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PAPER ELECTROPHORESIS OF THYROXINE IN TRIS-MALEATE BUFFER

BY N. B. MYANT AND C. OSORIO

From the Medical Research Council Experimental Radiopathology Research Unit, Hammersmith Hospital, London, W. 12

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When thyroxine, in the absence of serum, is submitted to electrophoresis on filter paper in barbiturate buffer at pH 8·6, all the thyroxine remains at the origin. If, however, the electrophoresis is carried out on filter paper that has been soaked in phenylalanine, or if phenylalanine is added to the paper with the thyroxine, the thyroxine moves towards the anode at a speed between that of the α_1- and α_2-globulins of human serum (Myant & Osorio, 1960). The term 'phenylalanine effect' was suggested for this action of phenylalanine, and for that of any other substance that exerts the same effect on the electrophoretic mobility of thyroxine on paper. The cause of the phenylalanine effect is unknown. We suggested, however, that it might be due to blocking of thyroxine-binding sites on the filter paper, which normally prevent thyroxine from moving towards the anode.

It has recently been shown (Ingbar, 1958) that when radioactive thyroxine, in the presence of human serum, is submitted to paper electrophoresis in tris-maleate buffer at pH 8·6, most of the activity is distributed between two peaks, one at the inter-alpha zone and the other in front of the albumin band (pre-albumin zone). Our observations with barbiturate buffer raised the question as to whether the phenylalanine effect also occurs in the presence of tris-maleate buffer and, if so, how far it could account for Ingbar's observations. We have therefore studied the effect of phenylalanine on the electrophoretic mobility of thyroxine in tris-maleate buffer at different hydrogen-ion concentrations with and without human serum. Observations were also made on the partition of thyroxine between the inter-alpha and pre-albumin zones at different concentrations of thyroxine. In an attempt to explain why thyroxine does not move to the pre-albumin zone in the presence of serum in barbiturate buffer, observations were made on the movement of thyroxine on paper electrophoresis in two dimensions.

METHODS

Paper electrophoresis was carried out at room temperature by methods already described (Myant & Osorio, 1960). The tris (2-amino-2-hydroxymethylpropane-1:3 diol) buffer was made by mixing solution *A* (equal volumes of 0·4 M tris and 0·4 M maleic acid) and solution *B* (0·2 N-NaOH) in the following volumes (ml.), and diluting to 1 l. with water: pH 7·6, 300 of *A* + 333 of *B*; pH 8·6, 250 of *A* + 405 of *B*; pH 9·2, 233 of *A* + 425 of *B*. The pH was checked with a pH-meter at room temperature and, if necessary, brought to the exact value by addition of the acid or alkaline component. The borate buffers were prepared as already described (Myant, 1957), the volume of 0·2 N-NaOH being adjusted to give the required pH. The method used for proteolytic digestion of serum has been described elsewhere (Myant & Osorio, 1960). In the experiments on the effect of thyroxine concentration upon the distribution of thyroxine in serum, the amount of thyroxine carried at each of the three zones was estimated from the proportion of the total radioactivity at each peak, and the concentration of added thyroxine in the serum. The endogenous thyroxine already present in the serum was ignored in the calculations. The radioactive L-thyroxine, of specific activity 30 μc/μg, was obtained from Abbott Laboratories Pacific Ltd., Chicago. Radioactivity on the paper strips was measured with a continuously recording scanner. The positions of the radioactive spots after two-dimensional electrophoresis were found by making autoradiographs with Ilford X-ray film.

RESULTS

Effect of pH on mobility of thyroxine in the presence of serum or phenylalanine

In each experiment two strips of filter paper were submitted to electrophoresis in parallel. Radioactive thyroxine in human serum (1 μg/100 ml. serum) was put on one of the papers; the other was soaked in a solution of phenylalanine (1 mg/ml.) and received radioactive thyroxine dissolved in the buffer (1 μg/100 ml.).

At pH 8·6 the bands of protein, shown by staining the paper, were similar in position to those observed after electrophoresis in barbiturate buffer at pH 8·6. No staining was visible anywhere in front of the albumin band. On the paper to which thyroxine had been added in serum the radioactivity was distributed in three peaks, one in the inter-alpha zone, one in the albumin and one in the pre-albumin zone (Fig. 1*a*). At this concentration of added thyroxine the inter-alpha zone always carried the highest proportion of the total activity on the paper, the proportion at each of the three peaks averaging 56, 8 and 34% respectively. On the phenylalanine-treated paper with thyroxine alone all the activity moved as a single peak to a position corresponding to the inter-alpha zone (Fig. 1*b*).

