THE EFFECT OF PROGRESSIVE IODINATION ON THE THYROIDAL ACTIVITY OF IODINATED CASEIN*

By E. P. REINEKE, M. B. WILLIAMSON, and C. W. TURNER

(From the Department of Dairy Husbandry, University of Missouri, Columbia)

(Received for publication, January 6, 1942)

During recent years, the fact that substances with thyroidal activity can be formed by the simple iodination of proteins has become well established. Abelin and Florin (1) and Abelin (2) reported the recovery of highly active acid-insoluble fractions from barium hydroxide hydrolysates of iodoproteins. Abelin and Neftel (3) demonstrated that both the type of protein and the iodination method used are important factors in determining the activity of the final product. Hydrolytic products of iodinated native proteins showed marked activity. Direct iodination of peptone and ereptone, on the other hand, failed to yield substances with thyroidal activity. Iodination in bicarbonate yielded better results than in ammonia solution.

Ludwig and von Mutzenbecher (4) confirmed by Harington and Rivers (5) succeeded in isolating thyroxine from iodinated casein after hydrolysis with barium hydroxide.

Lerman and Salter (6) reported the correction of myxedema in human patients and in thyroidectomized rabbits by the administration of iodinated serum protein and its acid-insoluble degradation products. In our own laboratory the administration of iodinated casein in graded doses to young thyroidectomized goats arrested the development of cretinism and stimulated growth in proportion to the amount of material given (Reineke and Turner (7)).

Earlier work (unpublished) indicated that the iodination of casein or skim milk by a variety of procedures led to large differences in activity, apparently due to variations in several factors or combinations of factors. This suggested the desirability of establishing more definitely the conditions influencing the reaction whereby substances with thyroidal activity are produced.

* Contribution from the Department of Dairy Husbandry, Missouri Agricultural Experiment Station, Journal Series, No. 798.

EXPERIMENTAL

Iodination Procedure

Two different series of iodinated proteins were prepared in order to determine the effect of progressively increasing iodine on their thyroidal activity.

Series I was prepared by the direct iodination of skim milk. 700 ml. of skim milk were placed in a glass jar, 5 gm. of sodium bicarbonate were added, and the container was placed in a water bath maintained at 38–40°. The mixture was agitated vigorously and continuously by means of a glass stirring rod with a triangular foot, attached to a motor stirrer. Finely powdered iodine was then added at intervals, and in small amounts, over a period of 3 to 4 hours. When the required amount of iodine had been added, the jar was closed with a rubber stopper having a glass bearing to accommodate the stirrer. The closed container and stirring apparatus were placed in an incubator at 38°, with continuous stirring, for 18 to 20 hours. pH readings were taken with a glass electrode pH meter after addition of the bicarbonate, after addition of all the iodine, and after the incubation period. The iodinated protein was recovered by careful addition of dilute HCl until the point of maximum precipitation was reached (pH 3.8 to 4.0). After several washings with water adjusted to pH 4.0, the greater part of the iodinated protein was removed by filtration, dried at room temperature, ground in a laboratory mill, and assayed without further treatment. A small portion from each member of the series was resuspended with a minimum amount of NaOH and dialyzed across a cellophane casing in order to eliminate loosely combined iodine. The iodoprotein was then precipitated by the addition of HCl, filtered, and dried. Iodine analyses were made on both the dialyzed and the undialyzed portions by the method of Kendall, as described by Harington (8).

Series II was made up with casein that had been prepared in the laboratory from fresh, unpasteurized milk. 21 gm. of casein were placed in 700 ml. of distilled water to which 5 gm. of sodium bicarbonate had previously been added, with vigorous stirring until the casein dissolved. The remainder of the process was then carried out exactly as in Series I.

Methods of Assay

The method of Kreitmair (9), based upon the weight loss of guinea pigs, has been used extensively for the assay of thyroid-active materials. Dressler and Holling (10) have published a method of assay based upon the increase in oxygen consumption of guinea pigs stimulated by a given dose of material.

