CXLVII. STUDIES IN SYNTHETIC IMMUNOCHEMISTRY

III. PREPARATION AND ANTIGENIC PROPERTIES OF THYROXYL DERIVATIVES OF PROTEINS, AND PHYSIOLOGICAL EFFECTS OF THEIR ANTISERA

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This communication deals with the application of the general method devised by Clutton *et al.* [1937] to the preparation of thyroxyl derivatives of various proteins, and with the serological study of the products. It has also proved of interest to investigate the pharmacological properties of the antisera raised against the thyroxylproteins.

Owing to the great insolubility of thyroxine and its derivatives the direct introduction of thyroxine into the protein molecule is hardly a practicable proposition; the difficulty can be overcome, however, by proceeding in a stepwise manner. 3:5-Diiodothyronine methyl ester can readily be converted into the *N*-carbobenzyloxy derivative; this yields by the usual methods in turn the hydrazide and azide and the latter can be coupled with a protein in aqueous dioxane solution at *p*H 8·9. The *N*-carbobenzyloxy-3:5-diiodothyronylprotein thus obtained is then treated with iodine in ammoniacal solution, which converts the 3:5-diiodothyronyl residues into thyroxyl residues and at the same time converts the tyrosine of the original protein into 3:5-diiodotyrosine.

The method outlined above has been applied to the preparation of thyroxyl derivatives of horse serum albumin and globulin and of ox and human thyroglobulins. As will be seen from the results of the serological study reported below these products are all powerfully antigenic, the specific nature of their serological reactions being, as is to be expected, largely conditioned by the thyroxyl groups which have been introduced; moreover, the antisera react not only with artificial thyroxine-containing proteins but in some cases at least with thyroglobulin itself. The latter observation raises the question as to whether such antisera exercise an antagonistic effect against thyroglobulin or circulating thyroid hormone in the intact animal. The final portion of the present paper deals with the experiments which have been made to test this possibility and it will be seen that evidence has been obtained that a state of resistance to the normal physiological effect not only of thyroglobulin but of thyroxine itself can be produced in an animal by passive immunization with antiserum raised against a thyroxylprotein.

Experimental

I. *Preparation of compounds*

N-Carbobenzyloxy-3:5-diiodothyronine methyl ester. 4·2 g. diiodothyronine methyl ester [Ashley & Harington, 1928] were suspended in 80–100 ml. of anisole and 1·32 g. of benzylcarbonyl chloride were slowly added. After a few seconds' shaking 40 ml. of a solution containing 1·6 g. $NaHCO_3$ were added, and the

whole was shaken vigorously until the evolution of CO_2 had ceased. A further addition of 1·32 g. of the chloride was then made, followed by 1·6 g. of $NaHCO_3$ (40 ml.), and shaking was continued until the ester had passed completely into solution.

The anisole layer was separated and the aqueous phase washed with chloroform. The anisole and chloroform solutions were combined and evaporated under reduced pressure to a syrup which was removed from the flask with a minimum of chloroform and precipitated with light petroleum. After a few hours in the ice-chest the oily mass hardened to a yellow granular powder which was collected and dried. The yield was 4 g.

The product was insoluble in water and light petroleum, fairly soluble in chloroform, benzene and hot alcohol, and very soluble in anisole. A sample recrystallized from hot benzene by slow cooling formed elongated prisms, M.P. 164·5°. (Found: C, 42·8; H, 3·14; I, 37·2 %. $C_{24}H_{21}O_6NI_2$ requires C, 43·2; H, 3·12; I, 37·8 %.)

N-*Carbobenzyloxydiiodothyronyl hydrazide.* 4 g. of the carbobenzyloxy ester were suspended in 20 ml. of absolute alcohol and treated with 1·36 g. of pure hydrazine hydrate. The solid was dissolved by heating on a steam bath and the solution transferred to an incubator at 38°. After 2 hr. a crystalline mass had started to appear; the mixture was again brought to the boil and returned to the incubator for 12 hr.; it was then diluted with 10 ml. alcohol and kept for 24 hr. in the ice-chest. The product, consisting of large prisms, was collected, washed with alcohol and ether, and dried. Yield 4 g.

The hydrazide was insoluble in alcohol, water and ether, but soluble in pyridine and dioxane. After recrystallization from pyridine and methyl alcohol the compound had M.P. 141°. (Found: N, 6·23; I, 38·0 %. $C_{23}H_{21}O_5N_3I_2$ requires N, 6·24; I, 37·9 %.)

N-*Carbobenzyloxydiiodothyronyl azide.* 1 g. of the hydrazide was dissolved with heating in a mixture of 12 ml. glacial acetic acid, 8 ml. water and 0·3 ml. conc. HCl, and the clear solution was cooled to 0°. 1 ml. of 10 % aqueous $NaNO_2$ was added drop by drop with vigorous mechanical stirring. After 5 min. the azide had partially separated as an amorphous mass; precipitation was completed by slow dilution with 30 ml. of ice-cold water, and after a few minutes the mixture was centrifuged; the azide was washed twice with 30 ml. of ice-cold water and taken up in 15 ml. dioxane, the solution being kept as cool as possible until required for coupling.

