The specificity of this absorbed antibody for *A. carolinensis* crystallins, the lens-specific proteins, was determined by its lack of reactivity (in immunoelectrophoretic and immunodiffusion analyses) with extracts (10 mg/ml) of other *A. carolinensis* tissues such as liver, brain, skin, serum, and eye minus lens. Immunoelectrophoresis of this antibody with its homologous antigen confirmed the presence of three main classes of crystallins—α, β, and δ crystallins—in the lateral eye lens of this organism, as reported for birds and reptiles (*7*). Such an antibody is suitable for use in immunofluorescence detection of corresponding antigens, that is, crystallins, in tissue sections. Alcohol-fixed sections (3 μm) through the median eye of *A. carolinensis* were processed, subjected to the antibody to the crystallins in the indirect or "sandwich" immunofluorescence technique (*8*), and examined as described (*6*). A positive immunofluorescence reaction for *A. carolinensis* lateral eye lens crystallins, with the apple-green fluorescence of fluorescein isothiocyanate as antigen marker, was detected in the median eye. The fluorescence observed is significant not only because of its presence, but also because of its intensity and specific localization; it was restricted to those cells comprising the lens of the median eye (Fig. 1B). Specific immunofluorescence was absent from other median eye tissues, including the retina and the cornea. Controls, consisting of substituting nonimmune rabbit gamma globulin for the antibody to total lens protein, also gave negative results for crystallins.

The lateral eye lens contains proteins shared by widely divergent vertebrate classes, a characteristic which is indicative of its immunologic "organ specificity" (*9*). These results suggest that this evolutionary conservatism of lens proteins (crystallins) may also be shared with other tissues in the same organism, that is, there may exist an exception to their acknowledged "tissue specificity" (*6, 10–12*). The fact that crystallins are found in a vestigial structure (*1*) thought to share functional properties (light-sensitivity), with its homolog (the lateral eye), lends credence to current hypotheses of gene action based upon the concept of variable gene activity. Supporting the latter view are observations (*13*) that (i) differences in DNA sequences in differentiated cells cannot be detected; (ii) only approximately 10 percent of the genome is engaged in DNA or RNA synthesis; and (iii) a distinct spectrum of genes is active in each tissue.

A possible relationship between such gene versatility and crystallin appearance is suggested by the well-known ability of the dorsal margin of the iris to regenerate a lens after lentectomy in the newt (*14*). In this case, a tissue not having or producing crystallins becomes one that does so (*11, 12*). It has also been reported (*15*) that larval *Rana pipiens* lens epithelial cells, which do not normally produce detectable amounts of γ crystallins, can be stimulated to produce this lens-fiber specific protein (*6, 12, 16*) by placing them in hanging-drop cultures, thus changing the normal cellular milieu.

Presumably, the lens tissue of the median eye has a similar gene complement to that of lens tissue of the lateral eye. Expression of these genes, that is, production of crystallins, is demonstrated by both tissues, whose functions are ostensibly the same, but whose anatomical location and embryonic origins differ significantly.

