THE INFLUENCE OF THYROXINE ON THE ENZYMIC ACTIVITY OF RAT TISSUES

by

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INTRODUCTION

The experiments reported in this paper are concerned with the influence of thyroxine, both *in vivo* and *in vitro* on the succinoxidase, choline oxidase and adenosine triphosphatase (ATP-ase) activity of the liver and the hexokinase activity of skeletal muscle and the brain in rats. Since there is a *prima facie* resemblance between certain of the over-all physiological effects of thyroxine and 2:4 dinitrophenol (DNP) following the administration of these substances to intact animals, the influence of DNP on certain of the above enzymes has also been investigated.

METHODS

Animals and diet

The animals used in these experiments were either hooded rats obtained from the Medical Research Council or albino rats obtained from Glaxo Ltd. In short termed experiments the animals were taken from a stock colony which had been fed a diet of rat cake *ad libitum*. In all long termed experiments involving the administration of substances over a period of four days or longer, the animals were pair fed with a vitamin supplemented diet the composition of which is shown in Table I. The animals were allowed drinking water *ad libitum* and were fed, weighed and injected at the same time each day.

Estimation of enzyme activities

Succinoxidase activity was determined at 37° exactly according to the method of SCHNEIDER AND POTTER[24]. ATP-ase activity was determined at 37° in the presence of 0.0025 *M* $CaCl_2$ by the method of DUBOIS AND POTTER[5]; the enzyme activity was always determined using two different concentrations of tissue. Choline oxidase activity was determined as follows: the tissue was removed from the animal, chilled on ice and weighed after chopping with scissors. It was then homogenised with 2 vols of ice cold glass distilled water using a previously chilled homogeniser. The resulting homogenate was strained through a layer of muslin and an aliquot (0.50 ml) added to Warburg flasks containing 1.06 ml 0.1 *M* sodium phosphate buffer. To each flask was also added 0.50 ml of either a 1% solution of choline chloride in 0.1 *M* phosphate buffer p_H 7.8 or phosphate buffer alone. The total volume in the main compartment was made up to 2.50 ml with water, cytochrome *c* or additions. 0.20 ml of 20% KOH and filter paper roll was placed in the centre inset. The oxygen uptake was determined manometrically after 10 min temperature equilibration at 38° with air as

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the gas phase. The choline oxidase activity was computed by subtracting the oxygen uptake in the presence of choline from that observed in the absence of added substrate ("endogenous respiration"). The oxygen uptake over the first 20 min was used for these estimations, the choline oxidase activity over this period being linear.

The hexokinase activity of hind limb muscles was estimated using aqueous extracts prepared exactly according to COLOWICK, CORI AND SLEIN[2]. The activity was computed from measurements of the glucose consumption by the method of NELSON[21]. The extracts (0.80 ml) were incubated for 10 min at 28° in open test tubes containing the following constituents in a final vol. of 2.35 ml: veronal-acetate buffer p_H 7.9 0.014 *M*; $MgCl_2$ 0.0085 *M*; NaF 0.056 *M*; glucose 0.0022 *M*; ATP 0.0042 *M* (added last to start the reaction). In a preliminary note describing some of these investigations[25], the concentrations of buffer, glucose and ATP stated are erroneous. 0.9 mg of glucose was added to each tube so that in control experiments approx. one quarter to one third of the total glucose was utilized. The activities observed are thus a reflexion of large changes in the galvanometer readings. The ATP concentration used was not limiting.

The hexokinase activity of brain was measured under the same conditions. The tissue was removed immediately the animal had been killed and was homogenised in 5 vols ice cold glass distilled water. 0.20 ml of the brain homogenate was added to each tube.

Dry weights were determined by heating to constant weight at 105°.

