Use of Thyroxine-Displacing Drugs in Identifying Serum Thyroxine-Binding Proteins Separated by Starch Gel Electrophoresis.* (28506)

RICARDO SQUEF,† MODESTO MARTINEZ, AND JACK H. OPPENHEIMER‡
(Introduced by L. Leiter)
Endocrine Service, Medical Division, Montefiore Hospital, New York City

It is generally agreed that 3 thyroxine-binding fractions can be identified by paper electrophoresis of normal human serum: (1) thyroxine-binding globulin (TBG), which has a mobility intermediate between alpha 1 and alpha 2 globulin; (2) serum albumin; and (3) thyroxine-binding prealbumin (TBPA), an unstained fraction with an anodal mobility greater than that of albumin. On the other hand, conflicting reports have appeared in the literature regarding the partition of radioactive thyroxine (T4-I-131) among serum proteins separated electrophoretically in a starch gel medium. The early studies of Rich and Bearn(1) and Allison(2) suggested that the principal thyroxine-binding protein in starch gel had an electrophoretic mobility similar to prealbumin 1 (Smithies(3)). Blumberg *et al.*(4,5), however, indicated the presence of 4 distinct bands in autoradiographs of starch gel patterns: band 1, corresponding to prealbumin 1; band 2, to the leading edge of the stained albumin zone; band 3, to the slowest half of the albumin zone; and band 4, to the immediate post-albumin area. Two-dimensional gel and paper studies led these authors to the conclusion that band 1 is identical to the TBPA observed in the paper system, that band 2 resembles albumin, and that band 4 corresponds to TBG. Band 3 could not be identified. Tata(6) regularly identified 3 radioactive bands (pre-albumin, albumin, post-albumin) with a low concentration of added T4 and an additional relatively slowly migrating band in the presence of high concentrations of T4.

It occurred to us that certain drugs which are known to displace T4 from specific thyroxine-binding proteins in paper electrophoretic systems might be useful in attempting a qualitative correlation between paper and starch gel electrophoretic systems. 5,5 diphenylhydantoin (Dilantin) (DPH) displaces T4 from TBG(7,8); 2,4 dinitrophenol (DNP) inhibits the binding of T4 by TBPA (7); and more recently the effect of penicillin in dislodging T4 from TBPA was also demonstrated(9). Accordingly, these drugs were added to serum in an effort to compare their effect on the electrophoretic partition of T4-I-131 in paper and starch gel media.

Methods. T4-I-131 in 50% propylene glycol was obtained from Abbott Laboratories, North Chicago, Ill. Radiochromatographic purity was tested in a descending system (butanol : dioxane : 2N NH_4 OH- 4:1:5). All samples contained less than 6% contaminating iodide-I-131, and no other radioactive impurities were found. To normal sera, 0.03 μg/ml T4-I-131 was added and allowed to equilibrate for 30 minutes at room temperature before the start of electrophoresis.

Vertical starch gel electrophoresis was performed by slight modification of the method of Smithies(10). Starch gel was prepared according to the instructions of the manufacturer (Connaught Medical Research Laboratories, Univ. of Toronto, Canada), employing a borate buffer at pH 8.6 (boric acid 0.0260 mol/l, NaOH 0.104 mol/l). Electrophoresis was carried out in an apparatus manufactured by Buechler Instruments, Fort Lee, N. J. Instead of the direct insertion of serum into the 10 slots at the origin, precut filter paper (Filter Paperwicks No. 319329, Spinco, Palo Alto, Calif) saturated with the serum preparation to be studied was placed into the origin slot. By this modification of the procedure the lateral spread of the serum albumin band was prevented. Electrophoresis

* Supported by grants from Nat. Inst. of Health and from Nat. Foundation for Neuromuscular Diseases.

† Recipient of a research fellowship from the Dazian Foundation for Medical Research.

