ther to decreased body retention of iodine nor to foreign protein reaction, but seems to take place either at organification of the iodine stage or at subsequent stages that lead to secretion of thyroid hormone. It has been shown that thyroxine or desiccated thyroid gland(7) as well as triiodothyronine(8) given either orally or intravenously produce an immediate depression in thyroidal uptake of iodide ion but leaves the level of protein-bound iodine (PBI) unchanged. The depressive effect lasts beyond removal of exogenous thyroxine. It does not therefore seem probable that the depressed conversion ratio is a result of thyroxine or triiodothyronine bound to the glycoprotein component of fraction VI.* We are currently carrying out fractionation procedures to separate or at least concentrate this factor.

Summary. Serum fraction VI from a bovine source showed on paper electrophoretic separation the presence of a glycoprotein band capable of binding I^{131} labeled thyroxine to the exclusion of other proteins present. On being given intravenously in the tail of rats, bovine fraction VI gave a marked depression in conversion ratio of inorganic to protein-bound iodine. This effect was shown to be due neither to a foreign protein reaction nor to a decreased body retention of iodine. The uptake of iodide ion by the thyroid gland did not appear to be the cause of this depressed conversion ratio.

* In fact, we have examined samples of BFrVI for iodine content and have found the complete absence of iodine. These results have been presented for publication in this Journal.

1. Larson, F. C., Deiss, W. P., Albright, E. D., *J. Clin. Invest.*, 1954, v33, 230.
2. Robbins, J., Petermann, M. L., Rall, J. E., *J. Biol. Chem.*, 1955, v212, 403.
3. Freinkel, N., Dowling, J. T., Ingbar, S. H., *J. Clin. Invest.*, 1955, v34, 1098.
4. Ingbar, S. H., Dowling, J. T., Freinkel, N., *Endocrinology*, 1957, v61, 321.
5. Schmid, K., *J. Am. Chem. Soc.*, 1953, v75, 60.
6. Köiw, E., Gronwall, A., *Scand. J. Clin. Lab. Invest.*, 1952, v4, 244.
7. Sharrer, N. E., Asper, S. P., *J. Clin. Endocrin. Metab.*, 1956, v16, 1311.
8. McConahey, W. M., Owen, C. A., *ibid.*, 1956, v16, 1480.

Received June 9, 1958. P.S.E.B.M., 1958, v99.

Thyroid Depressant Activity in Bovine Fraction VI. (24422)

BERNARD RABINOVITCH, FRANKLIN J. STAR AND DWIGHT E. CLARK
(With technical assistance of Felicia Carreon)
Argonne Cancer Research Hospital, USAEC, Departments of Biochemistry and Surgery, University of Chicago, Chicago, Ill.

We reported(1) the presence of a thyroxine-binding protein (TBP) in bovine fraction VI (BFrVI) which was identified as a glycoprotein. It was also reported that BFrVI has a depressant effect on thyroid activity, as measured by the conversion ratio, when given intravenously to the rat. It was suggested that this effect might be attributable to thyroxine(2) accumulated by the TBP. We have since obtained many samples of BFrVI which the present paper shows have markedly higher thyroid depressant activity than those previously described, and have demonstrated that the activity is not due to presence of thyroxine nor to versene or heavy metals which may be present as impurities. The method for assaying the BFrVI for thyroid activity has been described(1). Sprague-Dawley female rats, weighing 250 to 300 g each, were used unless otherwise stated. Table I gives evidence of the markedly increased thyroid depressant activity of 2 samples of the same lot of BFrVI,* a single dose of 0.3 ml of a 20% fraction VI solution giving a conversion ratio of 16.8 compared with a value of 16.1 $\pm$ 5.4 previously obtained with 4 doses of 0.5 ml of a 20% solution(1). *Determination of*

* Obtained from Pentex Corp.

TABLE I. Comparison of Different Samples of Bovine Fraction VI.