N-*Carbobenzyloxydiiodothyronylglobulin.* Stock horse serum globulin solution containing 1·5 g. protein [cf. Clutton *et al.* 1938] was diluted with water and dioxane to make a final concentration of 1·5 % in 50 % dioxane, and the mixture was made just alkaline to phenolphthalein and cooled to 0°; a dioxane solution of 1 g. of the azide was added over 20 min. to the cooled and stirred protein solution, the alkalinity (*p*H 8–9) being maintained by addition of 2*N* NaOH.

The solution was then acidified to *p*H 5 with 10 % acetic acid, and kept for 30 min. in the ice-chest. The flocculus was centrifuged off and purified by twice or thrice repeated solution in 150 ml. of 50 % methyl alcohol with the minimum of alkali, followed by precipitation with acetic acid. Some preparations, through partial denaturation, failed to dissolve completely in 50 % methyl alcohol; in such cases 50 % alkaline dioxane was employed instead. The product was finally dissolved in 100 ml. of alkaline 50 % methyl alcohol and analysed for nitrogen and iodine (Table I).

The compound was then flocculated with acetic acid and preserved in the ice-chest pending the next step.

Iodination of N-*carbobenzyloxydiiodothyronylglobulin.* In this step the aim was to iodinate exclusively in the positions *ortho* to the OH-groups of tyrosine and diiodothyronine. The theoretical quantity of iodine required was therefore calculated from the tyrosine content of the original globulin (6·8 %).and the quantity of diiodothyronine introduced, a 10 % excess being allowed.

The diiodothyronylglobulin from 1·5 g. serum globulin was dissolved in 180 ml. of strong ammonia and the solution cooled to 0°. 2·72 *N* iodine solution was then run in, drop by drop, with mechanical stirring. Fresh quantities of iodine were not added until all traces of nitrogen iodide had disappeared. At the theoretical end point the reaction became sluggish and when the 10 % excess had been added the whole turned to a grey-brown colour. In later experiments the diiodothyronylglobulin was brought into solution in ammoniacal dioxane and ammonia was added until the concentration of dioxane was 10 %. (By this variation of technique better solution and iodination of the proteins were obtained.)

After 10–15 min. the opalescent solution was precipitated by pouring into a slight excess (1200 ml.) of ice-cold 10 % acetic acid. A few drops of sodium metabisulphite were then added to remove any unchanged iodine. After several hours at 0° the supernatant liquid was removed, first by decantation and finally on the centrifuge.

The precipitate was washed twice with 400 ml. of water and made acid to Congo red, and then dispersed in the same quantity of water just alkaline to phenolphthalein. Precipitation and washing were repeated as before. Finally the product was treated with 100 ml. of 30 % alkaline methyl alcohol, when solution generally occurred. The whole was precipitated with 10 % acetic acid and treated with 200 ml. of water alkaline to phenol red. The product formed a thick opalescent solution. Iodine and nitrogen determinations were made (see Table I). (Earlier preparations were not subjected to treatment with 30 % methyl alcohol and were obtained as coarse suspensions.)

N-*Carbobenzyloxythyroxylalbumin.* This was prepared from crystalline horse serum albumin [cf. Clutton *et al.* 1938] and the procedure was the same as that employed with the corresponding globulin derivative, except for the following modifications.

(1) After the coupling, reprecipitation was carried out once from 20 to 30 % methyl alcohol; the product was then found to be soluble in water at *p*H 8 and water was therefore used in place of aqueous alcohol for purification.

(2) The coupled product was extremely soluble in concentrated ammonia and iodination was done in an ammoniacal solution containing 2 % protein. The tyrosine content was erroneously taken as 4·7 %, but as iodination was rapid and the end point sharp, iodine was added until the mixture assumed a permanent grey-brown colour. This involved the addition of as much as 20 % excess over that required for complete iodination of the 6·3 % tyrosine actually present [Holiday, 1936].

The properties of the albumin derivative were in striking contrast to those of the globulin and thyroglobulin complexes. This product was readily soluble in water at *p*H $> 8·0$ and $< 3·9$; in acid solutions, however, traces of salt induced precipitation.

N-*Carbobenzyloxythyroxylthyroglobulin.* Thyroglobulin was prepared from frozen ox thyroids and from frozen normal human thyroids; the latter were obtained *post mortem* from patients dying of diseases not involving the thyroid. The thyroglobulin was isolated by the method of Harington & Salter [1930] and was purified by two reprecipitations.

The coupling with *N*-carbobenzyloxydiiodothyronine and the subsequent iodination were conducted exactly as in the case of serum globulin, the tyrosine content of the thyroglobulin being assumed to be 5·4 % [Eckstein, 1926]. The product formed a much more stable solution than did the serum globulin derivative and in several preparations the final treatment with 30 % methyl alcohol could be omitted.

The analytical data relating to the various preparations of thyroxylproteins are contained in Table I.