‡ Career scientist, Health Research Council of N.Y.C.

was carried out at 125 v for 16 hours.

Upon completion of the electrophoresis, the path of migration of each radioactive sample was cut into serial 0.5 cm full-thickness sections. The slices were assayed for radioactivity in a Packard Autogamma Spectrometer. On either side of each radioactive sample, a sample of serum without added radioactivity was run to serve as a protein marker and to allow a generous lateral separation from next radioactive sample. Transverse markings demarcating the 0.5 cm sections were carried sufficiently far into the non-radioactive strips to allow the establishment of a point-to-point correspondence between the radioactive count and the position of the stained protein bands. This procedure was necessitated by the shrinkage of the non-radioactive strips in the course of the staining procedure which was carried out according to the recommendations of Smithies(3).

Paper electrophoresis was performed in a glycine acetate buffer (pH 8.6, ionic strength 0.15) in a Durrum-type apparatus as previously described(8). Radioactive areas were measured by an automatically-integrating recorder (Servo-Riter, Texas Instruments, Houston).

5,5 diphenylhydantoin was obtained as Dilantin Sodium (Parke, Davis and Co., Detroit, Mich.), benzylpenicillin as Penicillin G (Parke, Davis and Co.) and 2,4 dinitrophenol as supplied by Matheson, Coleman and Bell, Norwood, Ohio. Sera of normal euthyroid subjects were employed in these studies. All experiments cited in this study were repeated at least once with a different serum sample. Essentially similar results were always obtained.

Results. In the starch gel patterns 3 peaks of radioactivity (P1, P2, and P3) could be distinguished (Fig. 1). P1 corresponded to the stained area of prealbumin 1 and was well separated from P2 and P3. P2 was associated with the leading two-thirds of the stained albumin band, whereas, P3 was situated in the trailing third of albumin and in the immediate unstained post-albumin area. Although P2 and P3 were in general poorly separated when electrophoresis was performed

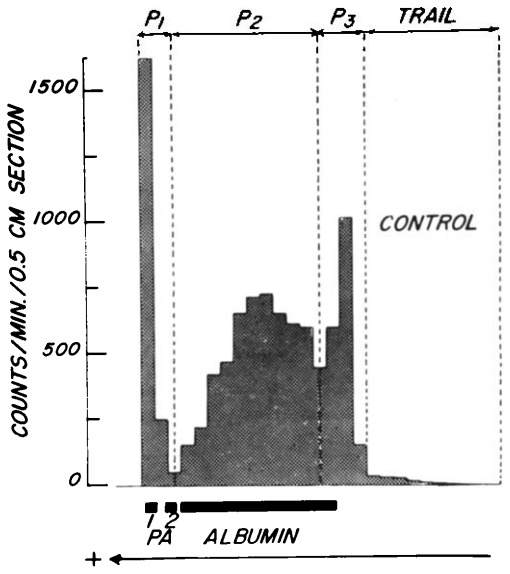

FIG. 1. Distribution of T4-I-131 (3 μg/100 ml) among serum proteins of normal subjects separated by starch gel electrophoresis. Areas of stained protein are indicated as well as direction of protein migration.

with serum containing only small quantities of T4-I-131, the distinction between these peaks became readily apparent when larger quantities of T4 and thyroxine-displacing drugs were added to the serum before electrophoresis. A variable amount of radioactivity (T) trailed behind P3.

Addition of 1.29×10^{-6} M T4 (100 μg/100 ml) (Table I) resulted in a shift of radioactivity from P3 to P1 in the starch gel medium and from TBG to TBPA and albumin in the paper medium. Although no well-defined binding maxima could be determined for P3, the direction of this shift suggested that P3, like TBG, had a smaller net binding capacity than other binding proteins. It appears probable that the failure to achieve a plateau concentration of T4 in P3 with addition of increasing quantities of T4 was due to the contamination of P3 with binding protein species other than TBG.

