the impure carboxylase preparations available contained a suggestively high content of nicotinic acid in addition to the thiamin already known to be present. The viruses investigated were found to be nearly devoid of B vitamins, and in this respect they appear to resemble "inanimate" rather than "animate" matter.

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[FROM THE CHEMICAL LABORATORIES OF HARVARD UNIVERSITY AND RADCLIFFE COLLEGE]

Some Experiments on the *in vitro* Formation of Thyroxine from Diiodotyrosine

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Following the report of von Mutzenbecher[1c] that thyroxine is readily formed *in vitro* from diiodotyrosine as well as from iodinated proteins,[2] considerable interest has been expressed in the nature of the reaction. As normally carried out, slightly alkaline digestion of diiodotyrosine over a period of two weeks gives a very small quantity of thyroxine. Block[3] repeated von Mutzenbecher's work using completely synthetic diiodotyrosine in order to eliminate the possibility that a trace of impurity, responsible for the thyroxine formation, had accompanied the tyrosine from its natural source. Johnson and Tewkesbury[4] made a further study of the reaction, obtaining a slight increase in the yield of thyroxine on the addition of hypoiodite to the solution and establishing pyruvic acid and ammonia as further products. As a result of their work, they postulated a mechanism evolved from the work of Pummerer[5] on the oxidation of *o*- and *p*-substituted phenols in alkaline solution. Their paper may be consulted for the proposed mechanism. We have made additional observations on the nature of the reaction, which throw further light on several points.

It appeared plausible that thyroxine may have been formed by the oxidizing action of the iodine solution originally used for the preparation of the diiodotyrosine rather than during the incubation of the diiodotyrosine. This possibility was eliminated when a sample of crude diiodotyrosine, as it was obtained from the iodination reaction, was subjected to butyl alcohol extraction for thyroxine. No thyroxine was found.

It was equally possible that a small amount of precursor was formed during the iodination and that this and not diiodotyrosine was converted into thyroxine during the long incubation period. A quantity of diiodotyrosine was therefore very carefully purified, but the yield of thyroxine after digestion was not altered.

To further eliminate the role of a small amount of accompanying impurity, diiodotyrosine which had already yielded thyroxine by incubation, was recovered, purified and re-incubated. Again thyroxine was formed.

In order to prove or disprove that oxidation, presumably by air, is involved in the formation of thyroxine, a solution of diiodotyrosine was incubated in an oxygen-free system under the usual conditions; it yielded no thyroxine. Whereas diiodotyrosine recovered from experiments carried out in the presence of air contain much brown, amorphous material, the diiodotyrosine recovered from this experiment was crystalline and only slightly darker than the starting material.

Conversely, the yield of thyroxine from a given amount of diiodotyrosine was increased by passing a slow stream of carbon dioxide-free air through the solution during the incubation.

In view of the work of Pummerer, *et al.*,[6] it was thought that incubation in the presence of a small amount of potassium ferricyanide might increase the yield of thyroxine. This appeared to be too drastic an oxidizing agent, however, as no thyroxine whatever was formed and the diiodotyrosine was converted to a dark brown amorphous substance.

A possible intermediate in the formation of thyroxine would be 3,5-diiodo-4-hydroxybenzoic acid, formed through the oxidative degradation of diiodotyrosine. An incubation carried out in the presence of this compound, instead of increasing the yield of thyroxine, completely prevented the conversion.

It was also found that 2,4,6-triiodophenol would not act as an intermediate to increase the

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(1) (c) von Mutzenbecher, *Z. physiol. Chem.*, **261**, 253 (1939).

(2) Ludwig and von Mutzenbecher, *ibid.*, **258**, 195 (1939).

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(4) Johnson and Tewkesbury, *Proc. Natl. Acad. Sci.*, **28**, 73 (1942).

(5) Pummerer and Rieche, *Ber.*, **59**, 2161 (1926).

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yield. The normal amount of thyroxine was obtained in the presence of this substance.

Experimental

3,5-Diiodotyrosine Dihydrate—This substance was prepared from *l*-tyrosine by the method of Bauer and Strauss.[7] It crystallizes as the dihydrate, which fact was overlooked by these authors but was pointed out by Savitskii.[8] From 32.6 g. of tyrosine, in a typical experiment, 62 g. (66%) of diiodotyrosine dihydrate of m. p. 198° (dec.) was obtained.

Thyroxine from the Alkaline Digestion of Diiodotyrosine.—The procedure of earlier workers[1,3] was adopted for our experiments. This involved digestion of 14 g. of diiodotyrosine in 325 cc. of 0.1 *N* sodium hydroxide at *p*H 8.8 for two weeks at 37.5°, followed by isolation of the thyroxine through its butyl alcohol-soluble potassium salt. From 25 to 40 mg. of thyroxine was normally obtained.