Table I. *Analytical data regarding thyroxylproteins*

Protein g.	Hydrazide g.	Coupled solution: mg. N per 100 ml.	Coupled solution: mg. I per 100 ml.	Iodinated solution: mg. N per 100 ml.	Iodinated solution: mg. I per 100 ml.
		A. *N*-Carbobenzyloxythyroxylglobulin			
1·5	1·0	230	235	—	—
1·5	1·0	232	188	182	355
1·5	1·0	224	204	172	290
0·87	0·5	252	147	179*	303
		B. *N*-Carbobenzyloxythyroxylalbumin			
0·48	0·4	252	206	174	391
2·0	1·2	370	240	246	382
		C. *N*-Carbobenzyloxythyroglobulin			
		Thyroglobulin A_1–Ox, contained 0·5 % I. Thyroglobulin A_2–Ox, contained 1·0 % I. Thyroglobulin B–Man, contained 0·5 % I			
2·5 A_1	2·0	190	96	144	155
1·5 A_2	0·9	114	100	120*	252
1·5 B	1·0	239†	294†	220*	462

* Iodination carried out in a mixture of dioxane (1 part) and conc. ammonia (9 parts).
† Poor reprecipitation.

The following additional compounds were prepared for use in the serological tests.

Iodoglobulin. Horse serum globulin (10 ml. of 15 % solution) was mixed with concentrated ammonia (20 ml.; sp. gr. 0·880) and the whole treated at 0° with iodine (0·95 ml. of 2·72*N*); further treatment was exactly as described above for thyroxylglobulin. The product contained about 6·0 % iodine; it still gave a faint Millon reaction.

Tryptic digest of thyroglobulin. About 60 g. of ox thyroglobulin were heat-coagulated and suspended in 400 ml. of water together with 10 ml. of 2*N* HCl. 0·5 g. of pepsin (Parke Davis 1 : 10,000) was added and the whole kept for 48 hr. at 38°. Another 0·5 g. of pepsin was added and digestion continued for 48 hr. longer. At this stage most of the protein was in solution; the whole was brought to *p*H 8 with 2*N* NaOH and treated with 0·5 g. trypsin (Merck).

After 48 hr. the solution was acidified to Congo red, the precipitate which formed being centrifuged off and washed with 500 ml. water. The dark brown sediment was suspended in 300 ml. of water and brought to *p*H 8. The whole was re-digested with 1·5 g. trypsin for 36 hr. at 38°, and with a further 1·5 g. trypsin for 48 hr. longer.

At this stage the amino-N amounted to 38 % of the total N.

The solution was again brought to Congo red point with HCl, the precipitate spun off, washed several times with 50 % acetone and finally with dry ether. Yield 6·0 g. Iodine 5·6 %.

The product was made up in 1 % solution and used for inhibition experiments.

II. *Immunological experiments*

In all cases 3 injections were given per week unless severe loss of weight necessitated a short rest. In all cases the rabbits were bled from 8 to 10 days after the completion of their course of injections.

(1) *Antigen thyroxyl-serum globulin.* Two groups of rabbits were used. The first group received 4 doses of 10 mg., 2 of 20 and 5 of 30 mg., followed by a month's rest, then one further dose of 20 mg. and 7 of 30 mg. Two rabbits in this group of three died, one from protein shock and one from excessive loss of weight.

The second group received 3 doses of 10 mg., 3 of 20 and 3 of 30 mg. All remained well during the course.

(2) *Antigen thyroxylalbumin.* One group of rabbits was used, which received 3 doses of 10 mg., 3 of 20 and 3 of 30 mg. All the rabbits lost weight and one of the group died from excessive loss of weight.

(3) *Antigen thyroxylthyroglobulin.* Three groups of rabbits were used. Each group received 3 doses of 10 mg., followed by 3 doses of 20 mg. and then 3 doses of 30 mg. Two of the groups received 4 and 5 additional doses respectively, after a week's rest, the doses varying from 30 to 50 mg.

Two of these rabbits died from protein shock and nearly all lost weight. The most severe loss of weight amounted to about a quarter of the original body weight.

Certain of the antigens used were in the form of suspensions and were unsuitable for precipitin tests. Two techniques could be used to obtain them as solutions.

(*a*) *Pyridine technique.* 20 mg. of the antigen were completely precipitated with acetic acid. The precipitate was centrifuged off and dissolved in 1 ml. of

Table II. *Precipitin reactions with antiserum from rabbit No. 38 prepared against thyroxylglobulin*

Antigen	Antiserum	Dilutions of antigen 1/100	1/500	1/1000	1/10,000	1/100,000	Time of reading min.
Thyroxylglobulin (pyridine)	38	.	++++	+++	++	+	60
	Normal serum	.	+++	±	−	−	60
Thyroxylthyroglobulin (pyridine)	38	.	++++	++	+++	++	90
	Normal serum	.	+±	±	−	−	90
Iodoglobulin	38	++	.	+++	++	0	60
Thyroxylalbumin	38	+	.	+	+	+	60
Diiodothyronyl-albumin	38	−	.	+±	±	+	60
Thyroglobulin	38	−	.	+	+±	−	60
Albumin	38	−	.	−	−	−	60
Globulin	38	−	.	−	−	−	60

Inhibition tests

A. 1 vol. serum 38 + 1 vol. diiodotyrosine 1·0% incubated at 38° for 1 hr.
B. 1 vol. serum 38 + 1 vol. saline 1·0% incubated at 38° for 1 hr.