The fact that thyroid material will stimulate precocious metamorphosis in frog tadpoles (Gudernatsch (11)) has been used widely both as a qualita-

tive and a quantitative measure of thyroid activity. Gaddum (12) reported that when tadpoles were exposed to thyroxine for 24 to 48 hours the decrease in body length bore a rough quantitative relationship to the amount of substance employed. Wokes (13) devised a method for the quantitative assay of thyroid substance on tadpoles.

Assays on Guinea Pigs—For the assay of the iodinated proteins reported in this paper, modifications of the three methods noted above were employed. Both the increase in oxygen consumption and the percentage decrease in weight loss of guinea pigs were determined on the same animals. Healthy male guinea pigs weighing from 230 to 280 gm., averaging approximately 250 gm., were used. In order to have the animals in a partially fasted state for daily measurements of metabolism, all food was removed from the pens at night and the animals were allowed access to food only during the day, after the oxygen consumption had been recorded. This plan of treatment was used during the experimental period and also during the week preceding it. Control animals handled similarly gained an average of 2 per cent per week. The diet consisted of 80 per cent of a grain mixture and 20 per cent Cerogras,[1] added as a vitamin supplement.

In preparation for administration, a weighed amount of the iodinated protein was put in solution in distilled water by addition of 2 or 3 drops of saturated sodium carbonate solution and triturated with a mortar and pestle. The dissolved material was then made up to a given volume and accurately measured quantities were given orally once daily for 6 days to guinea pigs by the method of Pugh and Tandy (14).

On the 4th and 5th day after beginning the dosage, measurements of oxygen consumption[2] were made in an eight chamber respiration apparatus of the same construction as that described by Winchester (15), but designed to accommodate animals between 150 and 300 gm. in weight. The percentage increase in oxygen consumption was calculated from the increase above normal controls of the same weight and maintained under the same conditions. Dosage of the animals was continued for 6 days and the final weights were taken on the 7th day after treatment was begun. Weight decreases were expressed as the per cent difference between the initial weight and the final weight of the animals.

Assays on Tadpoles—In contradistinction to the results obtained with thyroxine or thyroid substances it was found that tadpoles will give little or no response when placed in a solution or suspension of iodinated casein.

[1] Cerogras is a mixture of dried and finely ground immature cereal grasses. It was kindly supplied by Dr. W. R. Graham, Jr., Cerophyl Laboratories, Kansas City, Missouri.

[2] Grateful acknowledgment of the authors is given to H. H. Kibler, research assistant, for the determinations of oxygen consumption.

However, when the material was placed on the surface of the water as a fine powder so that the tadpoles could eat it, or when injected into the body cavity, the response was qualitatively indistinguishable from that of thyroid or thyroxine.

For the assays reported herein large frog tadpoles (*Rana pipiens*) of about 60 mm. in length were obtained by seining from a local pond. At this stage the rear legs were fully formed, but still non-functional, and th front limb buds had not yet emerged. Such tadpoles are extremely sensitive to stimulation and furthermore can be injected quite readily. Since the response obtained with tadpoles will vary widely from time to time, depending upon their stage of development, environmental temperature, and possibly other factors, all tadpoles to be used for the assay of a given series of preparations were injected on the same day. Final measurements of body length were made on the 4th day after injection. The same amount of iodoprotein from each member of the series was injected into the tadpoles of its respective test group. Therefore, the per cent decrease in body length provides an index of the relative potencies of the various members of the series.

Results

Data on the progressive iodination of skim milk and casein are presented in Table I. In Series I skim milk was iodinated directly by addition of iodine in concentrations ranging from 3.9 to 31.5 gm. per 100 gm. of protein. For purposes of calculation, the protein content of the milk used is assumed to be 3.5 per cent. While this assumption will introduce some error, it is believed to approximate closely the actual protein content of the mixed milk used.

Analyses of the iodoproteins before and after dialysis indicate that from 1 to 2 per cent of the iodine is in loose combination with the protein. This iodine can also be liberated by brief oxidation with hydrogen peroxide, potassium persulfate, or potassium permanganate in acid solution. After dialysis, however, no evidence of free iodine could be obtained after oxidation with the above reagents. Thus it is evident that the non-dialyzable iodine is in firm combination, presumably with the tyrosine of the protein. It is well established that the iodination of tyrosine proceeds by substitution according to the equation, tyrosine + $2I_2 \rightarrow$ diiodotyrosine + 2HI. In the present series somewhat less than one-half of the iodine added remained in firm combination with the protein after dialysis. Since some side reactions doubtless take place in the mixed systems used, this is in good agreement with the theoretical expectation.

