governing criterion for the formation of a given modification. Unfortunately, the insolubility of the compound in non-polar media prevented extension of the work to solvents of this character.

Obviously, observations on solutions are not necessarily characteristic of the situation which may exist in the solid state. Thus, solutions of tautomeric substances exhibit physical and chemical properties which may be expected on the basis of the relative proportions of the two forms present at a given time. Yet, in certain cases, the individual compounds comprising a tautomeric pair have been isolated and crystallized. When one deals with a substance which exists in more than one crystalline form for which classical structural theory provides no explanation, polymorphism is the customary interpretation regardless of the stability or persistence of identity of those forms. For example, Straus and Demus[8] found two forms of dibiphenylylcarbinol which were very stable, uninfluenced by seeding with each other and which were only slowly interconverted under various conditions. They exhibited different absorption characteristics in benzene. Similar cases of polymorphism have been cited by Naves and Korpi[9] in connection with their work on the oxalates of *dl*-menthol. Since various recorded cases of polymorphic forms show such marked differences in the degree to which their identity is retained, v. Auwers and Schaum[10] made a somewhat artificial classification of them into five groups. It is apparent that the physical and chemical behavior of many of these is such that they would be designated geometrical isomers if structural theory had so permitted. Obviously, where classical structural formulas are inadequate, it is necessary to find an explanation at the level of atomic arrangements within the molecule. This has been accomplished for the polymorphic forms of anhydrous oxalic acid[11] for which Raman spectra and infrared absorption indicated a *cis–trans* relationship at the carbon–carbon bond. This has been confirmed by Dunitz and Robertson[12] who deduced from an electron diffraction study of the hydrated acid that the carbon–carbon bond was slightly contracted and would possess attributes of the ethylenic linkage.

Infrared studies, which are expected to furnish more precise information on the discrete differences between the two solid modifications of 5-nitro-2-furaldehyde semicarbazone, have been initiated by these laboratories, and will be published at a later date. Without reference to the structural organization of the molecule which can produce two crystalline forms, the behavior of these modifications, particularly with reference to their interconvertibility and loss of identity in solution, is consistent with the simpler and more conventional cases of polymorphism.

(8) Straus and Demus, *Ber.*, **59**, 2427 (1926).
(9) Naves and Korpi, *Helv. Chim. Acta*, **30**, 1219 (1947).
(10) v. Auwers and Schaum, *Ber.*, **62**, 1675 (1929).

Summary

Two crystalline forms of 5-nitro-2-furaldehyde semicarbazone have been observed and their physical properties investigated. The gross behavior of the two forms is typical of that conventionally assigned to the more common examples of polymorphism.

(11) Duval and Lecomte, *Compt. rend.*, **212**, 389 (1941).
(12) Dunitz and Robertson, *J. Chem. Soc.*, 142 (1947).

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[CONTRIBUTION FROM THE DIVISION OF RADIOLOGY, UNIVERSITY OF CALIFORNIA MEDICAL SCHOOL, SAN FRANCISCO, AND THE RADIATION LABORATORY, UNIVERSITY OF CALIFORNIA, BERKELEY]

Paper Chromatography in Synthetic Organic Chemistry. Microgram Scale Syntheses of Labeled Monoiodotyrosine, Diiodotyrosine and Thyroxine[1]

BY R. M. LEMMON, WINIFRED TARPEY AND KENNETH G. SCOTT

The availability of labeled organic iodide of high specific activity is very desirable for tracer studies of thyroid metabolism. Investigations into some of the differences in the thyroid activity of normal and tumor-bearing animals are now under way at the University of California Medical School and, in connection with these studies, we have carried out *in vitro* syntheses of I^{131}-labeled monoiodotyrosine, diiodotyrosine and thyroxine on a microgram scale. In order to isolate and identify the products of these syntheses we have made use of the technique of paper chromatography.[2] This technique, which has already proved so valuable in amino acid work, provided us with a method by which our products could be isolated from their respective reaction mixtures without the addition of any inactive carrier. The positions of the amino acids on the chromatograms were located by preparing radioautographs of the chromatograms. The identity of the material in a given spot was established by preparing mixed chromatograms of the eluted material with a known sample of monoiodotyrosine, diiodotyrosine or thyroxine. Although a number of procedures for the quantitative estimation of

(1) The work described in this paper was sponsored by the Atomic Energy Commission. It was supported by a grant from the Henry, Laura and Irene B. Dernham Fund of the American Cancer Society and the Christine Breon Fund.