Sample No.	Dose (ml 20%)	C.R.
3-8205	.5 (2)	11.1 ± .2
	.3 (1)	16.8
2-8205	.5 (4)	9.2 ± .4
	none (2)	32.1 ± .2
	" (3)	32.7 ±2.7

Numbers in parentheses represent No. of rats used. C.R. represents Conversion Ratio defined as ratio of cpm on precipitated protein from 3 ml serum to that on 3 ml serum, 24 hr after intrav. inj. of 5 μc I^{131} iodide (carrier-free)(1).

The errors given in this and subsequent tables represent mean deviations from the means.

protein-bound iodine. To examine the possibility that thyroxine was present in these protein fractions, we determined the protein-bound iodine (PBI) by a method described by Moran(3). All water used in the determination and for washing glass apparatus was distilled prior to passing through an anion-cation exchange resin. Optical density measurements were made with 1-cm cuvettes on a Beckman Spectrophotometer, Model DU, at 420 mμ. Standard iodide solutions were used to establish a calibration curve in terms of PBI units, *i.e.*, γ per 100 ml serum. Such a curve is shown in Fig. 1, giving some idea as to reproducibility. A standard sample of Iodotrol† was examined for its iodine content,

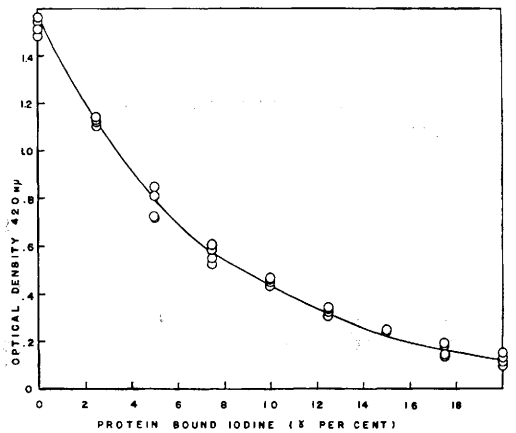

FIG. 1. Reproducibility of standard curve in determination of iodide by method of Moran(3).

† All glass apparatus and all chemical reagents specially prepared to be iodine-free and Iodotrol, a synthetic plasma preparation of known iodine content, supplied by Hormone Chemistry Lab.

and a sample of the same lot number returned to the Hormone Chemistry Laboratory for PBI determination. The recipients in the Hormone Chemistry Laboratory were unaware of the nature of the sample. At this time, 2 samples of the BFrVI, lot No. 8205, the first, 60 mg, the second, 600 mg, were examined for iodine content, and the plasma from 6 normal rats was examined similarly for comparison. The results are shown in Table II. We have taken the mean of the optical densities in 16 determinations on reagent blanks as most meaningful, since occasionally the apparatus on standing becomes slightly contaminated and this is reflected in the reagent blank. This mean is 1.32 ± 0.06 and this figure is used to determine PBI given in the last column; however, figures given in parentheses are the PBI determined using the reagent blank figures of column 3. The number returned to us from the Hormone Chemistry Laboratory was 6.3 ± 0.2 γ %. It is clear that the PBI determinations are valid, and that there appears to be no iodine in BFrVI. Schmidt(4) reports 10 g of protein in human fraction VI from 13 liters of plasma; we may therefore estimate that 600 mg of BFrVI represents 780 ml serum. The usual volume is 3 ml of serum for PBI determination by the Moran method.

TABLE II. Determination of Protein-Bound Iodine.

Sample	OD (420 mμ) Unknown	OD (420 mμ) Reagent blank	PBI (γ %)*
3 ml Iodotrol	.568, .570	1.19, 1.25	6.4 (5.85)
BFrVI			
60 mg	1.35, 1.35	1.40, 1.35	0
600 mg	1.29, 1.29	1.38, 1.39	.10 (.50)
3 ml rat serum			
1	.908, .925	1.34, 1.37	2.6 (2.8)
2	.740, .735	1.38, 1.39	4.2 (4.55)
3	.825, .805	1.31, 1.28	3.5 (3.4)
4	.900, .905	1.33, 1.35	2.65 (2.8)
5	.860, .845	1.18, 1.16	3.15 (2.3)
6	.890, .805		2.80

* Corrected for reagent blank.