When the pH of the buffer was changed from 8·6 to 7·6 or 9·2, the distribution of thyroxine was altered, both on the phenylalanine-treated paper in the absence of serum, and in the presence of serum. At pH 7·6, on the paper to which thyroxine had been added in human serum, 90% of the activity was present at the inter-alpha zone; the peak at the pre-albumin zone was either absent or only just detectable (Fig. 2*a*). On the

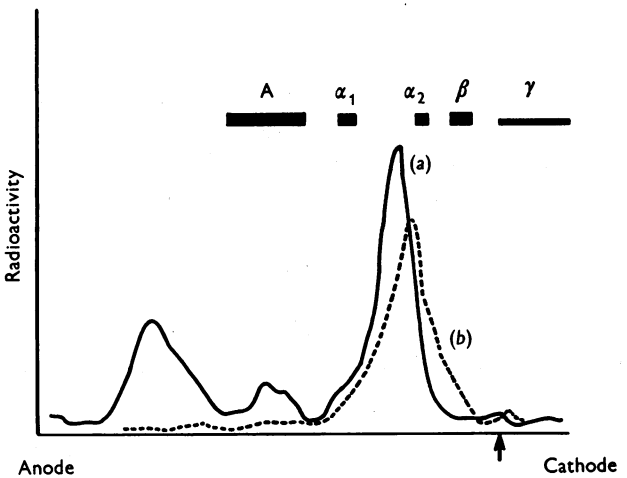

Fig. 1. Distribution of radioactivity along paper strip after electrophoresis in tris-maleate buffer at pH 8·6. *a*, radioactive thyroxine in human serum (1 μg/100 ml.) run on normal paper; *b*, radioactive thyroxine in buffer (1 μg/100 ml.) run on paper soaked in phenylalanine. The two strips of paper were run side by side in the same box. Arrow shows line of application of sample. Solid blocks show positions of protein bands (A = albumin). Note: the amount of radioactivity added to the paper in *a* was greater than in *b*.

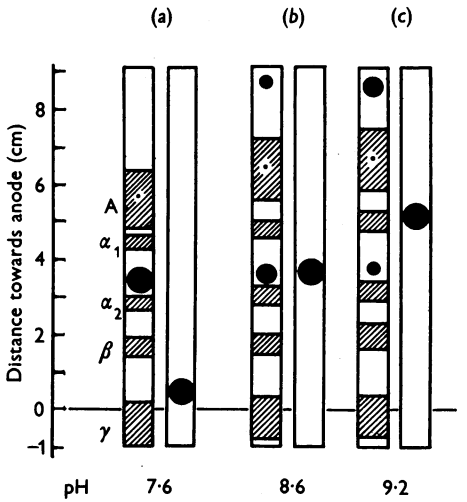

Fig. 2. Diagram to represent paper strips, showing effect of pH of tris-maleate buffer on distribution of thyroxine submitted to electrophoresis with serum on untreated paper and without serum on paper soaked in phenylalanine. The strip on the left of each pair was untreated and received radioactive thyroxine in serum (1 μg/100 ml.); that on the right was soaked in phenylalanine and received radioactive thyroxine in the buffer (1 μg/100 ml.). Shaded areas show positions of protein bands; solid circles show positions of peaks of radioactivity, the proportion of the total activity at each peak being shown roughly by the area of the circle. Vertical scale, distance towards anode from line of application of sample; horizontal scale, pH.

phenylalanine-treated paper all the activity remained within a few millimetres of the line of application of the sample.

At pH 9·2, the activity on the paper that had received the thyroxine in human serum was distributed in the same three peaks as at pH 8·6, but there was an increase in the amount of activity in the pre-albumin zone at the expense of that in the inter-alpha zone. At the concentration of added thyroxine used in these experiments (1 μg/100 ml.), an average of 40 % of the total activity was in the inter-alpha zone, 9 % was in the albumin band and 48 % in the pre-albumin zone. On the phenylalanine-treated paper with thyroxine alone all the activity was present as a single peak at, or just in front of, a position corresponding to the α_1-globulin (Fig. 2*c*).