(2) R. Consden, A. H. Gordon and A. J. P. Martin, *Biochem J.*, **38**, 224 (1944).

amino acids on paper chromatograms have been published,[3,4,5,6,7] we were not able to use any of them as they all involve a chemical reaction, usually the ninhydrin reaction, which leads to the destruction of the amino acid. Instead, we were able to make semi-quantitative estimations of our yields by spectrophotometry of the pure products. This technique is not feasible for most amino acids but is very satisfactory for the tyrosine derivatives due to the presence of the aromatic group; in alkaline solution the phenolate ions of these compounds have comparatively high molar extinction coefficients at their absorption maxima.

The presence of monoiodotyrosine in the thyroid has been reported by Fink and Fink.[8] The only *in vitro* synthesis of this compound (other than the hydrolysis of iodinated protein material) which hitherto has been reported is that of Harington and Rivers.[9] The present work shows that this compound may be synthesized easily, together with diiodotyrosine, by means of the direct iodination of tyrosine.

Experimental Part

Preparation of 3,5-Diiodo^{131}-L-tyrosine and Monoiodo^{131}-L-tyrosine.—The method of Harington[10] for the iodination of tyrosine was adapted to a micro-scale preparation. Radioactive iodide was oxidized to iodine with potassium iodate by the method of Horeau and Süe.[11] Micropipets were used to add aliquot portions of the reactants to the 0.2-ml. centrifuge tube used as the reaction vessel.

In a typical experiment, 3050 μc. (0.028 μg.) of carrier-free NaI^{131} in 1.0 ml. of a sodium bisulfite buffered solution was added gradually to 238 μg. (1.43 μmoles) of potassium iodide in 30 μl. of water. The solution was concentrated to approximately 10 μl. by means of a heat lamp and a stream of nitrogen blown over the surface of the liquid.

After the iodide solution was cooled in an ice-bath, 120 μg. (0.56 μmole) of potassium iodate in 20 μl. of water and 11 μl. of glacial acetic acid were added, giving 1.72 μeq. of iodine. Ninety micrograms (0.497 μmole) of recrystallized L-tyrosine dissolved in 20 μl. of concentrated ammonium hydroxide was added to the iodine solution with stirring; the iodine color slowly disappeared during this addition. To complete the reaction, 84 μg. (0.66 μeq.) of iodine in 20 μl. of 0.174 *N* potassium iodide solution was added to the reaction mixture. Thus the 3050 μc. of I^{131} was distributed among 6.13 μeq. of triodide (including the excess iodate which was reduced to iodide by the bisulfite). After it was allowed to stand for six hours with occasional stirring, the entire solution was transferred to the corner of a large filter paper and chromatographed (details below).

The monoiodotyrosine obtained after elution contained 79 μc. or 2.6% of the starting activity and the diiodotyrosine contained 355 μc. or 11.6% of the starting I^{131} (allowing for the radioactive decay of the iodine during the reaction). That the starting tyrosine was completely reacted was shown by spraying the paper with ninhydrin. No tyrosine could be detected (sensitivity limit about 5 μg.). From the known specific activity of the iodine used in the reaction (3050 μc./6.13 μeq. = 498 μc./μeq.) the specific activities of the mono- and diiodotyrosine products are known to be 498 and 996 μc./μmole, respectively. From these values and the total activities given above the yields of the two products were calculated to be 48 and 154 μg., respectively. These yields are 32 and 71% of the starting tyrosine. These calculations, as well as the work of Miller, *et al.*,[12] indicate that the exchange between the inorganic and organically-bound iodine is almost completed after the six-hour reaction period. A semi-quantitative determination by spectrophotometry (as described below) gave a value of 136 μg. for the yield of diiodotyrosine.