Results. Effect of sodium versenate. In preparation of BFrVI(4), the proteins are precipitated in the presence of excess barium ions and are subsequently removed by dialysis in presence of versenate over a period of sev-

TABLE III. Effect of Neutralized Versene and of Barium Ions on Thyroid Function.

Dose	C.R.
.10 mg Versene	30.6
.25 *Idem*	31.1
1.0 "	31.9
2.0 "	38.7
5.0 "	30.1
.1 ml 20% (4)	37.6 ± 5.9
.2 " " (3)	39.0 ± 5.4
Control (11)*	37.7 ± 4.3

* Control was performed on untreated rats of same strain and age as exp. rats. Numbers in parentheses represent No. of rats used.

eral days. To examine the possibility that some versenate had remained in the lyophilized fraction VI, which might account for the results obtained, a series of rats was given injections in the tail vein of 0.5 ml of a solution of sodium versenate at pH 7.4 so as to contain various quantities of the chelating agent. Simultaneously, they were given injections of 5 μc of I^{131} iodide, and a 24-hr conversion ratio was obtained. The rats used were 450- to 500-g Holtzman males. Results are given in Table III.

A normal conversion ratio determined on 11 of these male rats gave a value of 37.7 ± 4.3. It seems possible that the data may indicate a slightly depressed conversion ratio, but this could not account for the marked effects of the BFrVI. One-tenth mg has about the same effect as 5.0 mg of versene. This is not surprising since it has been shown that from 60 to 70% of the versene given intravenously to the rat is excreted in the urine in 2 hours and from 90 to 95% in 6 hours(5). Moreover, 5 mg would represent 5% by weight of the usual dose of BFrVI (0.5 ml 20%) given to the rat and one would have to reach an inordinately high versene content to obtain a conversion ratio of 10%.

Effect of heavy metal ions. We have received from Pentex Corp. samples of BFrVI (lot No. 8206), which are insoluble in a normal saline solution, unlike the usual samples which are readily soluble. The insoluble samples, however, are readily soluble in a neutralized versene solution (1%). We assume, therefore, that insolubility is due to presence of heavy metal ions, namely, barium. This suggested that low concentrations of such ions may even be present in the soluble BFrVI samples and that they may affect thyroid function in the rat. To test this possibility, 7 400 to 500 g Holtzman male rats were each given from 0.1 to 0.4 ml of a 20% suspension of an insoluble sample of BFrVI *via* the tail vein simultaneously with 5 μc of I^{131} iodide. The one given 0.4 ml died in a matter of seconds, that given 0.3 ml died within 2 minutes, and 2 others were dead in a matter of hours. In a group of 5 rats given 0.2 ml, 2 were dead by the end of 24 hrs, and in a group of 5 rats given 0.1 ml, one died by the end of 24 hrs. Those surviving at the end of 24 hrs were examined for thyroid activity, and results are shown in Table III.

It is obvious that barium ions do not account for the depressed conversion ratio found on treatment with BFrVI. To demonstrate the activity of this insoluble material, 2 g were dissolved in 25 ml of a 3% neutralized versene solution at pH 7.2 and dialyzed against running tap water for a total of 10 hr and against distilled water for 80 hr, at which time optical density of the dialysate was zero, down to a wavelength of 240 mμ. This dialyzed BFrVI solution was approximately 8% in protein and could be assumed free of both heavy metal ions and versene. Intravenous injections of 0.5 ml quantities of this solution (approximately one-third of normal dose) were given to groups of Holtzman male and Sprague-Dawley female rats for comparison, as previously described. Results are shown in Table IV. In addition, a separate com-

TABLE IV. Comparison of Holtzman Male and Sprague-Dawley Female Strains.

