ACTA CHEMICA SCANDINAVICA 15 (1961) 1165–1176

Methods for the Paper Chromatographic and Paper Electrophoretic Separation of Iodide, Iodotyrosines, Iodothyronines and their Derivatives

F. BJÖRKSTÉN, R. GRÄSBECK and B.-A. LAMBERG

The Minerva Foundation Institute for Medical Research, Apollogatan 5 B, Helsingfors, Finland

The authors present new techniques and a critical review of the techniques currently used for the separation and identification of thyroid hormones and related compounds. The solvent systems considered potentially useful were first classified into six groups and the general properties of these groups investigated. New chromatographic and electrophoretic systems were developed and these are described in detail. Special attention was paid to two-dimensional techniques. Solvent systems which continuously changed their composition were used, their action being analogous to the gradient elution employed in column chromatography. Staining methods are discussed.

Paper chromatography and paper electrophoresis, often in combination with isotope techniques, are widely used for the separation and identification of thyroid hormones and related compounds *. The methods have been reviewed by Roche, Lissitzky and Michel [2] and by Taurog and Chaikoff [3].

In spite of the large number of such techniques described, some of the analytical problems encountered in the authors' studies on the metabolic transformations of thyroid hormones could not be solved with the existing methods. For instance, no effective two-dimensional system seemed to have been evolved. A systematic investigation of the current techniques was therefore undertaken, and efforts were made to devise new and more satisfactory methods.

* Abbreviations: The abbreviations are those used by Solomon and Dowling [1]: DIT diiodotyrosine, MIT monoiodotyrosine, T_2 diiodothyronine, T_3 triiodothyronine, T_4 thyroxine, T_2A diiodothyroacetic acid, T_3A triiodothyroacetic acid, T_4A tetraiodothyroacetic acid, T_3F triiodothyroformic acid, T_4F tetraiodothyroformic acid, T_3P triiodothyropropionic acid, T_4P tetraiodothyropropionic acid.

PREPARATION OF SAMPLES

In nature, thyroid hormones and related compounds occur in very low concentrations. This frequently makes it necessary to purify samples before chromatography or electrophoresis, since only a limited amount of substance can be applied to the paper. Purification procedures have been discussed in the reviews mentioned [2,3] and many other articles [4,5]. A useful and widely employed method is extraction of the compounds with *n*-butanol, which is then concentrated to a suitable volume under reduced pressure. This simple technique has been used in our laboratory prior to the chromatography and electrophoresis of the iodine compounds contained in plasma, tissue homogenates, urine, faeces, bacterial incubation media [6], *etc.*

SOLVENT SYSTEMS FOR CHROMATOGRAPHY

For the paper chromatography of thyroid hormones and related compounds many different solvent systems have been recommended. It was found useful to classify these systems into groups (Table 1). The systems of each group are similar both in composition and resolving properties.

The results reported were obtained with Whatman No. 1 paper for chromatography. Unless otherwise stated, the ratios of the solvents in solvent mixtures are volume ratios (v/v).

Alkaline organic systems. Usually the solvent systems of this group are made up of the following two or three components:

1. An aliphatic C_3—C_5 alcohol is the main constituent.
2. Occasionally a smaller amount of an aliphatic C_1—C_4 alcohol or some other solvent of similar polarity, *e.g.* acetone or dioxane, is added.
3. The organic solvent(s) is (are) saturated with aqueous ammonia. The molarities of the ammonia solutions used vary within wide limits.

Other alkaline organic systems have also been used. A frequently utilized system is composed of collidine and aqueous ammonia.

In preparing the solvent systems the components are first mixed by shaking, and the two phases formed are then separated. The organic phase is used for chromatography, and the aqueous phase may be used for the saturation of the atmosphere in the chromatography tank.

All systems of this group separate the compounds in a similar manner. Thus the relative mobilities of the compounds in the different systems are fairly similar. The relative mobilities indicated by the R_F values given for two systems (Table 4) are typical. It was noted that the resolving efficiency and the R_F values are highly dependent on the ammonia concentration in the atmos-

Table 1. Grouping of chromatography solvent systems.

1. Alkaline organic systems
2. Neutral organic systems
3. Acidic organic systems
4. Alkaline aqueous systems
5. Neutral aqueous systems
6. Acidic aqueous systems

phere of the tank. Since ammonia readily escapes from the tank, accurate reproduction of conditions and results is difficult, and R_F values can only be taken as indicators of relative mobilities. In order to minimise variations in mobility, we found it necessary, immediately before each run, to check and adjust the ammonia content of the aqueous phase kept in the tank.

