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CHARACTERISTICS OF THE REACTIVITY OF THYRONINE, TYROSINE AND SOME IODINATED DERIVATIVES OF THESE SUBSTANCES IN THE PRESENCE OF THE FERRIC FERRICYANIDE–ARSENIOUS ACID REAGENT

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SUMMARY

The color response of several iodo derivatives of thyronine and tyrosine to the ferric ferricyanide–arsenious acid reagent of GMELIN AND VIRTANEN was studied stoichiometrically. The activity of the tested iodophenols depended on both their iodine content and the position of the halogen in the molecule. The reducing properties of phenolic hydroxyl as well as unknown properties of the ether bond also contributed to the color response and were responsible for the false positive reactions of thyronine and tyrosine with the GMELIN AND VIRTANEN reagent. Because multiple factors were involved in pigment production, the color response of iodophenols in contact with the reagent was not linear with respect to their iodine content. If these substances are to be quantitatively analyzed on the basis of their color production with the GMELIN AND VITANEN reagent, adequate standards are necessary. When the GMELIN AND VIRTANEN reagent is used for locating iodophenol spots in chromatography, the mobility of thyronine and tyrosine must be checked in advance to avoid errors arising from the ability of these amino acids to yield strong positive reactions and to chromatograph as some of their iododerivatives.

INTRODUCTION

In 1959 GMELIN AND VIRTANEN[1] described the ferrichloride–ferricyanide–arsenious acid (FFCA) reagent for the chromatographic detection of iodide and iodinated compounds. This reagent has been successfully employed for localizing thyroid hormones[2–9]. It is based on reduction of ferrichloride–(potassium)ferricyanide by arsenious acid in the presence of catalytic quantities of iodide and on the consequent precipitation of an insoluble pigment of Prussian blue and Turnbull's blue which is fixed on paper.

As originally recognized by GMELIN AND VIRTANEN, this reaction is not specific for iodide. Empirical observations demonstrate, in addition, that pigment production

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occurring when FFCA comes in contact with different iodophenols is not strictly related to the iodine content of each molecule. If only the iodine content influenced the reaction, equimolar quantities of iodothyronines and iodotyrosines would yield spots in which the color intensity increased linearly with the number of molecular halogen atoms. The pigment production of mono-, di-, tri- and tetraiodo-thyronine spots sprayed with FFCA fails to reveal a definite dependence on the halogen substitution (Fig. 1). This can be partially attributed to the activity of other chemical groups of these compounds which can also react in the presence of FFCA. The color response of thyronine which possesses the same basic structures as its iododerivatives, is considerable (Fig. 1) and corroborates this assumption. The incongruity between the halogen content of the iodophenols and the color response to FFCA could also be explained by assuming that the catalytic activity may vary according to the position of a iodine atom in the molecule.