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References and Notes

1. For reviews, see A. Oksche, *Progr. Brain Res.* **10**, 4 (1965); R. A. Hoffman, *Amer. Zool.* **10**, 191 (1970); R. M. Eakin, *Amer. Sci.* **58**, 73 (1970).
2. R. M. Eakin, *Rev. Suisse Zool.* **71**, 267 (1964); A. Petit, *Arch. Anat. Histol. Embryol.* **50**, 227 (1968).
3. R. M. Eakin and J. A. Westfall, *J. Biophys. Biochem. Cytol.* **8**, 483 (1960).
4. Results are based upon evaluation of 26 immunofluorescence analyses of 14 median eyes. A total of 42 lateral eye lenses provided protein for use in immunologic procedures.
5. D. S. McDevitt, *J. Exp. Zool.* **164**, 21 (1967).
6. ———, I. Meza, T. Yamada, *Develop. Biol.* **19**, 581 (1969).
7. M. Rabaey, *Exp. Eye Res.* **1**, 310 (1962).
8. T. H. Weller and A. H. Coons, *Proc. Soc. Exp. Biol. Med.* **86**, 789 (1954).
9. P. T. Uhlenhuth, *Robert Koch Festschrift* (Fisher, Jena, 1903), p. 49; L. Hektoen, *J. Amer. Med. Ass.* **77**, 32 (1921); W. Manski, T. P. Auerbach, and S. P. Halbert, *Amer. J. Ophthalmol.* **50**, 985 (1960).
10. C. Takata, J. F. Albright, T. Yamada, *Exp. Cell Res.* **34**, 207 (1964); J. Zwaan and A. Ikeda, *Exp. Eye Res.* **7**, 301 (1968).
11. C. Takata, J. F. Albright, T. Yamada, *Develop. Biol.* **9**, 385 (1964).
12. ———, *ibid.* **14**, 382 (1966).
13. B. J. McCarthy and B. H. Hoyer, *Proc. Nat. Acad. Sci. U.S.* **52**, 915 (1964); J. Paul and R. S. Gilmour, *J. Mol. Biol.* **34**, 305 (1968); M. Miyagi, D. Kohl, R. A. Flickinger, *J. Exp. Zool.* **165**, 147 (1967).
14. For reviews, see R. W. Reyer, *Quart. Rev. Biol.* **29**, 1 (1954); T. Yamada, *Curr. Top. Develop. Biol.* **2**, 247 (1967).
15. D. S. McDevitt and T. Yamada, *Amer. Zool.* **9**, 1130 (1969).
16. J. Papaconstantinou, *Biochim. Biophys. Acta* **107**, 81 (1965).
17. Supported by General Research Support Fund grant FR-05464-07 from the University of Pennsylvania School of Veterinary Medicine, and by grant NSF-IG-70-11 to the University of Pennsylvania. I thank Drs. R. M. Eakin, J. W. Lash, and W. C. D. Hare for advice and discussion.

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Triiodothyronine: The 3′-Iodine Is Proximal to the α-Ring in Crystal Structure Conformation

Abstract. *The crystal and molecular structure of the thyroid hormone* L-*triiodothyronine has been determined by x-ray diffraction. The two phenyl rings are almost perpendicular to each other, the acute angle between their normals being 82 degrees. The 3′-iodine is situated* proximal *to the* α*-ring, rather than* distal *as inferred from chemical studies. Theoretical calculations indicate this* proximal *conformation to be energetically favored over the* distal *one.*

The thyroid hormones L-thyroxine (T_4) and 3,5,3′-triiodo-L-thyronine (T_3) exhibit many important biological effects and are essential for normal growth and development and the control of oxidative metabolism. T_3 appears to be at least three to four times as potent as T_4 in most biological tests, and there is evidence that it is the more important hormone both in mammalian physiology and in most clinical situations (*1, 2*). It has been shown (*3, 4*) that more than one-third of circulating T_3 may arise from extrathyroidal conversion of T_4 to T_3 in normal human subjects, and this has given support to the speculation that T_4 may act primarily as a prohormone, and exert its effect only after transformation to T_3 (*2, 4*).

Although the biological importance of L-triiodothyronine is thus well established, the mechanisms of its actions are poorly understood. A great many thyroid hormone analogs have been prepared, and their hormonal activities have been measured in attempts to establish chemical features essential to thyromimetic action (*5*). It has been suggested that conformational structure, as well as chemical features, is of prime importance to hormonal action. Jorgen-

son *et al.* (*6*) recognized that the chemically identical 3′ and 5′ positions on the phenolic (β) ring of T_3 are not equivalent conformationally; they concluded from the synthesis and testing of "conformationally fixed" analogs of T_3 that, in order for triiodothyronine to exhibit regulatory activity upon cellular oxygen consumption, the β ring must be oriented with its 3′-iodine *distal* to the diiodotyrosine (α) ring and that the "free" (uniodinated) 5′ position must be *proximal* to the α ring.

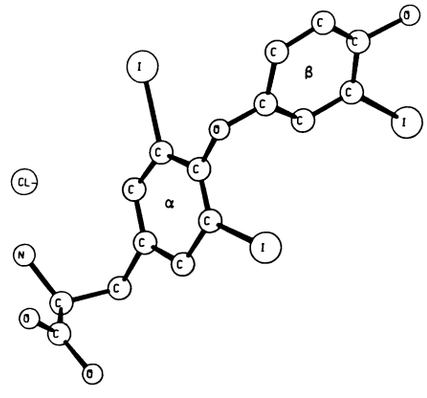
We have determined the crystal structure of 3,5,3′-triiodo-L-thyronine as part of an investigation of thyroid hormone structure, in the hope that a knowledge of molecular stereochemistry may be of help in understanding the ways in which hormonal functions are effected. This is the first thyroid hormone to have its three-dimensional molecular structure elucidated.