The following two empirical rules proved helpful when new systems were being developed and when it was found difficult to reproduce results reported in the literature:

1. An increase in the concentration of component No. 2 will counteract trailing and increase the R_F values. Particularly the R_F values for highly iodinated compounds like DIT and T_4 will increase.

2. An increase in the ammonia concentration has effects similar to an increase of component No. 2.

The chromatograms reproduced in Figs. 1 and 2 show how variations in ethanol and ammonia concentrations influence the results.

The alkaline organic system group is the most useful one in the separation of compounds of the present type. It is particularly well suited for the separation of different iodothyronine derivatives from each other and from iodide

Fig. 1. One-dimensional chromatogram run with the *n*-butanol — 0.5 M ammonia — ethanol — water system. Some pure compounds and a mixture of MIT, DIT, T_3 and T_4 were applied to the paper. The positions of the iodine substituents in the compounds are given in Table 4.

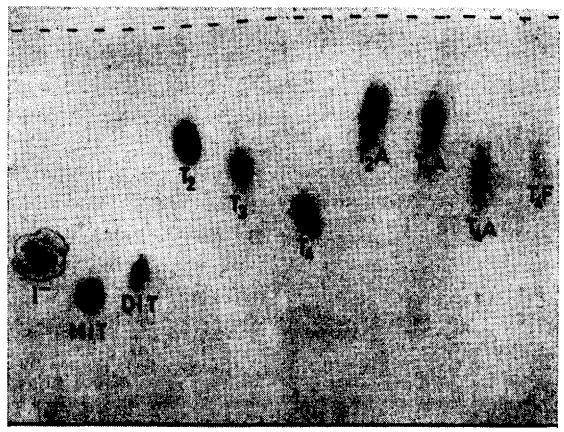


Fig. 2. One-dimensional chromatogram run with *n*-butanol — ethanol — 2 M ammonia (10:3:10). Some pure compounds were applied to the paper. The positions of the iodine substituents in the compounds are given in Table 4.

and the iodotyrosines. Some of the most popular systems are listed in Table 2. The best single system seems to be *tert*-amyl alcohol — 2 M ammonia.

Unfortunately this valuable group also has its shortcomings. As the relative mobilities of the compounds are similar in the different systems, it is difficult to check the results obtained with one system with the aid of another belonging to the same group. It is also inadvisable to use a combination of two alkaline

Table 2. Solvent systems generally used for the paper chromatographic separation of iodotyrosines, iodothyronines and their derivatives.

Group according to Table 1	Solvent system	References	Uses
Alkaline organic systems	*tert*-Amyl alcohol — 2 M ammonia (1:1)	3, 7	Particularly efficient for the separation of thyronine derivatives from each other and from iodide and iodotyrosines
»	*n*-Butanol — dioxane — 2 M ammonia (4:1:5)	2, 3, 8	General use
»	*n*-Butanol — ethanol — 2 M ammonia (5:1:2)	9	» »
»	Collidine — water (100:35) with ammonia in the atmosphere	2,3	» »
Acidic organic systems	*n*-Butanol — acetic acid — water (78:5:17)	2, 10	Separation of I^-, MIT and DIT from each other and from the iodothyronine group

organic systems for two-dimensional chromatography (see section on two-dimensional systems). Further, most alkaline organic systems fail to separate MIT and DIT efficiently. A few of the solvents have some drawbacks. Dioxane is said to cause the formation of artefacts [5]. This can to some extent be prevented by purification of the dioxane by distillation and by the addition of cyanide or some other reducing agent [5]. Collidine has an unpleasant odour.