Materials

For injection purposes, DL-sodium thyroxine (British Drug Houses Ltd.) was dissolved in water by adding the minimal amount of 2 *N* NaOH. The control animals were injected at the same time with the same vol. of dilute alkali. L-thyroxine ($(\alpha)_{20}^{D} = -5.42°$) was generously donated by Sir CHARLES HARINGTON. Thyroglobulin was prepared from sheep thyroid glands by the method of ROSSITER[23]. Iodinated casein was obtained from Boots Ltd. and was purified by isoelectric precipitation. DNP was obtained from Boots Ltd. and purified by repeated recrystallisation from hot water. ATP was obtained as the dibarium salt either from rabbit muscle by the method of NEEDHAM[20] or from Boots Ltd. Cytochrome *c* was prepared from ox heart by the method of KEILIN AND HARTREE[10] except that the final dialysis was made against glass distilled water; it was standardised spectrophotometrically. Veronal-acetate buffers were prepared according to MICHAELIS[18].

TABLE I

COMPOSITION OF THE DIET USED IN PAIRED FEEDING EXPERIMENTS

Constituent	g/100 g diet
80–90% extraction wholemeal flour	50.5
Casein	6.9
Dried milk	6.9
Animal fat	2.8
Calcium carbonate	1.0
Cod liver oil	0.7
Tap water	31.2

Roche "Becosym" vitamin tablets ground with constituents prior to addition of cod liver oil, animal fat and water so that each 30 g of final moist diet contained: thiamin 0.1 mg; riboflavin 0.2 mg; pyridoxine 0.2 mg; nicotinamide 2.0 mg; calcium pantothenate 0.3 mg. Diet stored at 2° for not more than two weeks.

RESULTS

a. *Enzymic activities in the tissues of thyroxine treated rats*

(i) *Hexokinase*

The administration of large doses of thyroxine over a period of eight days (total dose of 20 mg per kg) resulted in marked and statistically significant increases in the hexokinase activity of hind limb muscle extracts compared with the corresponding pair fed controls. Table II shows that the mean increase in hexokinase activity for six

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thyroxine treated rats was 67%. Six hours after the administration of 8 mg thyroxine per kg, however, the hind limb muscle hexokinase activity was within normal limits. Similarly, one to two hours after the administration of 40 mg DNP per kg the hexokinase activity of this tissue was unaltered.

TABLE II

THE INFLUENCE OF THYROXINE ADMINISTRATION ON THE HEXOKINASE ACTIVITY OF RAT HIND LIMB MUSCLE AND BRAIN

Long term experiments. Experimental animals injected with 1 mg DL-thyroxine on 1st, 3rd, 5th, 7th and 8th day. Animals killed on 8th day.

Short term experiments. Experimental animals injected with 2 mg DL-thyroxine 6–8 hours before sacrifice.

Group	Tissue	No. of animals	Initial weight (g)	Hexokinase activity (mg glucose/10 min/g tissue) ± Standard error of mean
Controls	Leg muscle	6	250	0.87 ± 0.04
Thyroxine injected (long term)	Leg muscle	6	234	1.44 ± 0.11
Controls	Leg muscle	4	231	0.79 ± 0.06
Thyroxine injected (short term)	Leg muscle	4	227	0.77 ± 0.03
Controls	Brain	6	225	2.95* ± 0.36
Thyroxine injected (long term)	Brain	6	228	2.70 ± 0.30

* Computed in terms of dry weight. Leg muscle hexokinase activity computed in terms of wet weight.

In contrast to the results obtained with muscle, the hexokinase activity of unfractionated, freshly prepared aqueous brain homogenates was not significantly affected by thyroxine administration over a period of eight days.

(ii) *Succinoxidase*

Table III summarises the results of experiments which confirm the finding of TIPTON AND NIXON[26] that thyroxine administration enhances the succinoxidase activity of rat liver. DNP administration either for short periods or for as long as 38 days did not, however, result in any significant change in the activity of this enzyme system.