3,5,3′-Triiodo-L-thyronine was dissolved in a mixture of methanol and HCl. Slow evaporation of the solvent yielded very small needle-shaped crystals. The crystals are monoclinic with $a = 29.080$, $b = 5.236$, $c = 17.047$ Å, $\beta = 115.85°$, space group $C2$ with $Z =$ four molecules per unit cell. Intensities of 1341 reflections to $2\theta = 100°$ (θ is the Bragg angle) for CuKα radiation were measured on an automated four-circle diffractometer. The intensities of reflections used as standards fell appreciably during the collection of the data, and the crystal slowly changed from colorless to yellowish, indicating that decomposition was taking place during exposure to x-rays, probably with the liberation of iodine. A linear corrective factor was applied to the data for the decrease in intensities with time, and the data were further corrected for x-ray absorption. The structure was solved by analysis of the Patterson function; the positions of two of the three iodine atoms were deduced from the three-dimensional Patterson map, and subsequent iterative Fourier methods revealed the positions of all the other atoms. It was discovered from the plots of electron density that the T_3 molecule had crystallized from the methanol-HCl solution as the hydrochloride, and during the refinement we also found that three molecules of water were present, and that they form a hydrogen-bond network with each other and with the carboxyl and phenol oxygens. After refinement by anisotropic full-matrix least-squares methods the discrepancy index $R = 0.07$. No attempt was made to locate the hydrogen atoms.

Table 1. EHMO Calculations on T_3.

Conformation	Total orbital energy (kcal)
3′-Iodine *proximal* to α-ring	−49,547
3′-Iodine *distal* to α-ring	−49,415

A perspective drawing of the T_3 molecular conformation is shown in Fig. 1, and a space-filling model of the structure constructed to fit the observed atomic positions is given in Fig. 2. Both aromatic rings are planar and are close to being mutually perpendicular; the acute angle between normals to the two rings is 82°. The inter-ring C-O-C angle of 125.6° is somewhat larger than the normal ether linkage angle, and is undoubtedly increased in order to alleviate steric interactions between the two rings. The other bond distances and angles in the molecule, although not exceptionally accurate because of the domination of the x-ray scattering by the iodines, exhibit normal values. The most surprising feature of the conformational structure is that the orientation of the β ring is such that the 3′-iodine is situated *proximal* to the α ring, and the uniodinated 5′ position



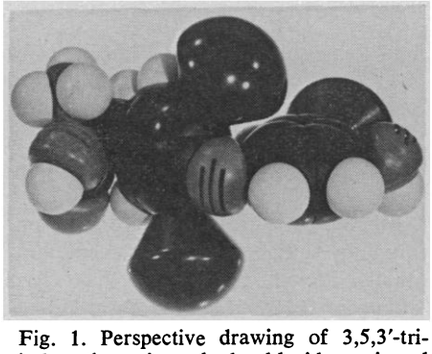


Fig. 1. Perspective drawing of 3,5,3′-triiodo-L-thyronine hydrochloride, viewed down the [010] crystal direction. Fig. 2. Photograph of space-filling model of the 3,5,3′-triiodo-L-thyronine molecule (Cl ion omitted).

distal, rather than the opposite orientation, which was expected from the chemical studies (*6*).

In order to test whether this conformation was an artifact of the crystal structure—that is, if it were favored because of strong intermolecular interactions existing when the molecules pack in the crystal with the 3′-iodine in this orientation—we calculated intermolecular distances between the atoms of one molecule and those of all its nearest neighbors. All distances correspond to normal van der Waals separations; thus no attractions of the charge-transfer I · · · O type, such as have been noted (*7*), are observed here to be a factor in stabilizing the 3′-iodine proximal orientation.