A decrease in the starting ratio of iodine to tyrosine led to a decreased quantity of diiodotyrosine. For example, in a preparation with a starting ratio of iodine:tyrosine of 2.1, the ratio of diiodotyrosine:monoiodotyrosine was 2.2. When the starting ratio was 1.9, the ratio for the products was 1.4. A reaction period shorter than six hours was found to give incomplete iodination of the tyrosine.

Chemical analyses were obtained for an inactive iodination reaction run on a 100-mg. scale under the same conditions as the micro preparation described. In this case, the 3,5-diiodo-L-tyrosine was precipitated from the reaction mixture by removing the excess ammonia *in vacuo* and adjusting the solution to a *p*H of 4. (The monoiodo-L-tyrosine was not recovered from the filtrates.) The product, which was purified by charcoal treatment and recrystallized from dilute alcohol, melted at 202° (dec.).

Anal. Calcd. for $C_9H_9O_3NI_2$: C, 24.96; H, 2.09; I, 58.62. Found: C, 24.92; H, 2.06; I, 58.40.

Chromatography and Spectrophotometry of Mono- and Diiodotyrosine.—The chromatography of the mono- and diiodotyrosine reaction mixture was carried out on Schleicher and Schuell filter paper number 589 (23″ × 23″). Excellent separations were obtained by developing the paper in one direction with a butanol–acetic acid–water (74:19:51 parts by volume) solvent and in the other direction with water-saturated phenol. Satisfactory separations also could be accomplished by developing the paper in a single direction with the same butanol-acetic acid solvent. The chromatography was accomplished in the usual fashion[13] by allowing the paper to hang down from a glass trough containing the developing solvent in which one edge of the paper is immersed; the operation was carried out in a closed cabinet which was placed in a room where the temperature was kept at 22–25°. The developed papers were allowed to dry in a hood at room temperature. A radioautograph of the chromatogram was prepared by placing a sheet of X-ray film (Eastman Kodak Co. "No-Screen," 14″ × 17″) directly in contact with the chromatogram for about one minute in a photographic dark room and then developing the film in the usual way. The radioautograph showed three distinct, well-separated spots. Two of these spots (together containing about 15% of the activity on the paper) were near each other in the far corner of the paper; their average R_F values[14] were 0.66 and 0.76 for the butanol–acetic acid solvent. The third spot, which contained most of the activity, was not developed nearly as far (R_F value approximately 0.32) and contained unused sodium iodide. The radioautographs from some of the syntheses also showed a small fourth area of activity which had an R_F value approaching unity. This area contained free iodine and/or iodinated phenol.

The two spots which contained radioactive organic iodine were cut out of the paper and eluted separately overnight with approximately 3 ml. of 50% alcohol. The

(3) H. B. Bull, J. W. Hahn and V. H. Baptist, THIS JOURNAL, **71**, 550 (1949).

(4) A. J. Landua and J. Awapara, *Science*, **109**, 385 (1949).

(5) A. J. Woiwod, *Nature*, **161**, 169 (1948).

(6) R. B. Fisher, D. S. Parsons and G. A. Morrison, *ibid.*, **161**, 764 (1948).

(7) L. Naftalin, *ibid.*, **161**, 763 (1948).

(8) K. Fink and R. M. Fink, *Science*, **108**, 358 (1948).

(9) C. R. Harington and R. V. P. Rivers, *Biochem. J.*, **38**, 320 (1944).

(10) C. R. Harington, *ibid.*, **22**, 1429 (1928).

(11) A. Horeau and P. Süe, *Bull. soc. chim. biol.*, **27**, 483 (1945).

(12) W. H. Miller, G. W. Anderson, R. K. Madison and D. J. Salley, *Science*, **100**, 340 (1944).

(13) For details of the chromatographic procedures see R. Consden, *Nature*, **162**, 359 (1948).

(14) The R_F value is the ratio of the distance which a substance has moved on a chromatogram to the distance which the solvent moved on the same chromatogram.

elution was effected by cutting the paper so that it had a tapered end, suspending the paper downward from a glass trough containing the eluting solvent, and allowing the tapered end to rest against the side of a sloping test tube—the operation was carried out under a glass jar. The elution was followed by observing the decrease of radioactivity on the paper strip as the solvent was drawn down the paper by capillary action.