(iii) *Choline oxidase*

The choline oxidase activity of hyperthyroid rat livers was essentially the same as that of the corresponding pair fed controls. The endogenous respiration of the hyperthyroid livers was elevated some 20–30%. It can be seen from Table IV that, as opposed to the choline oxidase activity, the endogenous respiration declined considerably over the first 20 min of the experiment. Previous investigations had shown that the endogenous respiration was unaffected by the addition of cytochrome *c* to a final concen-

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tration of 10^{-5} *M*, and Table IV shows that the oxygen uptake in the presence of choline by either normal or hyperthyroid rat liver homogenates was not influenced by 10^{-5} *M* cytochrome *c*.

TABLE III

THE INFLUENCE OF THYROXINE AND 2:4 DINITROPHENOL ADMINISTRATION ON THE SUCCINOXIDASE ACTIVITY OF RAT LIVER

Group	No. of animals	Initial weight (g)	Liver succinoxidase activity	
			Q_{O_2}	Total liver succinoxidase*
a. *Thyroxine administration*				
(Experimental animals received subcutaneous injection of 0.1 mg DL-thyroxine on 1st, 3rd and 5th day and 1 mg on 7th day. Animals killed on 9th or 10th day)				
Controls	6	260	54.2 ± 1.8	110.9 ± 7.5
Thyroxine injected	6	245	80.4 ± 3.8	154.3 ± 6.4
b. *Acute administration of dinitrophenol*				
(Experimental animals killed 1–2 hours after subcutaneous injection of 40 mg DNP/kg)				
Controls	5	244	47.4 ± 3.1	103.3 ± 12.4
DNP injected	5	239	49.9 ± 4.2	92.1 ± 8.1
c. *Prolonged administration of dinitrophenol*				
(Experimental animals injected subcutaneously with 10 mg DNP/kg/day for 19–38 days)				
Controls	5	198	50.1 ± 2.9	104.7 ± 8.8
DNP injected	5	200	48.0 ± 5.1	109.6 ± 8.6

* Q_{O_2} × total dry weight of liver (g) computed in terms of 250 g rat.

TABLE IV

THE INFLUENCE OF THYROXINE ADMINISTRATION ON THE CHOLINE OXIDASE ACTIVITY OF RAT LIVER

Experimental animals injected with 1 mg DL-thyroxine on 1st, 3rd and 5th day; sacrificed on 7th day

Group	No. of animals	Initial weight (g)	O_2 uptake (μl)/166 mg liver in:						Choline oxidase activity (μl O_2/166 mg/20 mi corrected for endogenous respiration
			10 min			20 min			
			End.	Chol.	Chol. + cyt. *c*	End.	Chol.	Chol. + cyt. *c*	
Controls	6	291	19.1	58.7	58.7	31.4	104.5	102.3	73.1 ± 3.2*
Thyroxine injected	6	292	24.6	62.3	61.3	38.3	116.1	108.8	72.8 ± 5.9

* Standard error of mean.

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(iv) *ATP-ase*

A few experiments were performed on the influence of prolonged thyroxine administration on the ATP-ase activity of rat liver. Although the liver ATP-ase activity of each thyroxine treated animal was greater than that of the corresponding pair fed control, the mean increase being 37% (Table V), the wide range of individual values found among the control series makes the increase in activity observed in the thyroxine treated animals of doubtful statistical significance. Two hours after the administration of 40 mg DNP/kg the ATP-ase of rat liver was within normal limits.

TABLE V

THE INFLUENCE OF THYROXINE ADMINISTRATION ON THE ATP-ase ACTIVITY OF RAT LIVER

Experimental animals injected with 1 mg DL-thyroxine on 1st, 3rd, 5th and 7th day. Animals killed on 8th day.

