metabolism of norepinephrine in the central nervous system is lacking. However, from experiments described here and elsewhere (*3*), it appears likely that O-methylation constitutes an important route for the metabolism of the norepinephrine in the brain.

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5. Under the conditions described above, metanephrine (*m*-O-methyl epinephrine) has markedly different R_f's from normetanephrine. No metanephrine could be detected in brain extracts of iproniazid-treated animals.
6. Normetanephrine and metanephrine were found to be present in the adrenal glands of iproniazid-treated rats.
7. Purified aldehyde dehydrogenase was prepared by a procedure described by H. Weissbach, B. C. Redfield, and S. Udenfriend (*J. Biol. Chem.*, **229**, 953 (1957) and kindly supplied by H. Weissbach.

3 December 1957

Extraction of an Osteogenic Inductor Factor from Bone

The hypothesis that osseous tissue contains an extractable substance which is capable of inducing the formation of new bone has been presented in detail by Bertlesen (*1*), Levander *et al.* (*2*), Willestaedt *et al.* (*3*), and La Croix (*4*) and has been denied by Heinen *et al.* (*5*) and Danis (*6*).

The percentage of all positive experimental results obtained to date, in rabbits and dogs, chiefly by alcoholic or acidic bone extracts (30.8 percent; that is, in 294 of 955 animals reported in the literature), when compared with the percentage of positive results in control animals (22.6 percent; that is, in 74 of 328 animals reported), while statistically significant, leaves room for refinement of techniques (*6*).

Intracerebral implantation (*7*) of a paste of bovine bone (*8*) in the young rat produced endocranial fusion of the normally patent coronal suture after 15 days. This paste had been stored for several months at 4°C in bovine plasma or physiological saline. Histologic examination of all paste samples showed that no living osteocytes or osteoblasts were present. In order to isolate the factors responsible for such osteogenic activity in a relatively resistant host, the following extractive procedures were undertaken.

Either 9 or 18 g of bone paste was incubated for 24 hours at 37°C in a solution consisting of 50 ml of Ringer–Tyrode and 20 ml of distilled water. The mixture was subsequently centrifuged and filtered. The resulting solution was metachromatic to toluidine blue. This reaction was lost after the solution was concentrated on a steam bath to one-fourth of its original volume. The clear brown solution was then stored at 4°C. Storage up to 30 days did not diminish its osteogenic activity.

Pieces of Gelfoam sponge were impregnated with the solution, either before or after concentration, and placed intracerebrally, under parietal bone flaps, in 22 seven-day-old Long-Evans rats. There were ten controls. Fifteen days after intracerebral implantation the animals were sacrificed, and the implantation sites were examined grossly and fixed with 10 percent Formalin. Decalcification with formic acid-sodium citrate solution was followed by paraffin embedding and hematoxylin and eosin staining of 10-μ serial sections.

The results were uniformly positive (Fig. 1). All cases of intracerebral implantation showed extensive osteogenic activity at the site of impregnated Gelfoam exclusively. Osteogenesis was present within the tissues which had invaded the sponge. This inductor activity was shown by all solutions tested, both concentrated and unconcentrated, of either strength, derived from plasma- or saline-stored paste. Grossly, the implants were firm and fused to the host calvaria. They gave a distinctly calcareous impression to a fine steel probe. Histologically, immature trabeculated bone was observed throughout the implant. Some of this new bone was fused to the host endocranial plate. Most of the bone was observed on serial section to have no such continuity. It consisted of isolated islands of osseous tissue in all stages of development. A very strong impression was obtained that these areas of bone had arisen *in situ*. At implantation sites the effective thickness of the calvaria was often four times that of adjacent areas. Active osteogenesis was underway on all surfaces of newly formed bone at sacrifice. The formation of new marrow spaces was frequent. The area of induced osteogenesis never extended beyond the area of implantation. Connective tissue and vascular infiltration and proliferation in the implant area was marked. The sponge had virtually disappeared at this state.

Control animals never showed the slightest osteogenic stimulation or induction following operative procedures alone, or following implantation of either plain, Ringer–Tyrode- or plasma-impregnated Gelfoam.