No Thyroxine from Undigested Diiodotyrosine.—A solution of 14 g. of diiodotyrosine dihydrate in 25 cc. of 2 *N* sodium hydroxide was extracted with twelve 25-cc. portions of wet *n*-butyl alcohol. On working up the combined butyl alcohol extracts by the usual procedure none of the potassium salt of thyroxine was obtained.

Digestion of Analytically Pure Diiodotyrosine.—Crude diiodotyrosine (30.2 g.) was dissolved in 175 cc. of 0.85 *N* hydrochloric acid, treated three times with activated carbon and crystallized by the addition of 20 g. of sodium acetate in 30 cc. of water. The dried product weighed 17.7 g. and melted at 202° (dec.).

Anal. Calcd. for $C_9H_9O_3NI_2$: I, 58.62. Found: I, 58.41.

Fourteen grams of this product digested in the usual way yielded 24 mg. of thyroxine; m. p. 228 (dec.).

Digestion of Recovered Diiodotyrosine.—A solution in 180 cc. of 0.12 *N* hydrochloric acid of 41.3 g. of diiodotyrosine recovered from previous runs was filtered free of brown, amorphous material, saturated with sulfur dioxide and treated twice with activated carbon. The diiodotyrosine was crystallized by the addition of 40 cc. of 50% sodium acetate. It was then recrystallized from 0.1 *N* hydrochloric acid by the addition of sodium acetate to give 17.1 g. of light yellow crystals melting at 200°.

Incubation of 14 g. of this recrystallized diiodotyrosine in the customary manner gave 39 mg. of thyroxine.

Digestion of Diiodotyrosine in the Presence of Potassium Ferricyanide.—Ten grams of diiodotyrosine dihydrate and 3.29 g. of potassium ferricyanide were dissolved in 231 cc. of 0.1 *N* sodium hydroxide, and the *p*H of the solution adjusted to 8.8. After the usual incubation period the solution was black. A dark brown amorphous precipitate was thrown out by acidification. This was examined in the usual way for thyroxine, but none was obtained.

A control experiment run simultaneously with the same diiodotyrosine gave a good yield of thyroxine.

Digestion of Diiodotyrosine in the Presence of 2,4,6-Triiodophenol and of 3,5-Diiodo-4-hydroxybenzoic Acid.—A. A mixture of 10 g. of diiodotyrosine dihydrate and 2.28 g. of triiodophenol, prepared by the method of Datta and Prosad,[9] was added to 280 cc. of 0.1 *N* sodium hydroxide. The solution, of *p*H 9.0, was placed in the incubator at 37.5° for fifteen days, although the triiodophenol was not entirely dissolved. The mixture was shaken daily to maintain saturation. After eleven days the *p*H had fallen to 8.7; it was raised to 8.9 and the solution digested five more days. The undissolved triiodophenol (1.68 g.) was filtered off and the solution worked up in the usual way for thyroxine, of which 29 mg. of m. p. 227° (dec.) was obtained.

B. A mixture of 10 g. of diiodotyrosine dihydrate and 1.56 g. of diiodohydroxybenzoic acid,[9] was added to 250 cc. of 0.1 *N* sodium hydroxide. All the diiodotyrosine dissolved but an appreciable amount of the benzoic acid remained undissolved. The system was incubated at *p*H 8.8, 37.5°, for fifteen days with daily shaking. The acids were precipitated by acidification and extracted in the usual fashion for thyroxine with a negative result.

Identification of Thyroxine.—The sample of our synthetic thyroxine employed for analysis and physiological testing was purified[10] by conversion into the potassium salt, followed by regeneration of thyroxine. This procedure was repeated three times. The material was obtained as microcrystalline sheaves and rosets, m. p. 233° (dec.). The sample was dried before analysis over phosphorus pentoxide for one hour at 100° (1 mm.).

Anal. Calcd. for $C_{15}H_{11}O_4NI_4$: I, 65.34. Found: I, 65.10.

The material gave a deep red color with nitrous acid and ammonia,[11] a specific reagent for ortho-iodophenols.

The method of Gaddum[12] for the qualitative assay of thyroxine and thyroid active substances by the use of tadpoles was employed for physiological testing. Our material proved to be fully as active as crystalline thyroxine, obtained from E. R. Squibb and Sons, in producing metamorphosis in tadpoles (*rana sylvatica*).

Summary

Some experiments on the *in vitro* formation of thyroxine from diiodotyrosine are reported.

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