	Antigens: Thyroxylglobulin 1/30,000	Thyroxylglobulin 1/60,000	Thyronylalbumin 1/1000	Thyronylalbumin 1/10,000	Thyroglobulin 1/100	Thyroglobulin 1/1000
A	+	±	+	+	−	±
B	++	++	+	+	−	+±

Table III. *Precipitin reactions of antisera from rabbits Nos. 54 and 55 prepared against thyroxylalbumin*

Antigen	Antiserum	Dilutions of antigen 1/100	1/500	1/1000	1/10,000	1/100,000	1/1,000,000	Time of reading min.
Thyroxylalbumin	54	+ +	.	+ +	+ +	+	±	60
	55	+ + ?	.	+	+	±	−	60
	Normal serum	−	.	−	−	−	−	60
Thyroxylthyroglobulin (pyridine)	54	.	+ +	±	±	−	.	60
Thyroglobulin	54	+ + + ppt.	.	+ ±	±	−	.	60
Iodoglobulin	54	+ +	.	+ +	+	−	.	60
Diiodothyronylalbumin	54	±	.	±	+ +	+	.	60
Thyroglobulin	54	+ ?	.	−	−	−	.	60
Albumin	54	−	.	−	−	−	.	60
Globulin	54	−	.	−	−	−	.	60
Thyroxylthyroglobulin (pyridine)	Normal serum	±	.	±	−	.	.	60

Inhibition tests

1 vol. serum 54 + 1 vol. diiodotyrosine 1·0 % incubated at 38° for 1·5 hr.
1 vol. serum 54 + 1 vol. saline 1·0 % incubated at 38° for 1·5 hr.

	Thyroxylalbumin 1/100	1/1000	1/10,000	Time min.
54 + Inhibitor	?	+	+	60
54 + Saline	?	+ ±	+ +	60

Table IV. *Precipitin reactions of antisera from rabbits Nos. 50, 51 and 53 prepared against thyroxylthyroglobulin*

Antigen	Antiserum	Dilutions of antigen 1/100	1/500	1/1000	1/10,000	1/100,000	Time of reading min.
Thyroxylthyroglobulin (pyridine)	50	.	+ + +	+ + +	+ +	−	60
	51	.	+ + + +	+ +	+	−	60
	53	.	+ + + +	+	+	−	60
	Normal serum	.	+	−	−	−	60
Thyroxylglobulin	51	+ + +	.	+ +	+ +	+	60
Iodoglobulin	51	+ + + +	.	+ + ±	+	−	60
Diiodothyronylalbumin	51	+ ?	.	−	−	−	60
Thyroxylalbumin	51	+ ?	.	−	−	−	60
Thyroglobulin	51	−	.	±	+	−	60
Thyroxylalbumin	Normal serum	+ ?	.	.	.	.	60

Inhibition tests

A. 1 vol. serum 51 + 1 vol. diiodotyrosine 1·0 % incubated at 38° for 1 hr.

B. 1 vol. serum 51 + 1 vol. thyroglobulin digest 1·0 % incubated at 38° for 1 hr.

$C_1 = C_2$. 1 vol. serum 51 + 1 vol. saline 1·0 % incubated at 38° for 1 hr.

	Antigens: Thyroglobulin 1/100	Thyroglobulin 1/1000	Thyroxylglobulin 1/30,000	Thyroxylglobulin 1/60,000	Thyroxyl-thyro-globulin	min.
A	−	−	−	−	.	60
C_1	−	+	+	+	.	60
	1/1000	1/10,000	1/1000	1/10,000	1/10,000	
B	−	−	Tr.	Tr.	±	45
C_2	+ ±	+	+	±	+	45

Bulk tests at 56° showed that the precipitin reaction between thyroxylthyroglobulin and its antiserum was inhibited much more strongly by a mixture of thyroxine (0·75%) and diiodotyrosine (1·0 %) than by diiodotyrosine alone.

warm pyridine; the resultant solution was made up to 10 ml. with warm water, filtered and diluted with saline as required.

(*b*) *Methyl alcohol technique.* The technique was identical with the above except that the precipitate was dissolved in 1 ml. of methyl alcohol alkaline to phenolphthalein.

All tests unless otherwise mentioned were made with the interfacial ring technique. The results are given in Tables II–IV.

Discussion

The methods employed in the preparation of the compounds call for little comment; the coupling with the azide of *N*-carbobenzyloxy-3:5-diiodothyronine is exactly analogous with that of proteins with glucosidotyrosylazide described in the preceding paper. It is evident from Table I that considerable latitude in the proportion of azide to protein can be allowed without greatly affecting the result of the coupling.

The conditions chosen for iodination were designed to favour substitution with iodine and to suppress oxidation; there seems little doubt that many so-called iodinated proteins which have been used in the past have been as much oxidized as iodinated; at least, their method of preparation, involving the use of dilute alkaline solutions at ordinary temperatures, has been such as to favour the action of hypoiodite which is an effective oxidizing agent for amino-acids.