It seems probable that part of the observed osteogenic response was due to stimulation of preexistent osteoblasts in calvarial implantation sites by some factor extracted from the bone paste. An additional inductive capacity of the extracted solution was also clearly indicated in the numerous sites of new bone formation, demonstrably unconnected with host bone. A spectrum of modulating cell types was noted, ranging from undifferentiated connective tissue cells to mature osteocytes. Intermediate cell types showed increasing basophilia with accompanying cytoplasmic and nuclear enlargement. The intermediate cell type associated with the onset of bone matrix formation possessed an eccentric nucleus, intense basophilia, and a somewhat vacuolated cytoplasm.

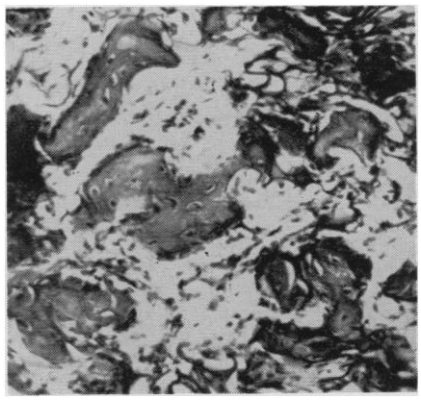

Fig. 1. Island of induced bone, 15 days after intracerebral implantation of Gelfoam impregnated with the extracted solution. Note the typical osteocytes and the sites of continued osteogenesis. This island is unconnected with and well removed from the host bone. In the immediate area several stages of connective tissue metaplasia were observed (× 750).

Biochemical analysis (*9*) of an osteogenically active solution derived from saline-stored bone paste demonstrated a concentration of 0.29 mg/ml of Chondroitin sulfate A or C, or both. This recovered material was redissolved in Ringer–Tyrode in a concentration of 0.1 mg percent. Gelfoam sponges impregnated with this solution were implanted as above and uniformly produced osteogenesis in eight rats. Additional fractions continue to be tested in a variety of heterotopic sites.

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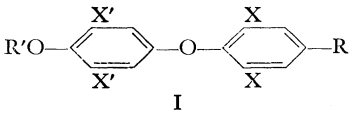
9. This analysis was carried out in the laboratory of Dr. Karl Meyer, Department of Medicine, College of Physicians and Surgeons, Columbia University. The methods have been described by K. Meyer *et al.* [*Biochem. et Biophys. Acta* 21, 506 (1956)] and by P. J. Stoffyn and R. W. Jeanloz [*Arch. Biochem. Biophys.* 52, 373 (1954)].

27 November 1957

Sterically Hindered Analogs of Thyroxine

In previous papers from this laboratory (*1–4*) the synthesis of various compounds of general structure I, related to thyroxine, was reported. In structure I, R = $-CH_2CH_2COOH$, $-CH_2CH(NH_2)COOH$, $-NH_2$, etc; X = iodine; X′ = iodine, methyl, etc.; and R′ = methyl or hydrogen.

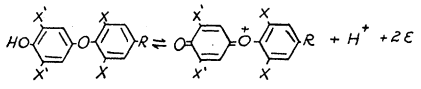


I

In an accompanying paper (*5*), an empirical correlation between structure and biological activity for 47 analogs of structure I was proposed.

The above correlation, while entirely empirical in nature, suggested significant deductions about the essential pharmacogen which is required for thyroxine-like activity. It also led to the conclusions that structural parameters, such as the electron-releasing abilities of X, X′ and OR′, the hydrogen bonding abilities of X and X′ and the *pK* values for the compounds (dependent on the nature of the ionizing side chain, R), are the probable factors which determine the comparative biological activity of these substances. Of major importance to our thinking in arriving at the correlative conclusions was the very striking fact (*5*) that the 3′,5′-dimethyl analogs of L- and D,L- thyroxine (structure I, X = iodine, X′ = methyl, R′ = H) were distinctly more active (*5*), in certain assays of thyroxine-like activity, than the corresponding stereoisomers of thyroxine. The suggestion that substitution of electron-releasing groups, such as methyl, in place of electron-attracting groups such as iodine, bromine, nitro, and so forth, can enhance thyroxine-like activity is a novel one and is in direct opposition to earlier considerations pertaining to such effects (*5, 6*).