We have felt the need for a simple system which would effectively separate the chief compounds found in the thyroid and the blood, *i.e.* I^-, MIT, DIT, T_3 and T_4. For this purpose we have modified [6] the *n*-butanol — ethanol — 2 M ammonia system listed in Table 2. 20 volumes of *n*-butanol are added to 20 volumes of 0.5 M ammonia and shaken, whereupon the phases are separated. To the organic phase are added 2 volumes of 94 % (w/w) ethanol and 1 volume of water. The entire aqueous phase and 40 % of the organic phase are used for the saturation of the atmosphere. The water phase must be 0.3—0.4 M in respect to ammonia. Note that the organic phase used in the run is not completely in equilibrium with the phases saturating the atmosphere. The solvent migrating into the paper will therefore undergo a gradual change until it is equilibrated with the atmosphere. Fig. 1 shows a paper run with the *n*-butanol — 0.5 M ammonia — ethanol — water system. For R_F values see Table 4. A fault of the system is that thyronine derivatives show moderate trailing which more or less precludes its use for two-dimensional chromatography. The system *n*-butanol — ethanol — 2 M ammonia (10:3:10) gives rounder and sharper spots but poorer separation (Fig. 3 and Table 4). This system is better suited for two-dimensional chromatography.

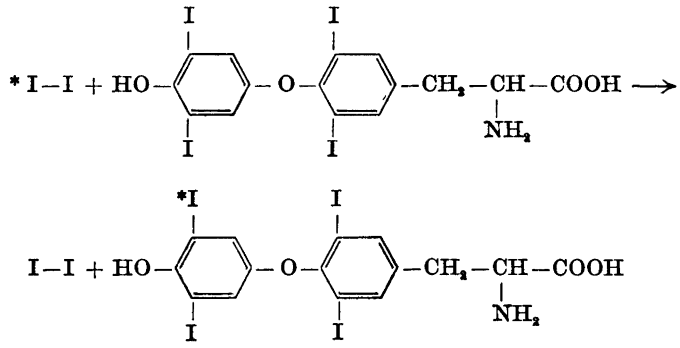
Acidic organic systems. The systems of this group in general use all have as their basis *n*-butanol to which varying amounts of acetic acid and water are

Fig. 3. One-dimensional chromatogram run with toluene — formic acid — ethanol (10:3:3). Some pure compounds and a mixture of T_4 and T_4A were applied to the paper. The positions of the iodine substituents in the compounds are given in Table 4.

added. In Table 2 a popular system is given as an example. The *n*-butanol — acetic acid — water systems are usually employed for the separation of I^-, MIT, DIT and the iodothyronines. The iodothyronines and their derivatives are not separated from each other.

Many authors have pointed out the risk of artefact formation when acidic systems are used [3,5,11,12]. Small amounts of iodide may be oxidised into iodine, and the free iodine may exchange with the bound iodine of the iodothyronine molecules [7]. The following equations show schematically how labelled T_4 may appear as an artefact:

$$*I^- + I^- \longrightarrow *I{-}I\,(=I_2) + 2\,e^-$$



Thus radioactive iodine may cause the labelling of non-labelled T_4. Previously non-iodinated substances may also be iodinated and labelled by the free iodine[11]. Attempts have been made to prevent the formation of artefacts by addition of reducing agents, such as sodium thiosulphate [5, 12], which keep the iodine in reduced iodide form. Dilution of the radioactive iodide with carrier will also decrease the formation of artefacts.

The acidic systems are poorly suited for quantitative separation of iodide from other compounds, since some iodide, in the form of hydrogen iodide, will evaporate from the paper.

The following new acidic organic system has been found useful: Just before use, 10 volumes of toluene are mixed with 3 volumes of formic acid and 3 volumes of 94 % (w/w) ethanol containing 1.5 g/l sodium thiosulphate. No phases separate. Ascending chromatography is used. The running time is 2 h, in which time the solvent front travels about 20 cm. Since the ethanol and the formic acid are rapidly esterified, the composition of the system changes during the run. At the end, about 50 % of the formic acid is esterified. The products formed in the reaction cause the appearance of two phases. The toluene — formic acid — ethanol system is a second example of a continuously changing chromatographic system. Deaminated thyronine analogues, *e.g.* T_4A and T_4P, move close to the solvent front and are not separated from each other. Iodide, iodotyrosines and iodothyronines have different R_F values, but the differences are too small to produce effective separation. (Fig. 2 and Table 4). The system may profitably be used for detecting deaminated thyronine analogues in a

Table 3. New solvent systems for paper chromatography. For details concerning preparation, see text.

Group according to Table 1	Solvent system	References	Uses
Alkaline organic systems	*n*-Butanol – 0.5 M ammonia – ethanol – water	6	Particularly suitable for the separation of I^-, MIT, DIT, T_3 and T_4
»	*n*-Butanol – ethanol – 2 M ammonia (10:3:10)		Two-dimensional chromatography
Acidic organic systems	Toluene – formic acid – ethanol (10:3:3)		Separation of deaminated thyronine derivatives, *e.g.* T_4A and T_4P, from other compounds
Acidic aqueous systems	Water – formic acid (5:1)		Particularly suitable for two-dimensional chromatography in combination with an alkaline organic system

preparation. The formation of artefacts is minimised by the addition of sodium thiosulphate and by the unusually short running time.