Relation between concentration of thyroxine and its electrophoretic distribution in serum

If the concentration of added thyroxine is increased from 1 to 500 μg/100 ml., there is a change in the partition of thyroxine between the inter-alpha, albumin and pre-albumin zones, the changes depending to some extent on the pH of the buffer. Figure 3 shows the amount of thyroxine carried in each of the three zones at different concentrations of thyroxine, as estimated from the distribution of radioactive thyroxine in serum submitted to paper electrophoresis.

When the pH of the buffer was 8·6, the inter-alpha zone acquired the highest proportion of the total thyroxine at the lowest concentrations. As the concentration was raised, however, the amount in the inter-alpha zone reached a maximum value corresponding to 20–30 μg/100 ml. serum, while that in the other two zones continued to increase to much higher values. When the total thyroxine concentration was 50 μg/100 ml., or higher, the pre-albumin zone always carried more than the inter-alpha zone. The amount in the pre-albumin zone rose to a maximum corresponding to 200–230 μg/100 ml. serum. The amount in the albumin zone increased slowly at first, but rose more steeply as the pre-albumin zone approached saturation, and eventually exceeded that in either of the other two zones.

At pH 9·2 the inter-alpha zone acquired a smaller proportion of the total thyroxine at low concentrations than was the case at pH 8·6. As the concentration was raised, however, the distribution of thyroxine at pH 9·2 became indistinguishable from that at pH 8·6. At both pH's the inter-alpha zone was saturated at a total thyroxine concentration of 20–30 μg/100 ml. and the pre-albumin zone at 200–230 μg/100 ml.

When the electrophoresis was carried out at pH 7·6, all the thyroxine was divided between albumin and the inter-alpha zone at all concentrations