In Series II more accurate computations are possible, because casein of known tyrosine composition was used throughout, and the intervals be-

tween amounts of iodine added were spaced more closely than in Series I. It is of interest to note that until 4 atoms of iodine were added or 2 atoms combined per mole of tyrosine, the amount of iodine remaining in combination after dialysis was close to the theoretical value. There was then a lag in the combination of additional iodine (dialyzed values) until excessive amounts were added. However, the total amount of iodine combined increased progressively with increasing addition of iodine.

TABLE I

Course of Progressive Iodination of Skim Milk Proteins and Casein

Series No.	Preparation No.	Iodine added per liter milk	Iodine* added per 100 gm. protein	Iodine† added per mole tyrosine	Per cent iodine combined		Iodine combined per mole tyrosine (after dialysis)	pH		
					Before dialysis	After dialysis		After bicarbonate addition	After iodine addition	After incubation
		gm.	*gm.*	*atoms*			*atoms*			
I. Direct iodination of skim milk	1	1.428	3.939	1.24	3.47	2.20	0.72	7.20	7.01	7.62
	2	2.857	7.878	2.47	5.34	4.23	1.41	7.21	7.23	7.23
	3	4.285	11.817	3.71	6.95	5.13	1.73	7.45	7.61	7.60
	4	5.714	15.756	4.95	8.08	6.21	2.11	7.47	7.21	7.03
	5	7.142	19.695	6.18	8.48	6.97	2.39	7.31	7.02	7.25
	6	8.571	23.634	7.42	9.57	7.57	2.62	7.54	6.89	6.81
	7	10.000	27.573	8.66	10.00	7.95	2.76	7.57		6.65
	8	11.428	31.512	9.89	10.41	8.33	2.90	7.63		6.33
II. Iodination of casein	1		7.904	2.00	4.12	3.77	0.99	7.47	7.51	7.41
	2		11.904	3.00	6.10	5.17	1.38	7.47	7.09	7.07
	3		13.857	3.50	7.35	6.02	1.62	7.61	7.48	7.98
	4		15.809	4.00	8.62	6.80	1.84	7.61	7.10	7.21
	5		17.619	4.45	9.16	7.22	1.97	7.78	7.28	7.42
	6		20.000	5.05	9.56	7.51	2.05	7.78	7.05	7.85
	7		21.904	5.53	10.38	7.50	2.05	7.20	7.30	7.65
	8		24.285	6.14	12.25	8.78	2.43	7.20	7.13	7.19

* Skim milk was assumed to contain 3.5 per cent protein.

† Calculated on the basis of 4.5 per cent tyrosine in the mixed proteins of skim milk and 5.65 per cent tyrosine in casein. Tyrosine was determined by the method of Lugg (16).

In both series the pH of the reaction mixtures remained fairly close to the physiological range throughout. The final low values of 6.3 to 6.8 in Series I are probably due to depletion of the buffer capacity of the solutions during excessive iodination by the HI formed as a side product in the reaction.

Thyroidal Activity—Results of the assay of preparations in Series I by three different methods and of Series II by the tadpole method are given

in Fig. 1. The test animals for each assay in a given series were dosed with the same amount of material. Therefore, the actual per cent response gives a comparative measure of the potency of the respective preparations in a series.

Thyroidal activity increased with increasing iodine concentration, attaining a maximum when 4.5 to 5.0 atoms of iodine had been added per mole of tyrosine in the protein, an amount slightly in excess of that theo-