The postulate of the Bruice-Kharasch-Winzler correlation, that electron-releasing groups in the 3′,5′-positions of structure I can enhance thyroxine-like activity, finds a possible rationale in the hypothesis of Niemann (*7*) that oxidation of thyroxine to a quinoid form, as shown below, may somehow be involved in its action. Essentially, this oxidation



appears to involve removal of the elements of a hydride group ($H^+ + 2e$) from the thyroxine analog, and this reaction should be enhanced by substituting electron-releasing groups into the back ring of thyroxine.

The above predictions of activities, and the possible relations to the Niemann hypothesis, can be tested by synthesis and biological evaluation of suitable compounds (such as L- and D,L- 3,5,3′-triiodo-5′-methylthyronines and others) in which electron-releasing groups are incorporated into the structures related to compound I. Compare, for example, reference *3*, for the synthesis of initial substances for these purposes.

We now also wish to report our studies toward the synthesis of compounds related to structure I, in which the X′ groups have favorable electron-releasing abilities, but the steric characteristics of which should be such as to cause significant steric complications toward an in vivo oxidative reaction, which may be involved in converting the analog of I to a quinoid form, as illustrated in the equation above. The first compound of this type which we wish to report is the 3′,5′-di-tertiarybutyl analog (X′ = *t*-butyl) and with R = $-CH_2CH_2COOH$. This compound was synthesized by the route shown in Fig. 1.

Compound III melted at 94° to 95°C and gave the following analysis: Calcd. for $C_{25}H_{32}N_2O_8$: C, 61.48; H, 6.56; N, 5.74; found: C, 61.68; H, 6.66; N, 5.57. The conversion of III to I (X = iodine; X′ = *t*-butyl; R′ = H; R = $-CH_2CH_2COOH$) was carried out by reducing to the diamine, diazotization and use of the Sandmeyer reaction to introduce iodine at the 3,5-positions, and hydrolysis of the intermediate ester; m.p. 120° to 121°C. Analysis (for the ester): Calcd. for $C_{25}H_{32}O_4I_2$: C, 46.16; H, 4.96; I, 39.03; found: C, 46.55; H, 4.97; I, 39.29. The final product, as the free acid, was obtained as excellent colorless needles, from aqueous ethanol, which melted at 197° to 198°C. Analysis: Calcd. for $C_{23}H_{28}O_4I_2$: C, 44.39; H, 4.53; I, 40.79; found: C, 44.53; H, 4.60; I, 40.60.

In the above compound, if the *t*-butyl groups are sufficiently large to block the in vivo oxidative reaction to the quinoid form, and if they are not removable in vivo (a question of general interest, which has yet to be resolved), then the possibility of finding a true competitive inhibitor to thyroxine is implied in this approach. The synthesis of various such molecules (the so-called "hinderins") is therefore a major objective of our studies (*8*).

A sample of "hinderin A" (structure I: X′ = *t*-butyl; X = iodine; R′ = H; R = $-CH_2CH_2COOH$) has been submitted for biological assay (effect on metabolism of glucose in *Aerobacter* aerogenes) by W. Marx and M. Gutenstein, who have reported interesting results for the initial screening.

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10 October 1957

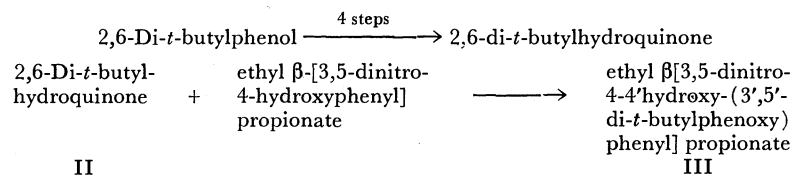

Fig. 1. Synthesis of the 3′,5′-di-tertiarybutyl analog of structure I.

On the Composition of Zymosan

Zymosan is the name given by Pillemer and Ecker (*1*) to a yeast fraction having the specific immunological property of inactivating the third component of complement, C′3. Later work indicated that zymosan adsorbs properdin, a radiation-sensitive serum protein reported to protect mammals from the spread of microbiological infection (*2*). Further interest in zymosan is derived from the finding that its injection into mice, rats, and rabbits results in an immediate decrease in the properdin titer followed by an increase to levels sometimes three times greater than the initial properdin level (*3*). Consistent with this property are the observations that zymosan decreases the lethal effect of x-radia-