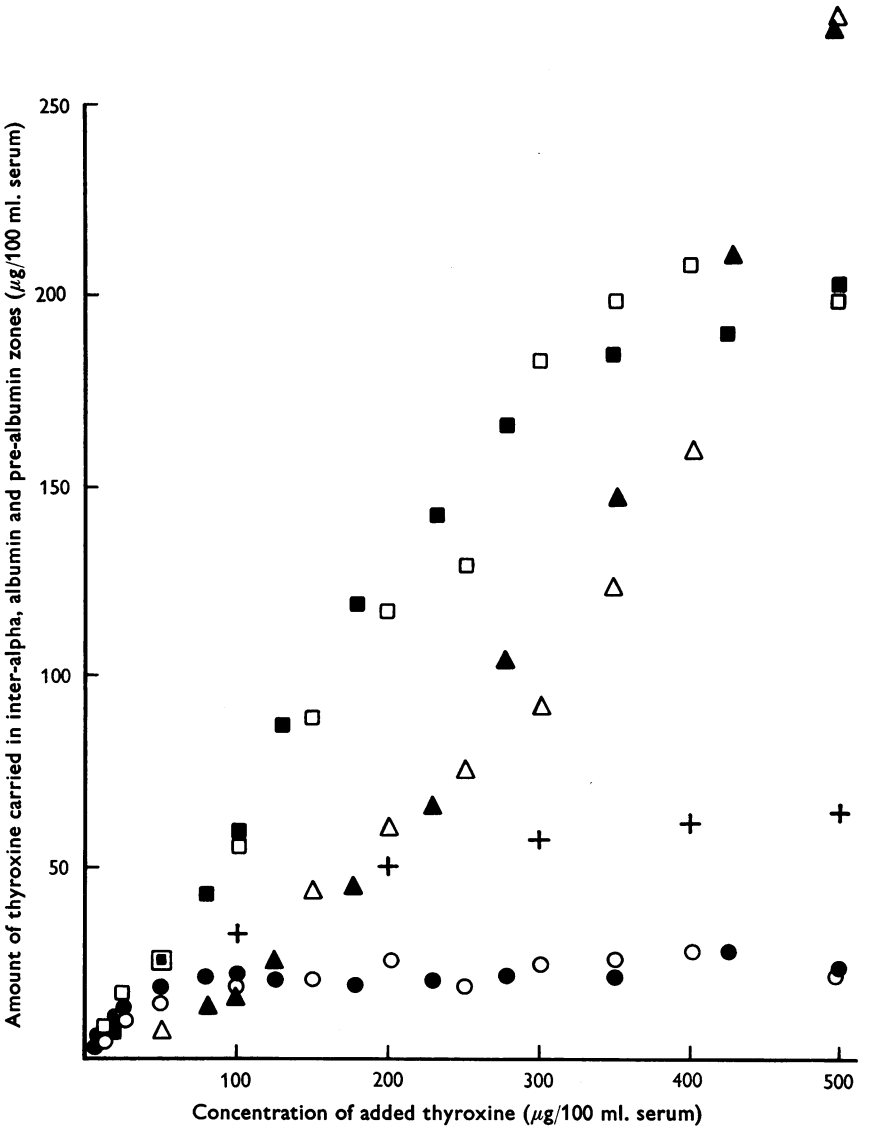

Fig. 3. Relation between concentration of added radioactive thyroxine in serum and amount of thyroxine carried at the inter-alpha (circles), albumin (triangles) and pre-albumin (squares) zones after electrophoresis in tris-maleate buffer at pH 9·2 (open symbols) and 8·6 (filled symbols). Crosses (+) show amount of thyroxine carried in inter-alpha zone in tris-maleate at pH 7·6. Each value averaged from several observations on different samples of serum from the same normal adult human being. The endogenous thyroxine in the serum (about 5 μg/100 ml.) has been ignored in all calculations.

from 1 to 500 μg/100 ml., none moving in the pre-albumin zone at any concentration tested. The amount carried in the inter-alpha zone increased throughout the whole of this range, reaching a value of about 60 μg/100 ml. when the concentration of added thyroxine was 500 μg/100 ml.

Electrophoretic distribution of endogenous thyroxine

A single observation was made on the distribution of endogenous thyroxine in the serum of a thyrotoxic patient. A sample of blood was taken from the patient 4 days after she had been given a dose of 7 mc of ^{131}I, and the serum was submitted to paper electrophoresis in tris-maleate buffer at pH 8·6. The distribution of radioactivity was similar to that in serum to which radioactive thyroxine had been added at a concentration of 1 μg/100 ml., the proportions of the total activity at the inter-alpha, albumin and pre-albumin zones averaging 57, 6 and 35% respectively. It was possible to demonstrate that nearly all the activity on the paper was due to radioactive thyroxine, by showing that 90% of the activity at each of the three peaks was removed by washing the paper with ethanol.

Electrophoretic distribution of thyroxine in borate buffer

Since the electrophoretic behaviour of thyroxine in tris-maleate buffer differs so markedly from that in barbiturate buffer, it was of some interest to see how thyroxine behaves in the presence of another buffer. Accordingly, all the experiments described up to this point were repeated with borate buffer. All the results obtained were similar to those obtained with tris-maleate buffer.

The distribution of thyroxine in the presence of serum, and when tested by itself on phenylalanine-treated paper, was the same in borate as in tris-maleate buffer at pH 7·6, 8·6 and 9·2; so, also, was the effect of raising the concentration of thyroxine on the partition of thyroxine between the inter-alpha, albumin and pre-albumin zones. Finally, the distribution of endogenous thyroxine in serum tested in borate buffer at pH 8·6 did not differ significantly from that observed in tris-maleate at pH 8·6.

Movement of thyroxine to the pre-albumin zone during electrophoresis in two dimensions

The failure of any thyroxine to move ahead of the albumin in tris-maleate at pH 7·6 or in barbiturate at pH 8·6 calls for some explanation. One possibility is that under these conditions the binding substance, presumed to be responsible for carrying thyroxine to the pre-albumin zone, moves ahead of the albumin, but is unable to bind thyroxine. In order to test this possibility paper electrophoresis of thyroxine in serum

was carried out in two dimensions, first in barbiturate, and then in tris-maleate at pH 8·6.