Acidic aqueous systems. As far as we know this type of chromatography system has not previously been used for the separation of thyroid hormones and related compounds. However, an investigation of this group showed that the system water – formic acid (5:1) with 0.3 g/l sodium thiosulphate added had many valuable properties. It is very rapid; the solvent front travels 35 cm in 3 h. The compounds are also well separated, and the R_F values are quite different from those obtained with other systems. This makes the water – formic acid system particularly well suited for two-dimensional chromatography in conjunction with an alkaline organic system (Fig. 4).

In the first experiments with acidic aqueous systems some difficulty was experienced because of the low solubility of many of the iodinated compounds. In particular, the tetraiodothyronine derivatives showed poor solubility. However, good results were obtained when the compounds were applied to the paper in sufficiently small quantities. An amount of the order of 0.1 μg of each compound is usually suitable. Such quantities are still easily detected with the sensitive colour reagents available [13,14].

The main disadvantage of the water – formic acid system is its acidity, as acidic systems may cause the formation of artefacts and evaporation of some iodide (see section on acidic organic systems). The undesirable tendencies are minimised by the short running time needed and by addition of sodium thiosulphate to the solvent system and carrier iodide to the sample.

Neutral organic, alkaline aqueous and neutral aqueous systems. These systems proved less useful for our purposes. Neutral organic systems have been employed by some investigators. Two such systems are mentioned as examples 95 % ethanol – 0.2 M ammonium carbonate (2:1) [3,15] and *n*-butanol – ethanol

Table 4. R_F values and electrophoretic mobilities of iodide, iodotyrosines, iodothyronines and their derivatives. R_F values are given for the new solvent systems listed in Table 3. The electrophoretic mobilities were obtained in paper electrophoresis with a diethylbarbiturate buffer with a pH of 8.0 and an ionic strength of 0.06. Serum albumin was used as reference in the electrophoresis, and its mobility was given the value 1.

Compound	R_F values				Electrophoretic mobilities
	n-butanol — 0.5 M ammonia — ethanol — water	*n*-butanol — ethanol — 2 M ammonia (10:3:10)	toluene — formic acid-ethanol (10:3:3)	water — formic acid (5:1)	
I^-	0.27	0.44	0.03	0.79	3.4
3-MIT	0.17	0.34	0.14	0.77	0.59
3,5-DIT	0.10	0.40	0.17	0.67	1.09
3,5-T_2	0.71	0.72	0.18	0.68	0.23
3,5,3'-T_3	0.70	0.65	0.22	0.54	0.10
3,5,3',5'-T_4	0.54	0.53	0.29	0.35	0.20
3,5-T_2A	0.80	0.78	0.94	0.57	0.96
3,5,3'-T_3A	0.79	0.75	0.98	0.36	0.88
3,5,3',5'-T_4A	0.64	0.63	0.98	0.14	1.34
3,5,3'-T_3F	0.81	0.74	0.98	0.20	0.74
3,5,3',5'-T_4F	0.70	0.67	0.99	0.12	1.16
3,5,3'-T_3P	0.79	0.75	0.97	0.28	0.80
3,5,3',5'-T_4P	0.66	0.72	0.98	0.08	1.15

— water (4:1:5) [16]. A few experiments with alkaline aqueous systems indicated that the compounds travel close to the solvent front and are not separated from each other. Some systems belonging to the neutral aqueous group have been employed. The best known is methanol — 0.2 M ammonium acetate (2:5) [3,13]. This system separates iodide and the iodotyrosines from each other and from the iodothyronine group.

PAPER ELECTROPHORESIS

Paper electrophoresis has frequently been used by French workers [17–19] and occasionally by others [20–23]. Both high-voltage and low-voltage techniques have been employed. Although buffers within the pH range 5.8—9.5 have been used, in our experience the best separation is obtained with a pH of about 8. Diethylbarbiturate [18,20,22] and ammonium carbonate [17] buffers are commonly used. At pH 8, most of the compounds move towards the anode. Only the amines, *e.g.* thyroxamine, remain stationary or have cathodic mobility. Iodide migrates much faster than the organic compounds and can be separated from these by applying the current for a short time only. The other compounds separate when electrophoresis is continued for a longer time. The quality of the separation is similar to that obtained with paper chromatography. Since the electrophoretic mobilities differ from the chromatographic ones, the two techniques supplement each other, and may be combined into two-dimensional chromatography-electrophoresis [17,19]. Electrophoresis is particularly to be recommended because of its mild conditions.