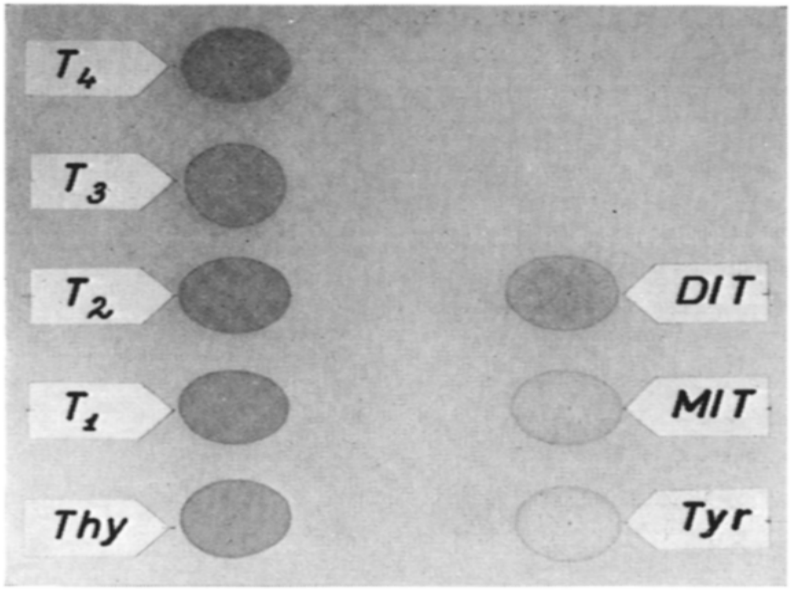


Fig. 1. Pigment production by equimolar quantities of some iodophenols and their parent substances in contact with FFCA. Each spot corresponds to 20 μl of a $5 \cdot 10^{-4}$ M solution fixed on filter paper and sprayed with the reagent. Thy, T_1, T_2, T_3 and T_4 correspond respectively to thyronine, mono-, di-, tri- and tetraiodo-thyronine. Tyr, MIT and DIT correspond to tyrosine, mono- and diiodo-tyrosine.

In addition to the influence of the halogen content, other factors influencing the color response of iodophenols to the GMELIN AND VIRTANEN reagent are obtained by comparing the reactivity of corresponding thyronine and tyrosine derivatives. The intensity of the spots of mono- and diiodo-thyronine is stronger than those of mono- and diiodotyrosine (Fig. 1). This difference arises from the different reactivities of the parent substances of both series to FFCA.

The reactivity of eight different iodinated and noniodinated phenolic amino acids were stoichiometrically compared in the presence of FFCA in order to correlate the observed features with the chemical structure of these substances. Other aims of this study were to facilitate the application of the reagent for the localization as well as for the quantitation of thyroid hormones in chromatography, as proposed by different authors[1,5,10].

MATERIAL AND METHODS

L-Tyrosine, 3-monoiodo- and 3,5-diiodo-L-tyrosine, DL-thyronine, 3-monoiodo-, 3,5-diiodo-, 3,3',5-triiodo- and 3,3',5,5'-tetraiodo-DL-thyronine were tested. Tyrosine and diiodotyrosine were purchased from Serva-Entwicklungslabor, Heidelberg, G.F.R.; monoiodotyrosine from Fluka AG Chemische Fabrik, Buchs SG, Switzerland; thyronine and diiodothyronine from Dr. Theodor Schuchardt GmbH & Co., München, G.F.R. Monoiodothyronine was donated by Gödecke & Co. Chemisches Fabrik, Freiburg, G.F.R.; triiodothyronine by Farbwerke Hoechst AG, Frankfurt (M), G.F.R. and tetraiodothyronine by Deutsche Hoffmann-La Roche AG, Grenzbach-Baden, G.F.R.

The substances were dissolved in a mixture containing equal volumes of 1-butanol, methanol and 0.1 *N* NaOH in a concentration of $5 \cdot 10^{-4}$ *M* and were spotted with a micropipette Marburg (Eppendorfer Gerätbau, Metheler + Hinz GmbH, Hamburg, G.F.R.) in fixed volumes of 20 μl on electrophoresis paper strips (Schleicher & Schüll 2043). Thus 7.6–1.8 μg of the amino acids were homogeneously distributed in circular areas of approx. 1.5 cm^2. If no more than six spots were present in the strips, optical density was strictly proportional to the known quantities of substances.

After drying, the paper strips were clamped in a glass holder and were rapidly sprayed on both sides with FFCA. The reaction was abruptly stopped after 1 min by immersing the strip in a 5-l water bath for 10 min. Occasionally, the bath was gently swirled to eliminate excess reagent. A thorough washing or repeated rinsing of the strips resulted in an uncontrolled elution of the blue pigment which introduced considerable errors in the measured values. After being dried in a warm air current, the strips were made transparent and run in an Elphor Integraph densitometer (Dr. Bender & Dr. Hobein, München–Karlsruhe–Zürich). Extinction measurements of the spots on the strip were automatically recorded and expressed as a function of the surface of the corresponding peaks in the integrated curve. Usually absolute values were restated as relative expressions to obtain mean and standard deviations in the series. In one case, absolute values also were compared.

Three different series (A, B and C) each composed of twenty paper strips were analyzed. Series A (thyronine, mono-, di-, tri- and tetraiodo-thyronine), Series B (tyrosine, mono- and diiodo-tyrosine) and Series C (thyronine, mono- and diiodo-thyronine and tyrosine, mono- and diiodo-tyrosine) were spotted.