A sample of serum, without any added thyroxine, was put on to a strip of filter paper and submitted to electrophoresis in barbiturate buffer at pH 8·6. The paper was then removed and cut longitudinally into two pieces. One piece was dried and stained to show the positions of the protein bands. The other was then gently pressed down along the cathodal edge of a square sheet of filter paper prepared for electrophoresis in tris-maleate buffer at pH 8·6, and already in position in the box. About 50 μl. of a solution of radioactive thyroxine in tris-maleate buffer at pH 8·6 was put on the square sheet of paper along a line 1 cm in front of the edge of the strip of paper used in the first dimension, and extending along the whole of its length. Electrophoresis was then carried out for 18 hr. The distribution of radioactivity, as shown on an autoradiograph made from the dried paper (Fig. 4), showed that the thyroxine placed opposite the pre-albumin zone in the first dimension was carried towards the anode in the second dimension. The same result as that shown in Fig. 4 was obtained when tris-maleate at pH 7·6 was used in the first dimension, in place of barbiturate at pH 8·6.

Movement of thyroxine at the pre-albumin zone could be demonstrated in the second dimension only if the experiment was done in the way described above. If the radioactive thyroxine was put on the strip of paper used in the first dimension, instead of 1 cm in front of it on the second paper, none of the activity in the pre-albumin zone moved towards the anode during electrophoresis in tris-maleate buffer at pH 8·6. Presumably the buffer in the paper strip used for the first dimension prevented the binding substance in the pre-albumin zone from binding thyroxine; binding could only occur in conditions in which the binding substance first came into contact with the thyroxine in the presence of the tris-maleate buffer used for the second dimension.

Effect of proteolytic enzymes on the electrophoretic distribution of thyroxine in serum

In an earlier paper (Myant & Osorio, 1960) it was shown that when thyroxine is submitted to paper electrophoresis in the presence of a proteolytic digest of serum in barbiturate buffer, at pH 8·6 all the thyroxine moves to the inter-alpha zone, whereas at pH 7·6 it remains at the line of application. These experiments were repeated, with tris-maleate instead of barbiturate buffer. For each experiment two papers were run in parallel, one with radioactive thyroxine dissolved in normal serum (1 μg/100 ml.), and the other with radioactive thyroxine dissolved at the same concentration in serum that had been digested with papain.

At pH 8·6 the radioactivity in the proteolytic digest moved as a single peak to a position corresponding to the inter-alpha zone, there being no detectable activity in the pre-albumin zone. At pH 7·6 all the radioactive thyroxine added to the paper in the digested serum remained at the line of application of the sample; most of the radioactivity on the paper with normal serum moved as a single peak to the inter-alpha zone, as has already been shown in Fig. 2*a*.

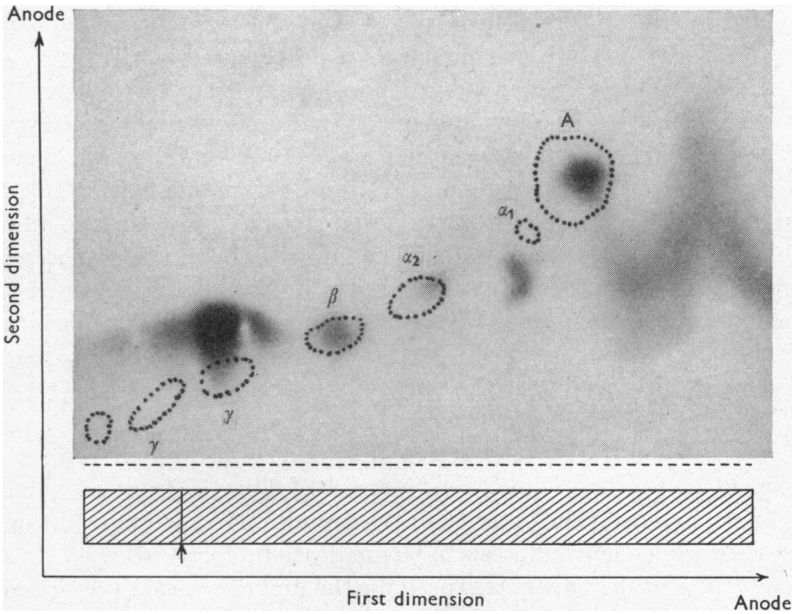

Fig. 4. Autoradiograph of two-dimensional paper electrophoresis of radioactive thyroxine in serum (10 μg/100 ml.). First dimension in barbiturate buffer at pH 8·6; second dimension in tris-maleate buffer at pH 8·6. Shaded area shows position of strip used for first dimension (line of application of serum shown by arrow), after being placed on square sheet of paper at start of run in second dimension. Radioactive thyroxine solution (50 μl.) was applied approximately along the horizontal dotted line for run in second dimension. Areas enclosed by dotted lines show positions of protein spots at end of run in second dimension, letters indicating serum protein fractions (A = albumin). Black spots show positions of radioactivity. Movement of radioactivity in pre-albumin zone shown by broad arrow-shaped area of blackening to right of albumin spot.