On addition of the thyroxine-displacing drugs, P1 behaved in a fashion analogous to TBPA, and P3, to TBG (Table I). Thus, DPH caused a progressive displacement of T4-I-131 from P3 to P1 in the starch gel system and from TBG to TBPA in paper

TABLE I. Effect of Thyroxine-Displacing Drugs and Thyroxine on Distribution T4-I-131 (3 μg/100ml) Among Serum Proteins Separated by Vertical Starch Gel Electrophoresis and Paper Electrophoresis.

Substance added	Conc. (M)	Endogenous PBI (μg/100 ml)	Distribution of radioactivity (%) Starch gel P1	P2	P3	Trail	Paper TBPA	Alb.	TBG
L-Thyroxine	0	5.3	19.77	57.43	20.93	1.87	35.52	9.31	55.17
	1.29×10^{-6}		30.74	54.71	11.48	3.07	68.88	14.46	16.66
	3.86×10^{-6}		25.92	60.64	8.59	4.85	69.25	23.27	7.48
	10.30×10^{-6}		15.07	68.83	11.13	4.96	40.31	56.64	3.05
2,4 Dinitrophenol	0	4.6	18.87	39.52	37.14	4.47	33.50	9.60	56.90
	1×10^{-5}		14.30	38.39	41.69	5.12	29.95	11.35	58.74
	1×10^{-4}		6.50	40.56	47.17	5.77	17.11	13.23	69.64
	1×10^{-3}		2.21	39.28	50.96	7.55	2.57	15.20	82.23
Benzylpenicillin	0	4.6	19.80	46.07	30.26	3.87	34.13	13.17	52.70
	3.4×10^{-4}		16.11	42.86	35.26	5.77	30.89	13.12	55.42
	3.4×10^{-3}		14.61	39.16	37.50	8.74	11.84	35.80	52.36
5,5 Diphenylhydantoin	0	5.3	15.33	50.26	30.63	3.87	28.27	10.84	60.89
	7.3×10^{-3}		19.52	49.50	24.75	6.23	52.69	9.96	37.35
	2.92×10^{-2}		25.78	50.69	17.66	5.87	66.44	9.41	24.15

electrophoresis. DNP, which displaces T4 from TBPA, induced a shift from P1 to P3 in starch gel. Similarly, penicillin caused a diminution of T4-I-131 associated with both P1 and TBPA. Two minor differences between the two systems are apparent with respect to the shifts of radioactivity induced by these drugs. (1) Whereas on paper T4-I-131 displaced by DNP from TBPA was accepted by both albumin and TBG, in starch gel only P3 accepted the T4 dislodged from P1. (2) In paper electrophoresis penicillin effected a diminution of radioactivity associated only with TBPA, whereas in a starch gel medium this drug displaced T4 from both P1 and P2.

Addition of the various drugs resulted in no visual alteration of the stained electrophoretic patterns except for a moderate increased mobility of the broad albumin band in the presence of high concentrations of penicillin. Similar alterations had previously been noted in stained paper electrophoretograms(9).

Discussion. It is evident from the effects of T4, DPH, DNP, and penicillin that P1 in the starch gel system corresponds qualitatively to TBPA in paper electrophoresis and P3 to TBG. However, marked differences in quantitative partition of T4-I-131 between these two systems were noted. Such differences may arise either from inherent differences in the binding characteristics of individual species of binding proteins in the two systems, or to differences in the electrophoretic separation of binding proteins. Thus, P2 contained a larger percentage of T4-I-131 in the starch gel system than albumin in paper electrophoresis. Either the albumin binds relatively more T4 in a starch gel or contaminating quantities of TBG and TBPA are present in P2.

Table II summarizes and compares the reports of a number of investigators who have studied thyroxine-binding by serum proteins separated by starch gel electrophoresis. The reason for the striking discrepancies in these investigations remains unclear. Certainly our

TABLE II. Summary of Reports on Distribution of T4-I-131 Among Serum Protein Separated by Starch Gel Electrophoresis.