Preparation of FFCA

Immediately before use, 5 parts of solutions A and B were mixed with 1 part of solution C. Solution A: 2.7 g of $FeCl_3 \cdot 6\ H_2O$ in 100 ml 2 *N* HCl. Solution B: 3.5 g $K_3Fe(CN)_6$ in 100 ml distilled water. Solution C: 5.0 g $NaAsO_2$ are dissolved in 30 ml 1 *N* NaOH, cooled and mixed at 0° under vigorous stirring with 65 ml of cooled 2 *N* HCl. The solutions should be stored in the dark.

RESULTS AND DISCUSSION

Densimetric evaluation of the strips in series A (in %, Table I) show that the reactivity of equimolar quantities of iodoamino acids in FFCA is a direct, but not a linear, function of their halogen content (Fig. 2). Most experimental points lie far from the theoretical line. To examine the influence of some reactive structures of thyronine

TABLE I

PERCENTAGE OPTICAL DENSITY VALUES OF THE ELEMENTS OF SERIES A
Mean and S.D. of 20 curves.

Thy	T_1	T_2	T_3	T_4
10.3 % ± 1.6	16.9 % ± 2.0	21.1 % ± 1.9	23.5 % ± 2.1	28.4 % ± 2.6

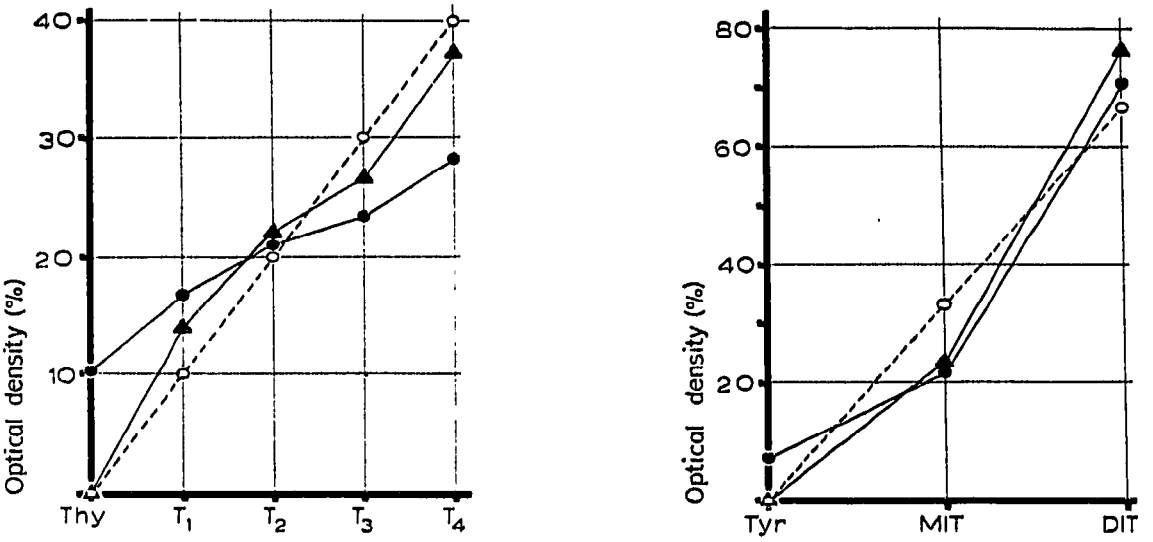


Fig. 2. The 100 % distribution of optical density produced by the five compounds of series A in contact with FFCA. Each point represents the mean of 20 values. ●—●, experimental points; ▲—▲, recalculated experimental points; O---O, hypothetical points according to the iodine content of each molecule. The same abbreviations as in Fig. 1 are used.

Fig. 3. The 100 % distribution of optical density produced by the three compounds of series B in contact with FFCA. Each point represents the mean of 20 values. ●—●, experimental points; ▲—▲, recalculated experimental points; O---O, hypothetical points according to the iodine content of each molecule. The same abbreviations as in Fig. 1 are used.