Group	No. of animals	Initial weight (g)	Liver ATP-ase activity (μg P liberated from ATP/mg wet weight tissue in 10 min)
Controls	5	277	10.4 ± 1.0*
Thyroxine injected	5	235	14.4 ± 1.8

* Standard error of mean.

b. *The influence of the in vitro addition of thyroxine, iodinated proteins and DNP on the enzymic activity of rat tissues*

Many attempts were made to duplicate the alteration in liver succinoxidase and muscle hexokinase activity observed in thyroxine treated rats by the *in vitro* addition of both DL- and L-thyroxine (concentrations up to $2 \cdot 10^{-4}$ *M*) under a wide variety of experimental conditions. Similar experiments were performed with thyroglobulin and iodinated casein (1–4 mg/ml). The immediate *in vitro* addition of these substances to the experimental test systems employed in the experiments described above did not result in any significant change in the activity of muscle and brain hexokinase, liver succinoxidase, choline oxidase and ATP-ase.

A similar lack of influence of thyroxine and iodinated proteins was observed under other experimental conditions. Thus the incubation of chopped muscle brei with thyroxine (10^{-4} *M*) for two hours at 38° in O_2, and the subsequent extraction of the tissue with ice-cold glass distilled water gave rise to extracts the hexokinase activity of which was no different from that of controls incubated without thyroxine. Thyroxine, thyroglobulin and iodinated casein incubated for as long as three hours at 38° with buffered liver homogenates in the absence of added succinate did not effect the O_2 uptake on subsequent addition of succinate from the side arms of the manometers; these experiments were carried out with incubated controls either at p_H 7.4 or at p_H 8.0. Ageing the liver homogenates (10%) for as long as seven days at 2°, or dialysis for 24 hours at 2°, failed to make the succinoxidase activity sensitive to thyroxine.

ALOISI AND CAVALLINI[1] observed that a crude, freshly prepared heart muscle succinoxidase preparation was unaffected by the *in vitro* addition of thyroxine, but that preparations aged by storing at 0° for many days were markedly activated by thyroxine.

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We performed a few experiments using the heart muscle succinoxidase preparation of KEILIN AND HARTREE[11] which was suspended and stored (at 2°) in 0.1 *M* phosphate buffer p_H 7.3. The activity of freshly prepared and aged preparations measured manometrically in the presence of methylene blue was not altered by the addition of 10^{-4} *M* thyroxine. However, if the activity was measured in the presence of $3 \cdot 10^{-5}$ *M* cytochrome *c*, thyroxine sometimes gave rise to small activations of the order of 15%. It was noticed that when activation of the enzyme activity did occur, thyroxine (added in alkaline solution to the suspension medium buffered at p_H 7.3) precipitated out in the flasks. Since the succinoxidase activity of similar preparations is markedly activated by substances which give rise to colloidal precipitates[12,13], little significance can be attached to such small activations, and it is suggested that the results of ALOISI AND CAVALLINI[1], which were obtained with much higher concentrations of thyroxine, were similarly unspecific.

10^{-4} *M* DNP, which almost completely abolishes the phosphate uptake associated with the oxidation of glutamate by washed kidney particles[9,16] had little influence on any of the enzyme systems investigated. Higher concentrations of DNP were however inhibitory to succinoxidase, the inhibition at a concentration of 10^{-3} *M* being 20–30%. Low concentrations of DNP (10^{-4} *M*) did nevertheless inhibit the endogenous respiration of rat liver homogenates 30–40%.

DISCUSSION

If it is assumed that the hexokinase reaction is the rate-limiting step in the utilisation of glucose by muscle[15,22], then the physiological observations of an enhanced rate of glucose utilisation in the peripheral tissues of hyperthyroid animals[19] indicate that the rate of the hexokinase reaction is increased under these conditions. Our findings concerning the influence of prolonged thyroxine administration on muscle hexokinase activity are clearly in accord with this deduction.