Even under careful working conditions we have ourselves been unable to obtain products of constant composition, as will appear from a study of Table I; this failure is due to difficulties of manipulation, many of the compounds having unfavourable solubility properties. Nevertheless, we have not failed in any instance to obtain a reasonably high degree of coupling with diiodothyronine and of subsequent uptake of iodine in proportion to the number of phenolic groups available.

The serological experiments have given results which are in general accordance with expectation. Thus, all the thyroxylproteins are powerful antigens (Tables II–IV). The unsatisfactory normal serum control in the case of the globulin derivative (Table II) is due to the pyridine technique which had to be employed to obtain a solution of the antigen; in spite of this the specific precipitation appears quite clearly at the higher dilutions of antigen. None of the thyroxylproteins retained any of the original protein specificity; this is to be expected in view of the drastic nature of the treatment to which they have been submitted.

The cross-reactions of the various sera and antigens are of considerable interest. Thus the antisera against thyroxylglobulin (Table II) and thyroxylthyroglobulin (Table IV) are extremely similar to one another; the thyroxylglobulin antiserum reacts as strongly with thyroxylthyroglobulin as with thyroxylglobulin itself, and the converse is true of the thyroxylthyroglobulin antiserum. Both sera also react strongly with ordinary iodoglobulin; a difference appears in the reactions of the sera with thyroxyl- and diiodothyronyl-albumin, which are fairly strong in the case of the thyroxylglobulin antiserum but weak in the case of the serum against thyroxylthyroglobulin. In accordance with this, the thyroxylalbumin antiserum (Table II), although precipitating to a high dilution with the homologous antigen, shows definitely less marked cross-reactions with thyroxylglobulin and thyroxylthyroglobulin.

One of the chief points of interest lies in the slight but definite reactions observed between thyroglobulin itself and the antisera against both thyroxylglobulin and thyroxylthyroglobulin. The question of the antigenicity of thyro-

globulin has not been satisfactorily settled. It has been known since the work of Hektoen *et al.* [1923] that preparations of thyroglobulin, when injected into a sufficiently remote species, give rise to precipitating antisera; in our opinion, however, this work has been too readily accepted as proof that thyroglobulin can itself act as an antigen. It has to be remembered that thyroglobulin is an extremely difficult protein to purify; it cannot be crystallized, and we at least have never been able to obtain preparations which we could regard as being free from tissue proteins. We are therefore inclined to ascribe the alleged precipitin formation by thyroglobulin to the presence of adventitious tissue proteins, and in this connexion the fact that precipitin formation can only be elicited between remote species seems to us to be significant. On general grounds it appears to us to be no more likely that thyroglobulin, with its specialized physiological function, should be antigenic than that insulin should be so. If this hypothesis is correct our thyroxylthyroglobulin must be antigenic by virtue of the alteration induced by the introduction of diiodothyronine residues followed by iodination, a supposition which is supported by the results of inhibition tests. Thus partial or complete inhibition of all cross reactions, including those involving thyroglobulin, is obtained with diiodotyrosine, and even more effective inhibition with a mixture of diiodotyrosine and thyroxine and with a digestion product of thyroglobulin containing thyroxine; this indicates that in all cases the determinant group is a complex involving both thyroxine and diiodotyrosine; it is particularly to be noted that there is a distinct difference between the effects of thyroxine and diiodotyrosine, suggesting that the whole of the aromatic portion of the thyroxine molecule plays a part in the immunological reactions just as it does in the physiological action of the compound; neither immunological nor physiological reactions are conditioned by the 3:5-diiodophenolic grouping alone.

Further evidence on this point is available from the facts that the antisera against thyroxylglobulin and thyroxylthyroglobulin do give slight precipitation with diiodothyronylalbumin and that this precipitation is not inhibited by diiodotyrosine; such an observation admits of no other explanation than that the 3:5-diiodophenolic ether linkage of thyroxine is involved.

It has been shown by Snapper & Grünbaum [1935] that the so-called serological reactions of thyroglobulin are not inhibited by thyroxine or diiodotyrosine; as has been pointed out above, however, these serological reactions have, in our view, probably nothing to do with true thyroglobulin and therefore the results of Snapper & Grünbaum are exactly what would be expected. Our own experiments, on the other hand, seem to prove beyond doubt that thyroxine and diiodotyrosine are capable of functioning as determinant groups in the molecule of thyroglobulin, as would be anticipated from what is known of their mode of combination in the protein and from the general importance of aromatic groups in serological reactions.

III. *Physiological experiments*

As pointed out in the introductory portion of this paper, the strongly antigenic character of the thyroxylprotein derivatives, the specific nature of their serological reactions and particularly the observation of cross-reactions between their antisera and thyroglobulin itself, raised the question of the possible inhibitory effect of such antisera on normal thyroid secretion. The method chosen to investigate this point was the observation of the effects of the antisera, alone and in combination with thyroglobulin and thyroxine, on the metabolic rate.