A semi-quantitative estimation of the amount of diiodotyrosine prepared was made by adding 1 drop of 0.1 *N* sodium hydroxide to the eluate and observing the optical density of the solution at 315 mμ, the wave length of the main absorption maximum, as determined with a Beckman spectrophotometer (Model DU). The molar extinction coefficient for diiodotyrosine in basic alcoholic solution at this wave length was found to be 6.58×10^3. In order to carry out the spectrophotometry it was necessary to pre-wash the paper and to use redistilled solvents for both the chromatography and the elution. The paper was prewashed by allowing the redistilled butanol–acetic acid developer to move all the way down the empty paper and drip from the bottom of the paper for about one day; the pre-washing otherwise was carried out in the same manner as the chromatography itself. A blank on the spectrophotometry was obtained by eluting an empty area of the chromatographic paper which was adjacent to the diiodotyrosine spot and equal to it in area; the volume of the eluting solvent was equal to that used for the diiodotyrosine spot. The optical density of this solution was subtracted from that obtained for the diiodotyrosine eluate in order to determine the optical density due to the amino acid alone. In this way it was found to be convenient to measure the optical density of solutions of 50–150 μg. of the amino acid in 2–3 ml. of solution. With 150 μg. of diiodotyrosine the estimated accuracy of the method is within five per cent.

The identity of the monoiodotyrosine was established by rechromatographing the radioactive material from the lower (R_F value 0.66, butanol–acetic acid) of the two adjacent spots with a sample of known monoiodotyrosine which was prepared by C. R. Harington and R. V. P. Rivers.[9] The radioactivity coincided exactly with the position of the amino acid as shown by spraying the paper with the ninhydrin reagent (0.5% in absolute alcohol) and by preparing a radioautograph of the chromatogram. The chromatographic identity was established using not only the butanol–acetic acid solvent but also when water-saturated 2,4-lutidine was used as the chromatographic developer. The identity of the upper spot (R_F value 0.76, butanol–acetic acid) as diiodotyrosine was established in exactly the same way (for the two different developers). Two different samples of known diiodotyrosine were used for the mixed chromatograms with the radioactive product. One was a recrystallized sample of the Eastman Kodak Co. white label product; the other was a sample of the product which was prepared on a macro-scale by the iodination of tyrosine. Its chemical analysis has been given above.

The rechromatographing of the pure mono- and diiodotyrosine products always showed that a small fraction of the labeled iodide was detached from the organic molecule during the elution and rechromatographing. This fraction varied from 2 to 4% for both products. No monoiodotyrosine spot could be observed when the diiodotyrosine was rechromatographed.

Preparation of I^{131}-Labeled Thyroxine.—Labeled thyroxine was prepared by means of the exchange reaction with inorganic iodide described by Frieden, *et al.*[15] The preparation was carried out several times and the quantitative data presented below are from a single representative experiment. One hundred and forty (±5) μg. of recrystallized thyroxine and 1.7 mc. of I^{131} (as carrier-free sodium iodide) were dissolved in 2 ml. of a 9:1 butanol–water mixture; hydrochloric acid was added in very small amount to bring the solution to a *p*H of 5–6 and to aid in dissolving the thyroxine. The solution was refluxed for twelve hours, cooled and evaporated under reduced pressure to about 0.5 ml. The residual solution was transferred to the corner of the chromatographic paper; a small amount of 0.1 *N* ammonium hydroxide was used to effect the transfer. Elution of the thyroxine from the developed paper followed by spectrophotometry of the eluate (as described below) showed that 120 μg. (83–89%) of the thyroxine was recovered. This thyroxine contained 11% of the labeled iodide used in the reaction (allowing for the radioactive decay of the iodine during the reaction). The specific activity of the thyroxine was 0.75 μc./μg.