An attempt was then made to compare the stabilities of the 3′-iodine proximal and distal orientations by calculating the total energy of each conformation directly and independently of crystal environment. We used the extended Huckel molecular orbital (EHMO) method (*8*), and for our calculations, we used (i) the T_3 atomic coordinates from the results of the crystal structure (omitting the Cl^- and water molecules, and using an uncharged T_3 molecule) and (ii) the T_3 atomic coordinates generated by rotating the β ring 180° about the axis of the ether oxygen and the β-ring carbon to which it is bonded (this generates the 3′-iodine distal conformation). In both cases, all hydrogen atoms were included in the calculations, reasonable positions for them being calculated from a knowledge of the expected bond distances and angles involved. The EHMO results are given in Table 1; the total energy for the T_3 molecule with the 3′-iodine in the proximal position (the observed crystal conformation) is lower than that calculated for a T_3 molecule with the 3′-iodine occupying the distal position. [Only qualitative significance should be attributed to the difference of 132 kcal calculated between the two conformations, as the EHMO method tends to lead to an overestimation of energy differences (*9*)].

Our investigations have resulted in the first three-dimensional structural determination of a thyroid hormone. They show that the conformation of the T_3 molecule in the crystal has the 3′-iodine *proximal* to the α ring, and theoretical calculations indicate that this conformation is energetically more stable than the *distal* 3′-iodine conformation; this is in contradiction to the conformation

concluded to be the pharmacologically active one, as judged from tests relating chemical structure to biological activity (*6*). This contradiction could be caused by (i) the chemical studies being inconclusive, or (ii) the 3′-iodine distally oriented conformation being necessary for hormonal activity and the total energy of T_3-receptor complex being favorable enough to easily effect rotation of the β ring.

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References and Notes

1. K. Sterling, D. Bellabarba, E. S. Newman, M. A. Brenner, *J. Clin. Invest.* **48**, 1150 (1969); K. Sterling, *Recent Progr. Hormone Res.* **26**, 249 (1970).
2. H. L. Schwartz, M. I. Surks, J. H. Oppenheimer, 53rd Meeting of the Endocrine Society, San Francisco, 24–26 June 1971, abstract 132. Interestingly, an inference that T_4 might function primarily as a precursor of T_3 was given as long ago as 1953 [J. Gross and R. Pitt-Rivers, *Biochem. J.* **53**, 652 (1953)].
3. L. E. Braverman, S. H. Ingbar, K. Sterling, *J. Clin. Invest.* **49**, 855 (1970); C. S. Pittman, J. B. Chambers, Jr., V. H. Read, *ibid.* **50**, 1187 (1971).
4. K. Sterling, M. A. Brenner, E. S. Newman, *Science* **169**, 1099 (1970).
5. T. C. Bruice, R. J. Winzler, N. Kharasch, *J. Biol. Chem.* **210**, 1 (1954); E. C. Jorgenson and J. Wright, *J. Med. Chem.* **13**, 745 (1970) and references therein; S. B. Barker, M. Shimada, M. Makiuchi, *Endocrinology* **76**, 115 (1965).
6. E. C. Jorgenson, P. A. Lehman, C. Greenberg, N. Zenker, *J. Biol. Chem.* **237**, 3832 (1962); E. C. Jorgenson, N. Zenker, C. Greenberg, *ibid.* **235**, 1732 (1960).
7. O. Hassel and C. Rømming, *Quart. Rev. (London)* **16**, 1 (1962); N. Camerman and J. Trotter, *Science* **144**, 1348 (1964).
8. The EHMO program employed originated from R. Hoffman [*J. Chem. Phys.* **39**, 1397 (1963)] and was somewhat modified for local use by S. A. Houlden and I. G. Csizmadia. The Slater orbital exponents (SOE) for the iodines were from E. Clementi, D. L. Raimondi, W. P. Reinhardt [*J. Chem. Phys.* **47**, 1300 (1967)], and for light atoms, from E. Clementi and D. L. Raimondi [*J. Chem. Phys.* **38**, 2686 (1963)]. The valence state ionization potentials (VSIP) for the light atoms were taken from H. Basch, A. Viste, H. B. Gray [*Theor. Chim. Acta* **3**, 458 (1965)], and for the iodines values were derived by extrapolation of curves through values for F, Cl, and Br given by L. C. Cusachs and J. E. Corrington [in *Sigma Molecular Orbital Theory*, O. Sinanoglu and K. B. Wiberg, Eds. (Yale Univ. Press, New Haven, 1970)]. Iodine unoccupied 5*d* orbitals were included in the calculations, with SOE and VSIP values obtained from linear extrapolation of similar values for Cl and Br from L. C. Cusachs and J. R. Linn [*J. Chem. Phys.* **46**, 2919 (1967)]. The *K* coefficient in the Wofsberg-Helmholtz formula for the off-diagonal matrix elements was 1.75.
9. I. G. Csizmadia, private communication.
10. We thank I. G. Csizmadia and J. Haines for help in setting up the EHMO calculations, and Smith, Kline and French Ltd. for a gift of triiodo-L-thyronine. Support was from the J. P. Bickell Foundation and from PHS research grant 1 RO1 NS-09839-01 BBCA from the NINDS.
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Tissue Factor (Thromboplastin): Localization to Plasma Membranes by Peroxidase-Conjugated Antibodies