The experiments were carried out on young adult male rats (180–300 g. in weight) and determinations of the metabolic rate were made in the apparatus of

Richards & Collison [1928] using the technique and precautions described by Gaddum [1929–30]. The animals were kept on a constant diet and were given water only for at least 17 hr. before each observation. It was found to be advantageous to restrict the movements of the animals within the metabolism chamber by placing them in a light wire cage of such size as to moderate their activity without causing discomfort. An element of training enters into the experiment, since the animals require some time to become accustomed to the procedure; with each animal therefore a preliminary series of observations was made under normal conditions and in no case was an animal used for experiment which had not given at least two (and generally three) consecutive results of reasonable constancy.

Under the conditions employed, the basal oxygen consumption of the normal rats varied in different animals between the approximate limits of 13·5 and 16·5 ml./kg./min.; the three last readings in the pre-experimental period were averaged and the resulting figures were taken to represent the basal level; the detailed course of two typical experiments is shown in the protocols in the Appendix to this paper.

The first experiments to be made were concerned with the administration of antiserum against thyroxylthyroglobulin to normal rats; the results of these were entirely negative, no effect on the metabolic rate being observed during the (relatively short) period over which the injections were maintained.

Table V. *Effect of previous treatment with antiserum prepared against thyroxylthyroglobulin on metabolic rate of rats injected with thyroglobulin*

Rat no.	Pre-treatment	Dosage	Maximum % rise in metabolic rate
A. 1	5 doses of 12 ml. antiserum	4 doses of 5 ml. thyroglobulin (0·5% solution)	15·2
2	,, ,, ,,	,, ,, ,,	29·0
3	,, ,, ,,	,, ,, ,,	7·7
4	,, ,, ,,	,, ,, ,,	2·8
Controls			
1	5 doses of 12 ml. normal rabbit serum	4 doses of 5 ml. thyroglobulin (0·5% solution)	62·5
2	,, ,, ,,	,, ,, ,,	62·4
B. 1	4 doses of 13 ml. antiserum	1 dose of 0·50 ml. thyroglobulin (3·5% solution)	11·3
2	,, ,, ,,	,, ,, ,,	9·1
3	,, ,, ,,	,, ,, ,,	9·1
Controls			
1	4 doses of 13 ml. normal rabbit serum	1 dose of 0·50 ml. thyroglobulin (3·5% solution)	58·8
2	,, ,, ,,	,, ,, ,,	40·8

Notes:

Rats	Serum given on days	Thyroglobulin given on days
Group A:		
1	1, 2, 3, 4, 6	8, 9, 10, 13
2	1, 2, 3, 4, 7	9, 10, 11, 14
3	1, 2, 3, 4, 5	8, 9, 10, 12
4	1, 2, 3, 4, 5	8, 9, 10, 12
Controls		
1	1, 2, 4, 5, 6	6, 7, 8, 9
2	1, 2, 3, 4, 5	7, 8, 9, 11
Group B:		
Control 2	1, 2, 3, 4	4
All others	1, 2, 3, 5	5

Table VI. *Effect of previous treatment with antiserum prepared against thyroxylglobulin on metabolic rate of rats injected with thyroglobulin*

Rat no.	Pre-treatment	Dosage	Maximum % rise in metabolic rate
A. 1	3 doses of 2 ml. antiserum (concentrated 1 : 3)	1 dose of 0·5 ml. thyroglobulin (3·5% solution)	12·8
2	,, ,,	,, ,,	2·2
3	,, ,,	,, ,,	13·7
Controls			
1	2 doses of 3 ml. normal rabbit serum (concentrated 1 : 3)	1 dose of 0·5 ml. thyroglobulin (3·5% solution)	44·0
2	,, ,,	,, ,,	28·6
B. 1	2 doses of 3 ml. antiserum (concentrated 1 : 3)	2 doses of 0·5 ml. thyroglobulin (3·5% solution)	18·7
2	,, ,,	,, ,,	34·0
Controls			
1	2 doses of 3 ml. normal rabbit serum (concentrated 1 : 3)	2 doses of 0·5 ml. thyroglobulin (3·5% solution)	60·2
2	,, ,,	,, ,,	77·3
C. 1	2 doses of 3 ml. antiserum (concentrated 1 : 3)	1 dose of 1 ml. thyroglobulin (3·5% solution)	29·0
2	,, ,,	,, ,,	44·6
Controls			
1	2 doses of 3 ml. normal rabbit serum (concentrated 1 : 3)	1 dose of 1 ml. thyroglobulin (3·5% solution)	24·0
2	,, ,,	,, ,,	26·6

Notes: Group A. Nos. 3 and 2, serum days 1, 2, 2 (evening). Thyroglobulin day 3.
No. 1, serum days 1, 2, 3. Thyroglobulin day 3 (evening).
Controls 1, serum days 1, 2. Thyroglobulin day 3.
Group B. All. Serum days 1, 2. Thyroglobulin days 3, 4.
Group C. All. Serum days 1, 2. Thyroglobulin day 2 (evening).