Investigators	Postalbumin		Albumin		Prealbumin
Rich and Bearn(1)					×
Allison(2)					×
Tata(3)	Fraction 1	Fraction 2	Fraction 3		Fraction 4
Blumberg *et al.*(4,5)	Trail	Band 4	Band 3	Band 2	Band 1
Squef *et al.*	Trail		P3	P2	P1

data would serve to confirm the observations of Blumberg *et al.*(4,5) and those of Tata (6) in suggesting that TBPA is not the only thyroxine-binding protein demonstrable in starch gel electrophoresis as was implied in the earlier reports of Rich and Bearn(1) and Allison(2).

It appears likely that band 1 of Blumberg corresponds to our P1 and that their bands 3 and 4 correspond to our P3. If so, our results corroborate the finding of these authors that thyroxine-binding prealbumin in starch gel is identical with the TBPA demonstrated by paper electrophoresis. Blumberg *et al.* also showed that their band 4 corresponded to TBG but they were unable to identify their band 3 in paper systems. It is of interest, however, that these authors found increased amounts of T4-I-131 associated with *both* bands 3 and 4 in pregnancy(5), a state associated with increased levels of circulating TBG. There thus appears to be a rough qualitative agreement between our results and those of Blumberg *et al.*

Although further studies are necessary to reconcile the conflicting reports, the present communication has demonstrated the usefulness of the thyroxine-displacing drugs in identification of individual binding proteins. An extension of this technique may materially assist in the solution of certain problems posed by the newer solid supporting media employed in electrophoretic separation of serum thyroxine-binding proteins.

That penicillin and DNP do not induce a visible change in the protein stain of prealbumin 1 indicates that the diminution of radioactivity associated with this protein fraction results from an actual inhibition at the binding site rather than from a drug-induced failure of protein separation.

Summary. Serum containing small quantities of radioactive thyroxine (T4-I-131) was subjected to vertical starch gel electrophoresis. Three radioactive peaks were recognized: P1, corresponding to prealbumin 1 in the protein-stained pattern; P2 to the leading two-thirds of the stained albumin band; and P3 to the trailing one-third of albumin and the immediate unstained post-albumin area. Various drugs known to dislodge T4 from specific thyroxine-binding proteins as identified in paper electrophoresis were added to the serum before electrophoresis. Benzylpenicillin and 2,4 dinitrophenol, drugs which displace T4 from thyroxine-binding prealbumin (TBPA) effected a reduction in the radioactivity associated with P1. 5,5 diphenylhydantoin, which displaces T4 from thyroxine-binding globulin (TBG) brought about a reduction in P3. Thus, it was concluded that P1 corresponds qualitatively to TBPA and P3 to TBG.

1. Rich, C., Bearn, A. G., *Endocrinology,* 1958, v62, 687.

2. Allison, A. C., *Experientia.,* 1959, v15, 281.

3. Smithies, O., *Biochem. J.,* 1955, v61, 629.

4. Blumberg, B. S., Robbins, J., *Endocrinology,* 1960, v67, 368.

5. Blumberg, B. S., Farer, L., Rall, J. F., Robbins, J., *ibid.,* 1961, v68, 25.

6. Tata J., Widnell, C., Gratzer, W. B., *Clin. Chim. Acta.,* 1961, v6, 697.

7. Wolff, J., Standaert, M. E., Rall, J. E., *J. Clin. Invest.,* 1961, v40, 1373.

8. Oppenheimer, J. H., Tavernetti, R., *Endocrinology,* 1962, v71, 496.

9. Surks, M. I., Oppenheimer, J. H., *Endocrinology,* 1963, v72, 567.

10. Smithies, O., *Biochem. J.,* 1959, v71, 585.

Received April 17, 1963. P.S.E.B.M., 1963, v113.