TABLE II

RECALCULATED PERCENTAGE OPTICAL DENSITY VALUES OF THE ELEMENTS OF SERIES A
Mean and S.D. of 20 curves.

Thy	T_1	T_2	T_3	T_4
00.0 % ± 0.0	13.9 % ± 3.6	22.1 % ± 3.5	26.7 % ± 3.3	37.2 % ± 5.2

in the color response of its iodo derivatives, each curve was recalculated in percentages after substracting from each absolute optical density term the one corresponding to the parent substance. Mean and standard deviations are shown in Table II and are plotted comparatively in Fig. 2. The new data are in better agreement with the theoretical values but show that the increasing reactivity of iodothyronines is not linearly related to its iodination rate. As densimetrically estimated, the percentage of catalytic

TABLE III

PERCENTAGE OPTICAL DENSITY VALUES OF THE ELEMENTS OF SERIES B
Mean and S.D. of 20 curves.

Tyr	*MIT*	*DIT*
7.3 % ± 4.8	21.9 % ± 5.6	70.8 % ± 8.5

TABLE IV

RECALCULATED PERCENTAGE OPTICAL DENSITY VALUES OF THE ELEMENTS OF SERIES B
Mean and S.D. of 20 curves.

Tyr	*MIT*	*DIT*
00.0 % ± 0.0	23.7 % ± 6.1	76.3 % ± 6.1

activity produced by introducing the first iodine atom in a position *ortho* to the ether oxygen exceeds that by introducing the second atom in this position. Conversely, replacing the second hydrogen *ortho* to the phenolic hydroxyl with an iodine atom more greatly increases reactivity than does the substitution of the first hydrogen in this position. In series B (Tables III and IV) the introduction of two iodine atoms *ortho* to the phenolic hydroxyl of tyrosine influences the percentage response of the corresponding derivatives of FFCA similarly to its occurrence in the outer ring of thyronine (Fig. 3). This is clearly evident from comparing the slope of the curve of the recalculated values of mono- and diiodo-tyrosine with that of tri- and tetraiodo-thyronine (Fig. 2).

After considering the data from series A and B, the reactivity of analogous elements of both series were compared graphically. For this purpose, the identical values of optical density (in cm^2) produced by spots of one substance in the twenty

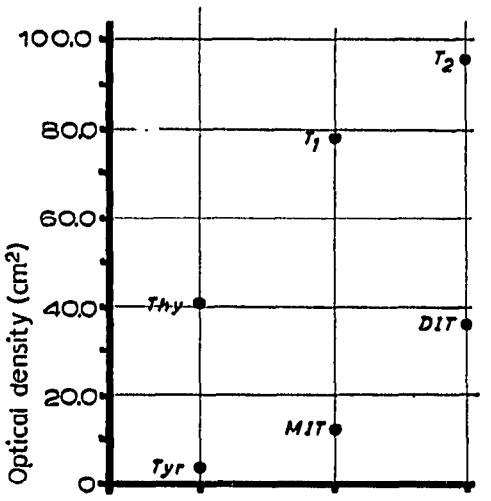


Fig. 4. Absolute values of optical density (as cm^2) of the elements of series C. Each point represents the sum of 20 terms. The same abbreviations as in Fig. 1 are used.

strips of series C were added. The resulting six absolute quantities were plotted on three coordinate axes corresponding to the parent substances, the mono- and diiodo-derivatives (Fig. 4). The wide separation between the points on each axis indicates the different pigment production of the compared substances in contact with FFCA. Under the same conditions, thyronine reacts more strongly than tyrosine. This difference is transmitted to the mono- and diiodo-derivative pairs with some fluctuations which are due to the influence of the halogen position on the reactivity of the molecule.

TABLE V

QUOTIENTS OF ABSOLUTE OPTICAL DENSITY VALUES OF THE EIGHT AMINO ACIDS IN RELATION WITH THE ABSOLUTE OPTICAL DENSITY VALUE OF THYRONINE

Mean and S.D. of 20 quotients except for Thy/Thy, T_1/Thy and T_2/Thy in Series A + C, which represent mean and S.D. of 40 values.