DISCUSSION

The observations described in this paper show that the phenylalanine effect occurs in tris-maleate buffer, as well as in barbiturate. It cannot, however, account for the electrophoretic mobility of thyroxine in human serum, as observed in tris-maleate. In the first place, phenylalanine does not cause thyroxine by itself to move to the pre-albumin zone at pH 8·6

or 9·2, although at both pH's some thyroxine moves to the pre-albumin zone in the presence of serum (Fig. 2). Secondly, if thyroxine is submitted to paper electrophoresis on phenylalanine-treated paper at pH 7·6, it remains at or near the origin, whereas at this pH it moves in the inter-alpha zone in the presence of human serum. Our observations are, therefore, consistent with the view, now generally held, that when thyroxine moves towards the anode on paper electrophoresis in the presence of serum, it does so because it is bound to a specific substance in the serum whose electrophoretic mobility determines that of the thyroxine. Although there is no proof for this hypothesis, no other satisfactory explanation has been put forward to account for the fact that there is a limit to the amount of thyroxine that can move in the inter-alpha and pre-albumin zones, and for the purpose of this Discussion we shall assume the hypothesis to be true.

The electrophoretic distribution of thyroxine in serum in tris-maleate at pH 8·6 and 9·2 raises the possibility, first suggested by Ingbar (1958), that there are two specific thyroxine-binding substances in human serum. It has been suggested (Tata, 1960) that there is only one binding substance —the one responsible for carrying thyroxine to the inter-alpha zone in barbiturate—and that in the presence of tris-maleate at pH 8·6 this splits into two fractions, one remaining in the inter-alpha zone and the other moving to the pre-albumin zone. A similar explanation might also be put forward to account for the difference between the behaviour of thyroxine in tris-maleate at pH 8·6 and at pH 7·6 (Fig. 2). The binding capacity at the inter-alpha zone is higher in barbiturate than in tris-maleate at pH 8·6 (compare Robbins (1956) with Fig. 3), and this certainly suggests that the buffer influences the relative amounts of binding substance in the two zones. However, this cannot be the whole explanation of the failure of thyroxine to move in the pre-albumin zone in barbiturate, since the inter-alpha zone in barbiturate always carries less thyroxine than is carried in the two zones together in tris-maleate at pH 8·6. A comparison between the results of Robbins (1956) and those of Fig. 3 shows, for instance, that when the total thyroxine concentration is 100 μg/100 ml., in barbiturate the inter-alpha zone carries 35 μg/100 ml. whereas in tris-maleate at pH 8·6 the inter-alpha and pre-albumin zones together carry more than 80 μg/ml.

Electrophoresis in two dimensions shows that a binding substance does move to the pre-albumin zone in barbiturate and in tris-maleate at pH 7·6, although it is unable to adsorb thyroxine in these conditions. This is best explained by supposing that there are, in fact, two binding substances in human serum with different electrophoretic mobilities, one of which moves to the pre-albumin zone (pre-albumin binding substance) and is prevented

from binding thyroxine under certain conditions. The existence of two different binding substances would also explain why extracting serum with alcohol suppresses thyroxine-binding in the inter-alpha zone, but not in the pre-albumin zone (Osorio & Myant, unpublished observations). This hypothesis does not explain the high binding capacity at the inter-alpha zone in barbiturate and in tris-maleate at pH 7·6. As was suggested by Robbins (1956), part of the albumin fraction may, in some conditions, move in the inter-alpha zone. Alternatively, the binding capacity of the inter-alpha substance itself may be increased in barbiturate and in tris-maleate at pH 7·6.

When serum has been digested with proteolytic enzymes it no longer enables thyroxine to move in the pre-albumin zone during paper electrophoresis in tris-maleate at pH 8·6. This suggests that the pre-albumin binding substance is a protein. If so, its concentration in the serum must be very low, since protein at a concentration of 6 mg/100 ml. was detectable with the dye used for staining the paper, yet no staining was ever seen in front of the albumin band.