Abstract. *Peroxidase-conjugated antibodies were used to determine the histologic and cytologic localization of bovine and human tissue factor (thromboplastin). Tissue factor antigen was found in highest concentration in the intima of blood vessels, particularly in the plasma membranes of endothelial cells and in human atheromatous plaques. Tissue factor was also found limited to the plasma membranes of many cell types. The presence of tissue factor in the plasma membranes of endothelial cells and atheromata suggests that it may play a significant role in hemostasis and thrombosis.*

Blood coagulation proceeds by two mechanisms in which proteins, lipids, and calcium ions interact to yield a fibrin clot (*1*). The intrinsic system is initiated in vitro by contact with any of a number of surfaces including collagen, although the nature of the physiological activator is uncertain. The extrinsic or tissue factor system is activated by a specific particle-bound lipoprotein found in extracts of many tissues. Nemerson and Pitlick have purified a protein solubilized from delipidated bovine lung powder; this protein, when combined with certain phospholipids, has the biological characteristics of tissue factor (*2*). We have now prepared specific rabbit antiserums to purified bovine lung and human placenta tissue factor. Using the techniques of Nakane and Pierce (*3*) and Avrameas and Bouteille (*4*) for localizing tissue antigens with peroxidase-conjugated antibodies, we have shown that tissue factor is present in the plasma membranes of many cell types. Significantly, it is in highest concentration in the plasma membranes of endothelial cells and in atheromata where it may interact with circulating clotting factors to initiate coagulation.

Antibodies were produced as follows. White New Zealand rabbits were given an initial intradermal injection of 1 mg of purified human or bovine tissue factor with complete Freund's adjuvant, and subsequent biweekly injections of 100 μg of the antigen; the antiserum was collected for the first time 2 weeks after the third injection, and then 2 weeks after subsequent injections. Gamma globulin was precipitated and washed with 33 percent saturated ammonium sulfate and dialyzed in phosphate-buffered saline, *p*H 7.2, ionic strength, 0.15. Serum from unimmunized rabbits was processed in the same way and used for controls.

The rabbit antibody to bovine tissue factor neutralized the coagulant activity of preparations derived from lung, brain, kidney, spleen, and liver. The antibody to human tissue factor neutralized tissue factor from human brain and placenta. When the soluble form of tissue factor from bovine lung and human placenta was studied by immunodiffusion (*5*), two precipitin lines were seen. These most likely correspond to the two species of tissue factor previously noted (*2*).

Horseradish peroxidase (Miles Laboratories) was conjugated to the γ-globulin fraction of sheep antiserum to rabbit globulin (GIBCO) by a slight modification of the technique of Avrameas and Bouteille (*4*). Sections (6 μm) were dried in air and fixed in 10 percent formaldehyde in phosphate-buffered saline for 5 to 10 minutes. Tissue sections were incubated for 30 minutes at room temperature in γ-globulin (1 mg/ml) either from unimmunized rabbits or from rabbits immunized against tissue factor; the sections were incubated with the peroxidase–sheep globulin conjugate and stained for peroxidase activity (*6*). An easily recognizable brown-black reaction product was found in sections incubated with globulin from immunized rabbits. A faint brown coloration of membranes and cytoplasm in controls was attributed to nonspecific binding of conjugated protein.

In all tissues studied, the intense brown staining of the tissue factor antigen was located on the plasma membranes of most cells (Fig. 1A). Blood vessels showed a typical staining regardless of their size or location. There was marked endothelial staining, particularly of the plasma membranes, with lesser amounts in the media (Fig. 1, C, D, and F). The adventitia was essentially devoid of antigen. The findings were identical in human and bovine tissues. Of particular interest was the concentration of tissue factor in the intimal and subintimal tissues of human atheromata (Fig. 1, H and I). Cholesterol crystals were completely surrounded by intensely staining cells.