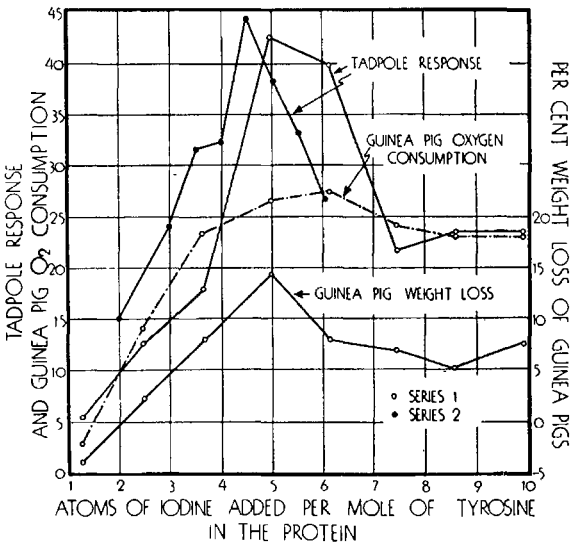

FIG. 1. The effect of progressive iodination on the thyroidal activity of casein and skim milk proteins. The results are expressed in terms of the amount of iodine added to the reaction mixture; this is somewhat more than twice the amount actually combined (see Table I). The weight losses for guinea pigs are based on eight animals, and metabolic results on four animals per assay. Iodinated protein was administered orally at the rate of 6 mg. daily per animal. The tadpole assays include five animals per preparation in Series I, and four per preparation in Series II. Each tadpole was given a single injection of 0.1 mg. of iodinated protein.

retically required for substitution of 2 atoms of iodine on the tyrosine ring. A striking feature of these results is that further iodination led to marked loss of activity.

In the assays of Series I, results of the tadpole test and the weight loss of guinea pigs are nearly parallel throughout the series, the only difference being one of degree of response. A second test run on groups of twenty artificially reared tadpoles given the preparations orally was in good agreement with the one illustrated. The t values were computed as described by Snedecor (17) in order to test the significance of the difference between

each member of the series and the preceding preparation. Results by the method of guinea pig loss in weight and the tadpole test revealed that both the rise in activity with progressive iodination and the decline after the maximum are statistically significant.

The increase in oxygen consumption of guinea pigs follows the same general trend as is shown by the other two methods. In this particular instance, however, the results are considered to be less quantitative than those obtained by the other methods, because it was observed during the course of the assays that the normal metabolism declined with the change from spring to summer conditions. This made it difficult to establish an accurate normal base from which to calculate the increase in oxygen consumption of the test animals. With this limitation in mind, it is believed that the metabolic data afford valuable confirmation of the results obtained by the other methods.

Results of the tadpole assay on the preparations of Series II are in good agreement with those of Series I, showing again an increase in activity up to a maximum when 4.5 atoms of iodine had been added per mole of tyrosine, followed by a decline in activity with further iodination. The results of this assay were confirmed by a second trial in which alternate members of the series were tested by injection into groups of twenty tadpoles. In both trials, computation of the statistical t values for the differences of response in succeeding members of the series indicated that both the rise in activity and the decline from the maximum are significant.

As judged by both the metabolic response and the weight loss of guinea pigs, preparations iodinated under optimal conditions show a potency of 0.01 to 0.005 of that of thyroxine when both materials are given orally. These results compare favorably with the activity of 1/300 that of thyroxine reported by Harington and Rivers (5) for iodinated casein. U.S.P. desiccated thyroid tested in the same way showed about 0.005 of the activity of thyroxine. Lerman and Salter (6) reported that iodinated serum albumin had a potency of about one-fifth that of thyroglobulin.

DISCUSSION

From the results, it is apparent that the degree of iodination of a protein is a controlling factor in the amount of thyroidal activity attained. Under the conditions of these experiments, thyroidal activity reaches a maximum when sufficient iodine has been added to substitute 2 atoms on the tyrosine ring. Further iodination leads to significant losses of activity. This is in agreement with the report (4) that excessively iodinated proteins fail to yield thyroxine after hydrolysis.

The iodination of serum albumin in a water-alcohol-ammonia medium by addition of compound solution of iodine has been reported by Muus,

Coons, and Salter (18). According to these authors thyroidal activity did not begin until 2 to 3 atoms of iodine per molecule of tyrosine were combined. Full activity was obtained only when 9 to 10 per cent of iodine or the equivalent of 4 atoms per molecule of tyrosine had been incorporated. No decrease of activity was noted with excessive iodination. The optimally iodinated preparations contained more iodine than could be accounted for by substitution on both the tyrosine and histidine of the protein.