Strain	Dose	C.R.
Holtzman ♂ (4)	.5 ml 8% solution dialyzed	35.1 ± 2.8
Sprague-Dawley ♀ (6)	*Idem*	19.0 ± 4.3
Holtzman ♂ (3)	.5 ml 20% solution untreated and undialyzed	19.3 ±13.7
Sprague-Dawley ♀ (3)	*Idem*	11.4 ± 5.2
Normal C.R.		
Sprague-Dawley ♀ (29)		39.6 ± 3.4
Holtzman ♂ (11)		37.7 ± 4.3

Numbers in parentheses represent No. of rats used.

parison was made on these 2 strains of rats using a normally soluble and, therefore, untreated sample of fraction VI. These results are also shown in Table IV.

It appears that although the Holtzman male rat is a less sensitive and less reliable rat for this bio-assay, it does respond to a high enough dose of BFrVI. Moreover, there is no doubt that the sample of fraction VI, after treatment with versene and prolonged dialysis, does in fact show a depressant effect on thyroid activity, but in the dosage given, only by the more sensitive Sprague-Dawley female rat.

Discussion. The fact that BFrVI contains a TBP identifiable as a glyco-protein as well as a component showing thyroid depressant activity may be fortuitous. However, the TBP of Ingbar, Freinkel, and Dowling(6), obtained from serum fraction IV-6 was also identified as a glycoprotein and it may be that ability to bind thyroxine is a property of carbohydrate-containing proteins in general. Thyrotropic hormone (TSH) has been identified as a glycoprotein by 2 independent means(7,8), and there is considerable evidence available to show that TSH is inactivated after reaching the thyroid gland(9,10,11), or simply by acetylation(12). Moreover, this acetylated product acts as a TSH inhibitor. It is also known that there is present in the urine of hypothyroid patients(13) a TSH inhibitor that was shown to be a protein of low N content and could in many respects be likened to TSH. It was suggested that this inhibitor was in fact an inactivated TSH. The possibility exists that the thyroid depressant present in BFrVI is an inactivated TSH, acting as a TSH inhibitor, and that may be capable of binding thyroxine.

Summary. Some very active samples of BFrVI have shown marked ability to depress thyroid function as measured by the conversion ratio. Careful examination of these samples has shown that there is little or no iodine in the serum fraction, so the presence of thyroxine or any other iodine-containing calorigenic substance cannot be used to explain these results. The possibility that either versene or barium ions, both used in preparation of fraction VI, are responsible for this activity has been discounted. The higher sensitivity of the Sprague-Dawley female rat in comparison with the Holtzman male rat in this bio-assay has been demonstrated. Further studies are in progress attempting to elucidate the nature of this thyroid depressant in bovine fraction VI.

1. Rabinovitch, B., Carreon, Felicia, PROC. SOC. EXP. BIOL. AND MED.,

2. Sharrer, N. E., Aspes, S. P., *J. Clin. Endocrinol. Metab.*, 1956, v16, 1311.

3. Moran, J. J., *Anal. Chem.*, 1952, v24, 378.

4. Schmid, K., *J. Am. Chem. Soc.*, 1953, v75, 60.

5. Freeman, H., Vies, M., Magee, Marguerite, *J. Biol. Chem.*, 1953, v203, 1045.

6. Ingbar, S. H., Dowling, J. T., Freinkel, N., *Endocrinol.*, 1957, v61, 321.

7. Fels, I. G., Simpson, M. E., Evans, H. M., *J. Biol. Chem.*, 1954, v213, 311.

8. Barrnett, R. J., Ladman, A. J., McAllaster, N. J., Siperstein, E. R., *Endocrinol.*, 1956, v59, 398.

9. Rawson, R. W., Money, W. L., *Recent Progress in Hormone Research,* 1949, v4, 43.

10. Savoie, J. C., *Ann. endocrinol.* (Paris), 1952, v13, 81.

11. Kassenaar, A. A. H., Kerkhofs, H., *Acta Endocrinol.*, 1955, v19, 244.

12. Sonenberg, M., Money, W. L., *Endocrinol.*, 1957, v61, 12.

13. Starr, P., Lepp, A., Katz, Y., *J. Clin. Endocrinol. and Metab.*, 1958, v18, 278.

Received September 4, 1958. P.S.E.B.M., 1958, v99.