We then proceeded to examine the effect of previous administration of antiserum on the action of a dose of thyroglobulin. The results in so far as the antiserum against thyroxylthyroglobulin are concerned are shown in Table V. Two sets of experiments were performed; in the first the thyroglobulin was given as four doses of a dilute solution at daily intervals and in the second as a single dose of a more concentrated solution; in both sets administration of thyroglobulin was preceded by treatment with antiserum and both sets were controlled by experiments in which similar amounts of normal rabbit serum and of thyroglobulin were given in the same time relationship to one another. It is evident from the figures that the protective effect of the antiserum against the physiological action of thyroglobulin is high.

The experiments were next extended to the antiserum against thyroxylserum globulin. In this and subsequent cases the antiserum was concentrated by precipitation of the total globulin with an equal volume of saturated ammonium sulphate; the precipitate was collected, dissolved in water and dialysed exhaustively against running tap water, after which sodium chloride to 0·9% was added to bring the euglobulin back into solution. The results in Table VI show that in two sets out of three a high degree of protection against the action of thyroglobulin was obtained with this concentrated antiserum also; the failure in the third set remains unexplained; it may be pointed out however that the

controls in this set gave a quite abnormally low response to a large dose of thyroglobulin.

The general success in obtaining protection against the action of thyroglobulin encouraged us to investigate whether a similar protective effect against thyroxine could be observed. Tables VII and VIII show that such is indeed the case. With antiserum against thyroxylthyroglobulin (Table VII) four out of five experimental rats showed very marked inhibition of the action of thyroxine as compared with the controls and a similar statement applies to the experiments with antiserum against thyroxyl-serum globulin (Table VIII).

Table VII. *Effect of previous treatment with antiserum prepared against thyroxylthyroglobulin on metabolic rate of rats injected with thyroxine*

Rat no.	Pre-treatment	Dosage	Maximum % rise in metabolic rate
1	3 doses of 3 ml. antiserum (concentrated 1 : 1·75)	3 doses of 0·75 mg. thyroxine	36·2
2	,, ,,	,, ,,	16·6
3	,, ,,	,, ,,	24·1
4	,, ,,	,, ,,	9·7
5	,, ,,	,, ,,	12·6
Controls			
1	3 doses of 3 ml. normal rabbit serum (concentrated 1 : 1·75)	3 doses of 0·75 mg. thyroxine	61·1
2	,, ,,	,, ,,	42·0

Note: All rats were given serum on days 1, 2, 3, and thyroxine on days 3 (evening), 4 and 5.

Table VIII. *Effect of previous treatment with antiserum prepared against thyroxylglobulin on metabolic rate of rats injected with thyroxine*

Rat no.	Pre-treatment	Dosage	Maximum % rise in metabolic rate
1	2 doses of 3 ml. antiserum (concentrated 1 : 3)	3 doses of 0·75 mg. thyroxine	14·3
2	,, ,,	,, ,,	15·9
3	,, ,,	,, ,,	19·4
4	,, ,,	,, ,,	21·9
5	,, ,,	,, ,,	52·9
Controls			
1	2 doses of 3 ml. normal rabbit serum (concentrated 1 : 3)	3 doses of 0·75 mg. thyroxine	65·8
2	,, ,,	,, ,,	42·3
3	,, ,,	,, ,,	46·0
4	,, ,,	,, ,,	30·0

Note: All rats were given serum on days 1, 2, and thyroxine on days 2 (evening), 3 and 4.

Discussion

There seems to be little doubt from the experiments which have just been described that the serological reactions recorded in the second section of this paper afford a true indication of the power of antisera against thyroxylproteins to react with and neutralize in the body exogenously administered thyroglobulin and thyroxine.

The failure to observe any effect of the antiserum upon the normal animal might be explained by a difference of the actual thyroid secretion from either thyroxine or thyroglobulin; since however these two compounds represent, so to

say, the extreme limits of simplicity on the one hand and complexity on the other which are conceivable for the composition of the thyroid secretion, and since both these compounds are physiologically neutralized by the antisera, such an explanation is unlikely. In any case the point will be put to direct test by examining whether the effect of thyrotropic hormone, which may be presumed to act by increasing the rate of output of normal thyroid secretion, is also neutralized by the antisera. It seems more likely that the failure of the antisera to affect the normal animal is due to the well-known power of the thyroid gland to respond to any call for increased secretion.

The inhibitory effect of the antisera on the action of exogenously administered thyroglobulin seems to be unequivocal, and indeed, in view of the serological results, is perhaps not surprising; more striking is the inhibitory effect on the action of thyroxine. At first sight the latter result seems unexpected, for whilst the neutralization of the protein thyroglobulin might be regarded as analogous with toxin-antitoxin neutralization, thyroxine is a low-molecular substance which would not be expected to take part in a reaction of this type. On the other hand it must be remembered that the serological inhibition reactions actually afford an analogy for the neutralization of thyroxine *in vivo*; the inhibition reactions are due to the power of thyroxine to combine with the specific antibodies and such combination occurring *in vivo* might well inhibit its physiological action.