Series	*Tyr/Thy*	*MIT/Thy*	*DIT/Thy*	*Thy/Thy*
A				1.00 ± 0.00
C	0.07 ± 0.00	0.29 ± 0.10	0.93 ± 0.12	1.00 ± 0.00
A + C	0.07 ± 0.00	0.29 ± 0.10	0.93 ± 0.12	1.00 ± 0.00

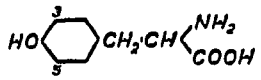
Series	*T_1/Thy*	*T_2/Thy*	*T_3/Thy*	*T_4/Thy*
A	1.68 ± 0.00	2.15 ± 0.47	2.40 ± 0.62	2.88 ± 0.58
C	2.00 ± 0.00	2.52 ± 0.73		
A + C	1.84 ± 0.40	2.34 ± 0.52	2.40 ± 0.62	2.88 ± 0.58

In order to compare the reactivity of the eight amino acids with a common reference standard, absolute optical density values of the elements of the curves corresponding to series A and C were proportionally related to those of thyronine present in each strip (Table V). The eight substances were not directly compared in a new series because of many difficulties in processing strips containing more than six spots. The quotient Tyr/Thy = 0.07 demonstrates the stronger reactivity of the former substance in contact with FFCA when compared with the latter one. The pigment formed by tyrosine can be attributed to the reducing properties of its phenolic hydroxyl, as recognized by GMELIN AND VIRTANEN in their original report. The existence in thyronine of another factor which further enhances the interaction with FFCA is evident. The comparison of the chemical structures of both amino acids suggests that this factor is the characteristic ether bond not present in tyrosine. The reactivity of thyronine even exceeds that of the iodo-derivatives of tyrosine, amounting to around one third of those of thyroxine (Table V).

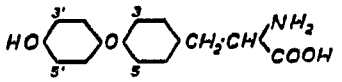
The mechanism causing the ether group of thyronine to enhance the reduction of ferric and ferricyanide ions through arsenious acid is not discussed here. In light of chemical analogies[10], it can be assumed that the activity of the iodine atom in iodophenols is catalytic and similar to that observed by BOWDEN *et al.*[11] and by BARKER[12] when some iodinated compounds came in contact with the reagent of Kolthoff and Sandell. MORREALE DE ESCOBAR AND GUTIÉRREZ RÍOS[13] studied the kinetics of the response of different iodophenols to the Kolthoff-Sandell reagent and stated that the position of the halogen atom with respect to the ether bond or to the phenolic hydroxyl

influences the reactivity of the tested compounds similarly with that observed here for the GMELIN AND VIRTANEN reagent. This further supports the assumption that the role of the iodine atom is the same in the reduction of ceric of ferric ferricyanide ions in the presence of arsenious acid.

As shown in the present experiment, the color response of the iodophenols to the GMELIN AND VIRTANEN reagent is due to the catalytic activity of the iodine and to the action of nonspecific pigment-producing factors: the ether bond and the phenolic hydroxyl. The intensity of the iodine reaction is proportional to the number of halogen atoms contained in the compound but is also influenced by their position in the molecule. The pigment production of the ether oxygen is quantitatively more important than that accorded to the reducing properties of the phenolic hydroxyl and is responsible for the stronger reaction of thyronine and its iododerivatives compared with that of the compounds of the tyrosine, when these substances come in contact with FFCA.



TYROSINE



THYRONINE

Fig. 5. Chemical structure of thyronine and tyrosine.

Consequently thyronine and tyrosine spots can be easily mistaken for those of some iododerivatives when FFCA is used for revealing chromatograms, because the migration rate of all these substances is very similar in many systems. Therefore in systems used for the separation or identification of iodophenols, it is convenient to check in advance the chromatographic behavior of their parent substances in order to avoid errors[14].

Thus each iodophenol has its own reactivity coefficient with FFCA. Hence, the quantitation of the substance or of the iodine present in a chromatographic spot can be attained only after the chemical identification of the reacting substances and the comparison with the corresponding standard.

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