As shown by the data in Table I, the iodination method used in the present work favors the substitution of iodine on the tyrosine ring, with little or no substitution in other parts of the protein molecule. Thus the marked differences in the course of formation of the thyroidally active product can probably be explained by the differences in the methods used.

The formation of thyroxine by the iodination of proteins could be accounted for (4, 5) by (*a*) the oxidative coupling of 2 molecules of diiodotyrosine with the elimination of one side chain or (*b*) the iodination of preformed thyronine which may exist as part of the protein molecule. The difficulties in the way of acceptance of both possibilities are pointed out by Harington and Rivers (5). In favor of the first mechanism is the fact that thyroxine has been formed directly from diiodotyrosine by prolonged incubation in alkaline medium (von Mutzenbecher (19), Block (20)). The increase in thyroidal activity with progressive iodination as reported herein is compatible with the first hypothesis. The loss of activity as excessive amounts of iodine are added could be explained by the coupling of a 3rd tyrosine molecule, forming an inactive compound such as the thyroxine analogue reported by Bovarnick *et al.* (21). As an alternative explanation, it appears possible that prolonged oxidation could result in changes in the remaining side chain of the thyroxine formed in the initial stages, resulting in degradation products with reduced activity.

SUMMARY

1. Casein and total skim milk proteins, buffered with sodium bicarbonate, were combined with progressively increasing amounts of iodine, and the iodine content and thyroidal activity of the resulting iodoproteins were tested.

2. It was found that under these conditions of iodination thyroidal activity reaches a maximum when sufficient iodine has been combined to substitute 2 atoms of iodine on the tyrosine ring. Further iodination results in a significant decrease in thyroidal activity.

3. From a consideration of the iodine contents of the dialyzed iodoproteins in relation to their original tyrosine content, it appears that the method of iodination used favors substitution on the tyrosine ring, with little or no substitution in other parts of the protein molecule.

BIBLIOGRAPHY

1. Abelin, I., and Florin, A., *Arch. exp. Path. u. Pharmakol.*, **171,** 443 (1933).
2. Abelin, I., *Arch. exp. Path. u. Pharmakol.*, **175,** 146, 151 (1934); **181,** 250 (1936).
3. Abelin, I., and Neftel, A., *Arch. exp. Path. u. Pharmakol.*, **189,** 473 (1938).
4. Ludwig, W., and von Mutzenbecher, P., *Z. physiol. Chem.*, **258,** 195 (1939).
5. Harington, C. R., and Rivers, R. V. P., *Nature*, **144,** 205 (1939).
6. Lerman, J., and Salter, W. T., *Endocrinology*, **25,** 712 (1939).
7. Reineke, E. P., and Turner, C. W., *Endocrinology*, **29,** 667 (1941).
8. Harington, C. R., The thyroid gland; its chemistry and physiology, London, 192 (1933).
9. Kreitmair, H., *Z. ges. exp. Med.*, **61,** 202 (1928).
10. Dressler, C., and Holling, K., *Arch. exp. Path. u. Pharmakol.*, **196,** 266 (1940).
11. Gudernatsch, J. F., *Arch. Entwicklungsmech. Organ.*, **35,** 457 (1913).
12. Gaddum, J. H., *J. Physiol.*, **64,** 246 (1927–28).
13. Wokes, F., *Quart. J. Pharm. and Pharmacol.*, **11,** 521 (1938).
14. Pugh, A. E., and Tandy, A. W., Jr., *J. Lab. and Clin. Med.*, **24,** 80 (1938).
15. Winchester, C. F., *Missouri Agric. Exp. Stat., Bull. 315* (1940).
16. Lugg, J. W. H., *Biochem. J.*, **31,** 1402 (1937).
17. Snedecor, G. W., Statistical methods, Ames, 68 (1937).
18. Muus, J., Coons, A. H., and Salter, W. T., *J. Biol. Chem.*, **139,** 135 (1941).
19. von Mutzenbecher, P., *Z. physiol. Chem.*, **261,** 253 (1939).
20. Block, P., Jr., *J. Biol. Chem.*, **135,** 51 (1940).
21. Bovarnick, M., Bloch, K., and Foster, G. L., *J. Am. Chem. Soc.*, **61,** 2472 (1939).