We have drawn attention elsewhere (Myant & Osorio, 1960) to the difficulty of correlating observations on the electrophoretic distribution of thyroxine in serum, if the observations are made by different methods. Rich & Bearn (1958) have shown that when thyroxine is submitted to electrophoresis in starch gel in the presence of serum, it moves in a zone ahead of the albumin. However, the substance responsible for this effect cannot be assumed to be the pre-albumin binding substance observed on paper electrophoresis in tris-maleate. On the contrary, Tata (1960) has suggested that the binding substance observed by Rich & Bearn is the same as that which carries thyroxine to the inter-alpha zone during paper electrophoresis.

It has generally been assumed that the substance that carries thyroxine to the inter-alpha zone on paper electrophoresis in barbiturate at pH 8·6 also binds most of the thyroxine circulating in the blood under physiological conditions (see, for instance, Robbins & Rall, 1957), though the evidence for this has always been indirect. Since it now appears that there are two specific binding substances in serum, this assumption must be re-examined. At pH 8·6 and 9·2 the pre-albumin substance has a binding capacity of more than 200 μg of thyroxine/100 ml. of serum, as shown by the maximum value approached at the highest concentrations tested (Fig. 3). This is about ten times the capacity of the inter-alpha substance. The binding affinity of the inter-alpha substance, however, must be at least ten times that of the pre-albumin substance. This is shown by the fact that with endogenous thyroxine, and at the lowest concentrations of added thyroxine, when the amount bound by each substance is

proportional to the product of its affinity and its capacity (Myant & Osorio, 1959), the thyroxine is shared about equally between the two substances.

Unfortunately, the conditions necessary for adequate separation of the serum proteins by paper electrophoresis are not physiological; nor is it easy to deduce how thyroxine is distributed between the different protein fractions *in vivo*, by extrapolation from observations *in vitro*. As the pH of the tris-maleate buffer is reduced from 9·2 to 8·6, there is a slight increase in the proportion of the thyroxine carried in the inter-alpha zone at low concentrations, but no change in the binding capacity at either the inter-alpha or the pre-albumin zone (Fig. 3). This suggests that a change in the pH towards the physiological level increases the binding affinity of the inter-alpha substance relative to that of the pre-albumin. The findings with tris-maleate at pH 7·6 are consistent with this, although some other effect, as discussed above, must be assumed in order to account for the high capacity at the inter-alpha zone at this pH.

The effect of pH might suggest that the pre-albumin substance plays little part in the transport of thyroid hormone *in vivo* and that the distribution of thyroxine observed by paper electrophoresis in barbiturate buffer is closer to the physiological than that observed in tris-maleate at pH 8·6. On the other hand, it may be relevant that at pH 8·6 the pre-albumin substance binds nearly half the thyroxine in serum in three different buffers—ammonium carbonate (Beierwaltes & Robbins, 1959), tris-maleate and borate. Until something is known about the mechanism by which the pH and composition of the buffer influence the behaviour of thyroxine-binding substances in serum, it would be unwise to draw any conclusions as to which results most closely reflect the distribution of thyroxine *in vivo*.

SUMMARY

1. In tris-maleate buffer the electrophoretic mobility of thyroxine alone on phenylalanine-treated paper differs from that of thyroxine in human serum, at pH 8·6 and at pH 7·6. It is concluded that the distribution of thyroxine in serum, as observed by paper electrophoresis, is not due to the serum exerting an action analogous to that of phenylalanine. It is more likely that it is determined by the presence of thyroxine-binding substances in the serum.

2. During paper electrophoresis in barbiturate or in tris-maleate at pH 7·6 no thyroxine moves ahead of the albumin, although in both conditions a binding substance may be demonstrated in the pre-albumin zone by electrophoresis in a second dimension.

3. If the pH of tris-maleate buffer is reduced from 9·2 to 8·6, there is

an increase in the binding-affinity of the inter-alpha substance relative to that of the binding substance moving in the pre-albumin zone. The binding of thyroxine by the serum proteins in physiological conditions is discussed in relation to this finding.

4. When thyroxine is submitted to paper electrophoresis in the presence of papain-treated serum, it no longer moves in the pre-albumin zone in tris-maleate at pH 8·6. This is consistent with the view that the binding substance responsible for carrying thyroxine in the pre-albumin zone is a protein.

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