Chromatography and Spectrophotometry of Thyroxine.—The chromatography of the thyroxine reaction mixture was accomplished in a fashion similar to that described for the mono- and diiodotyrosine except that in later work it was found to be unnecessary to run the chromatogram in the second (phenol) direction; consequently, unidirectional chromatograms were employed. In addition, a 7:1 butanol–water solution was used instead of the butanol–acetic acid developer as the R_F value for thyroxine (0.82) is lower in the former solvent and a more satisfactory separation from free iodine can be obtained.

The position of the labeled thyroxine was established by preparing a radioautograph of the chromatogram in the manner described above for mono- and diiodotyrosine. The radioautographs from the thyroxine preparations always showed three spots: the lowest spot (R_F approximately 0.20) was sodium iodide, the middle spot (R_F 0.82) was thyroxine and the uppermost spot (R_F approximately 0.95) was free iodine. The thyroxine spot was, for a given weight of amino acid, more diffuse than the mono- and diiodotyrosine spots, and, in general, showed some streaking. This streaking indicated that some iodine (principally as iodide) was detached from the thyroxine during the chromatography.

The thyroxine spot was cut out of the paper and eluted overnight with approximately 3 ml. of 50% alcohol which was made alkaline with 1% of ammonium hydroxide. One drop of 0.1 *N* sodium hydroxide was added to the eluate, the solution was transferred to the cell of a Beckman spectrophotometer and the optical density was determined at 326 mμ. Thyroxine in alkaline solution shows an absorption maximum at this wave length with a molar extinction coefficient of 6.05×10^3. A blank on the spectrophotometry was obtained in the same manner as described above for mono- and diiodotyrosine.

The identity of the thyroxine was established by rechromatographing a small fraction of the radioactive product together with about 50 μg. of recrystallized thyroxine (Roche–Organon, Inc., product). The developed chromatogram was sprayed with the ninhydrin solution in order to establish the position of the thyroxine spot and a radioautograph was prepared in order to locate the radioactivity. The positions of the ninhydrin color and of the activity coincided exactly.

The rechromatographing of the thyroxine showed that a considerable quantity of the iodine in the thyroxine reappeared as inorganic iodide. The fraction of I^{131} which reappeared as inorganic iodide and free iodine during the processes of elution, evaporation and rechromatographing (all carried out at room temperature) varied from 22–28%. The paper chromatography seems to indicate considerable instability of the thyroxine molecule with respect to its keeping iodine in organic combination.

Acknowledgment.—We are indebted to Dr. A. A. Benson who suggested the use of paper chromatography for this work and to Dr. Choh Hao Li who provided us with some of the sample of monoiodotyrosine which was given to him by Professor C. R. Harington. We are also grateful to Professors Melvin Calvin and Joseph G. Hamilton for making available to us the facilities of their laboratories.

(15) E. Frieden, M. B. Lipsett and R. J. Winzler, *Science*, **107**, 353 (1948).

Summary

Syntheses of I^{131}-labeled monoiodotyrosine, diiodotyrosine and thyroxine of high specific activity have been carried out on a microgram scale. The products have been separated from their reaction mixtures and identified through the use of paper chromatography. A semi-quantitative estimation of the products has been made by spectrophotometry.

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The Structures of Cyanamide and Carbodiimide

BY WILLIAM C. SCHNEIDER

In a recent paper by Hunter and Rees[1] it was suggested that a tautomerism between cyanamide and its isomer carbodiimide seems quite probable. Since the observed[2] electric moment of cyanamide, 3.8, is similar to other nitriles, it was felt that a redetermination of the electric moment of cyanamide combined with an evaluation of the electric moments of a dialkylcyanamide and some carbodiimide derivatives should indicate readily any tautomerism between cyanamide and carbodiimide. It was not possible to measure the electric moment of carbodiimide itself since the compound is not known to exist. In addition, the dipole moment of dicyandiamide was obtained as an aid in determining the structure of this important dimer of cyanamide.