A few experiments conducted with different buffers led to the use of a diethylbarbiturate buffer with a pH of 8.0 and an ionic strength of 0.06. A voltage gradient of 7 V/cm was applied. The relative mobilities of some compounds in this system are given in Table 4. Fig. 5 shows a two-dimensional chromato-electropherogram.

TWO-DIMENSIONAL TECHNIQUES

As the number of naturally occuring thyroid hormone analogues has turned out to be great, the necessity of resorting to two-dimensional techniques has become apparent. It is doubtful, for instance, whether the acetic acid analogues can be identified with the sole aid of one-dimensional chromatography [24]. Many combinations of the numerous existing systems have been employed for two-dimensional runs.

Practically all the chromatographic systems in general use belong to either the alkaline organic or the acidic organic group. The acidic organic solvent systems do not separate thyronine derivatives and their application is therefore limited to some special purposes. An alkaline organic system may profitably be utilised for chromatography in one direction, but it is not very advisable to use systems from this group for both directions, since the relative mobilities of the compounds are similar in all alkaline organic systems. When a combination of such systems is used, a chromatogram is produced on which all the compounds lie approximately along a straight line, and the separation is not much better than on a one-dimensional strip.

Two methods were found to give good results: The first one consists of two-dimensional chromatography with *tert*-amyl alcohol — 2 M ammonia (1:1) in one direction and water — formic acid (5:1) containing 0.3 g/l sodium thiosulphate in the other (Fig. 4).

The second method is a slight modification of the technique employed by the French school [17,19]. First, chromatography with *tert*-amyl alcohol — 2 M ammonia (1:1), then electrophoresis with a diethylbarbiturate buffer of pH 8.0 and an ionic strength of 0.06 in the second direction (Fig. 5). Care must be taken not to disturb the spot pattern on the paper when it is moistened with the electrophoresis buffer. The best way to apply the buffer is to place the dry paper on a glass plate and spray the buffer evenly over it with an atomiser operated with compressed air from an electric pump. The amount of buffer applied is not very critical.

COLOUR REAGENTS

Several colour reagents are suitable for the detection of thyroid hormones and related compounds on filter paper.

An alkaline solution of diazotised sulphanilic acid (Pauly's reagent) gives characteristic red and violet colours with phenolic compounds, and is useful for the detection of tyrosine and thyronine derivatives. The reagent may be conveniently applied by spraying the paper with a freshly prepared solution of diazotised sulphanilic acid and sodium carbonate in water [3]. Its principal disadvantage is the comparatively low sensitivity; 5—50 μg of the compound to be coloured is needed.

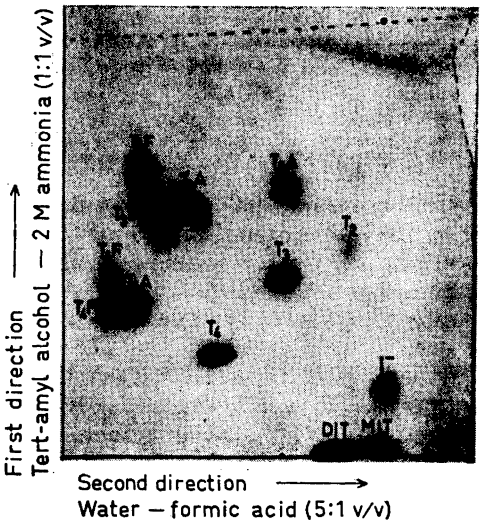


Fig. 4. Two-dimensional chromatogram run in the first direction with *tert*-amyl alcohol — 2 M ammonia (1:1) and in the second with water — formic acid (5:1). The positions of the iodine substituents in the compounds applied to the paper are given in Table 4.