The facts at any rate are clear and they seem to open up considerable possibilities since they suggest that immunization with a protein to which is attached a physiologically active group acting as a hapten may give rise to an antiserum with the power of neutralizing the normal physiological effect of the attached group *in vivo*. It must be pointed out that it is not safe to draw this general conclusion from the results obtained with thyroxine alone; thyroxine is an amino-acid and may have to be built up into the molecule of a protein before it can exercise its effect in the body; if such is the case it would be the thyroxine-containing protein which is neutralized by the antiserum and the reaction *in vivo* would fall into a recognized immunological category. Experiments are planned to test the validity of the general proposition by the use as hapten of a physiologically active group entirely unconnected with protein. It is also intended to attempt to produce a state of resistance to the action of thyroxine and other physiologically active groups by active immunization with proteins containing these groups as haptens.

It was anticipated at the outset of this work that the nature of the protein used as the basis for the artificial antigen might be of significance; actually, as between serum globulin and thyroglobulin, this does not seem to be the case, since antisera against derivatives of both these proteins are extremely similar both serologically and physiologically; it may be concluded from this that the iodine-containing groups introduced in the present experiments are so powerfully determinant in character as to override the effect of structural differences between the proteins used.

The preparation of large amounts of antisera of the type described in the present paper has been undertaken in order to test the possibility of their therapeutic application in human hyperthyroidism.

Summary

1. The preparation and properties of thyroxyl derivatives of horse serum globulin and albumin and of thyroglobulin are described.

2. The serological reactions of the derivatives have been studied and have been found to be conditioned by the thyroxine groups introduced which thus

act as haptens. The hapten property is a function of the whole aromatic portion of the thyroxine molecule and not of the diiodophenolic group alone.

3. Passive immunization with antisera against the thyroxylproteins protects against the normal physiological effects of exogenously administered thyroglobulin and thyroxine. The general implications of the latter observation are discussed.

The new derivatives of 3:5-diiodothyronine, described for the first time in this paper, were prepared some years ago in this Department by Dr S. Kishi. We desire to thank Messrs Hoffmann-la Roche and particularly Dr M. Guggenheim for generous gifts of 3:5-diiodothyronine; our thanks are also due to Sir Henry Dale and Prof. T. R. Elliott for the loan of the metabolism apparatus. One of us (M. E. Y.) is indebted to the Medical Research Council for a personal grant.

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APPENDIX

Protocol 1

Rat no. 1. Treatment: injections of antiserum to thyroxylthyroglobulin followed by thyroxine

Date	Time	Oxygen absorbed at N.T.P. ml./kg./min.	Injections	Weight g.
31. iii. 38	16.00	16·72	—	228
1. iv. 38	16.00	16·74	—	232
2. iv. 38	11.30	—	3 ml. antiserum (conc. 1 : 1·75) I.P.	—
3. iv. 38	11.30	—	3 ml. antiserum (conc. 1 : 1·75) I.P.	—
4. iv. 38	10.15	16·50	—	212
4. iv. 38	12.00	—	3 ml. antiserum (conc. 1 : 1·75) I.P.	—
4. iv. 38	16.00	—	0·75 mg. thyroxine I.M.	—
5. iv. 38	10.15	16·89	—	210
5. iv. 38	16.00	—	0·75 mg. thyroxine I.M.	—
6. iv. 38	10.15	17·16	—	210
6. iv. 38	16.00	—	0·75 mg. thyroxine I.M.	—
7. iv. 38	10.20	18·84	—	205
8. iv. 38	10.00	16·70	—	203
9. iv. 38	10.15	17·36	—	202

Average of first 3 readings = base-line reading = 16·652 ml. O_2/kg./min.
Highest percentage rise after thyroxine injections = 13·1.

I.P. = intraperitoneal. I.M. = intramuscular.

Protocol 2

Rat no. 2. Control for rat no. 1. Treatment: injections of normal rabbit serum followed by thyroxine.

Date	Time	Oxygen absorbed at N.T.P. ml./kg./min.	Injections	Weight g.
29. iii. 38	10.15	17·33	——	210
29. iii. 38	16.00	—	3 ml. normal serum (conc. 1 : 1·75) I.P.	—
30. iii. 38	14.15	15·25	—	210
30. iii. 38	16.00	—	3 ml. normal serum (conc. 1 : 1·75) I.P.	—
31. iii. 38	10.30	14·68	—	208
31. iii. 38	12.00	—	3 ml. normal serum (conc. 1 : 1·75) I.P.	—
31. iii. 38	16.00	—	0·75 mg. thyroxine I.M.	—
1. iv. 38	13.55	20·19	—	200
1. iv. 38	16.00	—	0·75 mg. thyroxine I.M.	—
2. iv. 38	09.45	21·88	—	192
2. iv. 38	11.30	—	0·75 mg. thyroxine I.M.	—
3. v. 38	10.15	22·57	—	188
4. iv. 38	10.15	24·89	—	178
5. iv. 38	10.00	21·88	—	196
7. iv. 38	10.00	17·97	—	198

Average of first three readings – base-line reading = 15·752 ml. O_2/kg./min.
Highest percentage rise after thyroxine injections = 57·8.