TABLE I

Compound	M. p., °C.
Cyanamide[a]	
Dicyandiamide[a]	
Diisopropylcyanamide[b]	B. p. 70° at 15 mm.
Diisopropylcarbodiimide[b]	B. p. 155°; 40° at 10 mm.
p,p'-Dichlorophenylcarbodiimide[b]	55–57°

[a] Purified as discussed by Salley and Gray, THIS JOURNAL, **70**, 2650 (1948). [b] Prepared by Dr. I. Hechenbleikner, Research Division, These Laboratories.

TABLE II

w	*E*	*d*	*w*	*E*	*d*
Cyanamide (dioxane)			Cyanamide (benzene)		
0.0	(2.1930)[a]	1.01627	0.0	2.2540	0.86304
.0004609	2.2203	1.01633	.0005396	2.2771	.86316
.0008338	2.2422	1.01639	.001077	2.2958	.86330
.001808	2.2980	1.01659	.001433	2.3104	.86337
.002442	2.3347	1.01664			
Dicyandiamide			Diisopropylcarbodiimide		
			0.0	(2.1974)	1.01624
0.0	2.1907	1.01637	.0005048	2.1996	1.01610
.0001633	2.2085	1.01651	.001102	2.2014	1.01590
.0003247	2.2205	1.01648	.0001630	2.2031	1.01577
			.002156	2.2053	1.01560
Diisopropylcyanamide			*p,p'*-Dichlorophenylcarbodiimide		
0.0	2.1968	1.10623	0.0	(2.2154)	(1.01424)
.0003577	2.2045	1.10606	.0004333	2.2152	1.01435
.0006434	2.2099	1.10592	.0008125	2.2154	1.01447
.0009944	2.2170	1.01589	.001009	2.2143	1.10448
.001282	2.2236	1.01592	.001804	2.2155	1.01470

[a] Values in parentheses obtained by extrapolation.

(1) Hunter and Rees, *J. Chem. Soc.*, 617 (1945).
(2) Devoto, *Gazz. chim. ital.*, **63**, 491 (1933).

Experimental

The apparatus and measuring technique have been described previously.[3] The compounds investigated are listed in Table I. Table II lists the experimental values of E, dielectric constant; d, density; and w, weight fraction, for dioxane solutions of the compounds at 35°. Dipole moments were calculated by a modified Hedestrand method similar to that introduced by Halverstadt and Kumler,[4] differing in that densities were used rather than specific volumes. The empirical equation used may be written as

$$ {}_{\infty}P_T = \frac{\epsilon_0 - 1}{\epsilon_0 + 2} \times \frac{1}{d_0}[1 - \beta/d_0] + \frac{3\alpha}{(\epsilon_0 + 2)^2 d_0} \quad (1) $$

where ϵ_0 = extrapolated dielectric constant of solvent
d_0 = extrapolated density of solvent
${}_{\infty}P_T$ = specific polarization at infinite dilution
α = slope of dielectric constant *vs.* weight fraction curve
β = slope of density *vs.* weight fraction curve

The total molar polarization, ${}_{\infty}P_T$, is obtained from the specific polarization by multiplying by the molecular weight. Atomic polarization was neglected, and molecular refractions were calculated from the atomic refractions listed in the "Landolt-Börnstein Tabellen." The values obtained from these calculations are listed in Table III, where P_D and P_O refer to the distortion and orientation polarizations, respectively.

Discussion

Any tautomeric equilibrium between cyanamide and carbodiimide, $\begin{matrix}H\\H\end{matrix}\!\!>\!N-C\equiv N \rightleftharpoons HN=C=N-H$ seems quite improbable if one compares the electric moments of cyanamide, 4.52*D*, diisopropylcyanamide, 4.76*D*, and diisopropylcarbodiimide, 2.08*D*. The close agreement between cyanamide and its diisopropyl derivative indicates essentially identical structures. Although one might argue that the small discrepancy, 0.24*D*, could be interpreted as resulting from tautomerism, it seems more probable that the observed increase from cyanamide to diisopropylcyanamide results from replacing the hydrogen atoms of the amide group by the more negative isopropyl group, causing a small

(3) Schneider and Halverstadt, THIS JOURNAL, **70**, 2626 (1948).
(4) Halverstadt and Kumler, *ibid.* **64**, 2988 (1942).