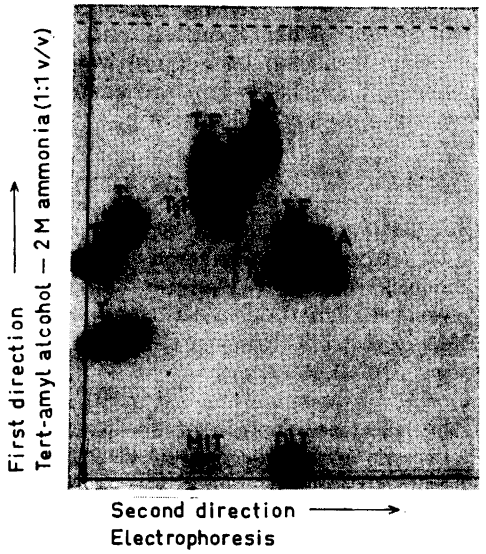


Fig. 5. Two-dimensional chromato-electropherogram. Chromatography in the first direction with *tert*-amyl alcohol — 2 M ammonia (1:1) and electrophoresis in the second, a diethylbarbiturate buffer with a pH of 8.0 and an ionic strength of 0.06 being used. The positions of the iodine substituents in the compounds applied to the paper are given in Table 4.

Iodide and many iodine containing compounds can be detected with a ceric sulphate — arsenious acid colouring agent. The original reagent [25] has been modified by several investigators [14,26-28]. It is fairly specific and highly sensitive; 0.005 μg of organic iodine can be detected [14]. Neither this reagent nor the following one is completely specific for iodine; some reducing substances also give the colour reactions.

A second spraying reagent based on the catalytic properties of iodine has been described by Gmelin and Virtanen [13], who use an acidic solution of ferric chloride, potassium ferricyanide and sodium arsenite. The limit of detection is 0.001 μg of iodide and 0.002 μg of thyroxine [13]. In our laboratory this reagent has been found very useful for the location of minute amounts of iodine containing compounds. The reagent is an important supplement to the water — formic acid chromatography system, which permits the application of only small amounts of the compounds on the paper. The high sensitivity necessitates cleanliness in the handling of the papers. The paper itself may contain impurities which cause undesirable background colouring. The impurities can be removed before the paper is used by descending "chromatography" with water — formic acid (5:1). This solvent is permitted to drip off the lower edge of the paper for 1—2 h.

Ninhydrin is suitable for the detection of iodotyrosines and iodothyronines [2], which are amino acids.

Palladous chloride is a specific reagent for iodide. A solution containing 0.1—1 % palladous chloride in dilute hydrochloric acid is suitable for the staining of paper chromatograms [29,30].

DISCUSSION

The purpose of this investigation was to develop effective chromatographic and electrophoretic techniques for a research program which requires reliable identification of thyroid hormone metabolites. In the course of the work it was found helpful to group all conceivably useful chromatographic solvent systems into six groups (Table 1). The systems in each group are similar in composition and separation properties. For instance, the relative mobilities of the compounds are usually similar in different systems belonging to the same group. After this systematisation, a study of the general properties of the different groups could be undertaken. Such a systematic approach would also be useful when solvent systems for other kinds of compounds are developed. In our case the alkaline organic, acidic organic and acidic aqueous systems were found to have the most useful properties.

Alkaline organic solvent systems are particularly efficient. A dependable system belonging to this group was developed for the one-dimensional resolution of the chief iodine compounds of the thyroid and blood, *i.e.* I^-, MIT, DIT, T_3 and T_4. Most acidic organic systems separate thyronine derivatives poorly. They are, however, useful for certain special purposes. There seemed to be a particular need for an efficient solvent system with properties different from those of the alkaline organic group. This gap has been filled by a system belonging to the previously unused acidic aqueous group.

A good two-dimensional separation procedure should resolve the compounds analysed into small, well-defined spots, distributed over a large area of paper. This can be accomplished with a combination of two good one-dimensional systems in which the compounds have different relative mobilities. When thyroid hormones and related compounds are analysed, an alkaline organic system, e.g. *tert*-amyl alcohol — 2 M ammonia, can be recommended for use in the first direction. For the second direction two acceptable methods are available. One consists of chromatography with the new acidic aqueous system water — formic acid. The second possibility is electrophoresis with a buffer having a pH of approximately 8.

Carefully stabilised solvent systems are usually employed in paper chromatography. However, it was found that continuously changing solvent systems sometimes offer advantages. Such systems can be produced by using solvent systems in which a reaction occurs during the run or which are not in equilibrium with the atmosphere of the chromatography tank. The process is to some extent analogous to the gradient elution technique used in column chromatography.

Acknowledgments. The skilled technical assistance of Mrs. Leena Kivistö is gratefully acknowledged. The authors wish to thank Glaxo Laboratories Ltd., Greenford, Middlesex, England, Dr. R. I. Meltzer of the Warner-Lambert Research Institute, Morris Plains,

N.J., U.S.A., and Drs. R. Pitt-Rivers and J. R. Tata of the National Institute of Medical Research, London, England, for gifts of pure thyroid hormone analogues. Mrs. Jean Margaret Perttunen kindly revised the English language. The study was aided by grants from the *Sigrid Juselius Stiftelse*, *Medicinska Understödsföreningen Liv och Hälsa* and *Statens Naturvetenskapliga Kommission*.

REFERENCES

1. Solomon, D. H. and Dowling, J. T. *Ann. Rev. Physiol.* **22** (1960) 615.
2. Roche, J., Lissitzky, S. and Michel, R. in Glick, D. *Methods of Biochemical Analysis*, Interscience, New York 1954, Vol. 1, p. 243.
3. Taurog, A. and Chaikoff, I. L. in Colowick, S. P. and Kaplan, N. O. *Methods in Enzymology*, Academic Press Inc., New York 1957, Vol. 4, p. 856.
4. Block, R. J., Werner, S. C., Mandl, R. H., Row, V. V. and Radichevich, I. *Arch. Biochem. Biophys.* **88** (1960) 98.
5. Shaw, E. I. and Goldsby, R. *Intern. J. Appl. Radiation and Isotopes* **3** (1958) 161.
6. Gräsbeck, R., Lamberg, B.-A. and Björkstén, F. *Acta Endocrinol.* **34** (1960) 113.
7. Gleason, G. I. *J. Biol. Chem.* **213** (1955) 837.
8. Gross, J., Leblond, C. P., Franklin, A. E. and Quastel, J. H. *Science* **111** (1950) 605.
9. Taurog, A., Wheat, J. D. and Chaikoff, I. L. *Endocrinology* **58** (1956) 121.
10. Roche, J., Jutisz, M., Lissitzky, S. and Michel, R. *Biochim. et Biophys. Acta* **7** (1961) 257.
11. Acland, J. D. *Nature* **170** (1952) 32.
12. Dobyns, B. M. and Barry, S. R. *J. Biol. Chem.* **204** (1953) 517.
13. Gmelin, R. and Virtanen, A. I. *Acta Chem. Scand.* **13** (1959) 1469.
14. Mandl, R. M. and Block, R. J. *Arch. Biochem. Biophys.* **81** (1959) 25.
15. Roche, J., Michel, R. and Nunez, J. *Bull. soc. chim. biol.* **37** (1955) 809.
16. Fletcher, K. *Biochem. J.* **67** (1957) 140.
17. Michel, R. *Am. J. Med.* **20** (1956) 670.
18. Roche, J., Michel, R., Closon, J. and Michel, O. *Biochim. et Biophys. Acta* **38** (1960) 325.
19. Lissitzky, S., Bénévent, M.-T., Roques, M. and Roche, J. *Bull. soc. chim. biol.* **41** (1959) 1329.
20. Taurog, A., Tong, W. and Chaikoff, I. L. *J. Biol. Chem.* **227** (1957) 759.
21. Matsuura, T. and Cahnmann, H. J. *J. Am. Chem. Soc.* **81** (1959) 871.
22. Tata, J. R., Rall, J. E. and Rawson, R. W. *Endocrinology* **60** (1957) 83.
23. Lybeck, H. *Acta Med. Scand.*, Suppl. 327 (1957).
24. Pitt-Rivers, R. and Tata, J. R. *The Thyroid Hormones*, Pergamon Press, London 1959, p. 157.
25. Bowden, C. H., Maclagan, N. F. and Wilkinson, J. H. *Biochem. J.* **59** (1955) 93.
26. Fletcher, K. and Stanley, P. G. *Nature* **175** (1955) 730.
27. Dragúnová, I. and Langer, P. *Nature* **178** (1956) 537.
28. Štolc, V. *Nature* **182** (1958) 52.
29. Gross, J. and Leblond, C. P. *Endocrinology* **48** (1951) 714.
30. Feigl, F. *Qualitative Analysis by Spot Tests*, Elsevier, New York 1947, p. 197.

Received February 27, 1961.