The failure to observe any significant changes in the activity of either liver succinoxidase or muscle hexokinase following the *in vitro* addition of either thyroxine or iodinated proteins or in short termed administration experiments raises the question of the mode of action of the thyroid hormone. On the basis of their observation of an altered sulphydryl content of rabbit myosin after thyroid treatment, GOL'DSHTEIN *et al.*[7] concluded that the thyroid hormone acts by rupturing thioether linkages in proteins with the liberation of free sulphydryl groups. Our finding that the choline oxidase activity of hyperthyroid liver is not significantly different from normal suggests that the thyroid hormone does not influence intracellular enzyme activities by restoring functional sulphydryl groups in enzyme molecules, for both succinoxidase and choline oxidase require free sulphydryl groups for their activity[3]. However, it must be mentioned that evidence is now at hand that folic acid is essential for choline oxidase[4] and it is well established that hyperthyroidism greatly increases the requirement for dietary folic acid[17]. Since the diet we employed in our paired feeding experiments was not supplemented with folic acid, our results with choline oxidase may have been complicated by an induced deficiency of this vitamin.

The experimental results obtained with DNP serve to emphasise the difference between the mode of action of this substance in enhancing the metabolic rate of the intact animal and that of the thyroid hormone. The response of liver succinoxidase to

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the prolonged administration of DNP and thyroxine appears to parallel that of cytochrome *c*, for TISSIÈRES[27,28] has demonstrated that although thyroxine administration increased the cytochrome *c* content of rat muscle, DNP administration was without effect. The mode of action of DNP (which does not exhibit a latent period of action like thyroxine) seems to be closely connected with its ability to dissociate oxidation from phosphorylation[16]; oxidative phosphorylation is not affected by the *in vitro* addition of either thyroxine or thyroglobulin[9]. Whether the inhibition of the endogenous respiration of rat liver homogenates (which is uninfluenced by thyroxine) by low concentrations of DNP is related to the very low respiratory quotient of such preparations[6] and is the result of the inhibition of fatty acid oxidation by such systems[14] can only be decided by further experimentation.

Although the possibility that the thyroid hormone directly influences the catalytic activity of certain enzyme proteins is still extant, the simplest interpretation of the available facts concerning the influence of the thyroid hormones on intracellular enzyme systems is that the thyroid hormone affects the concentration of enzyme proteins in various tissues. In this connexion our results with hexokinase deserve further comment. From Table II it will be seen that the hexokinase of the muscle extracts is expressed on the basis of the wet weight of tissue taken. In a further series of experiments with hyperthyroid rats, carried out under conditions identical with those used for the hexokinase experiments, the wet to dry weight ratio of the control and thyroxine treated rats was compared. The latter group showed a mean increase in the wet to dry weight ratio of 17%. This increase is clearly quite disproportionate to the very marked increase in the hexokinase activity of the extract. The wet to dry weight ratios of the homogenates used in the brain hexokinase and liver succinoxidase experiments (the results of which were expressed in terms of unit dry weight) were practically the same in the normal and thyroxine treated groups.

We considered that some light might be shed upon the question of the change in enzyme concentration in hyperthyroid tissues from studies of the schlieren diagrams obtained in the electrophoresis of normal and hyperthyroid rat muscle extracts. A few exploratory experiments have recently been carried out along these lines by one of us (R.H.S.) in collaboration with Miss B. A. ASKONAS. It is apparent from these experiments that an increase in the relative amounts of certain of the electrophoretic components of muscle extract attends the prolonged administration of thyroxine to the rat. Such an increase has been found in the instance of the slow-moving component designated *n* by JACOB[8] and of the "Group II" components of this author. These studies are being continued; it is hoped to obtain an improved resolution of the muscle components and to identify more of them with known enzymes.

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SUMMARY

1. The administration of thyroxine over a period of eight days resulted in a marked increase in the hexokinase activity of rat skeletal muscle extracts, but not of brain homogenates.

2. The hexokinase activity of skeletal muscle was unaltered a few hours after the administration of large doses of either thyroxine or 2:4 dinitrophenol.

3. Prolonged thyroxine administration enhanced the succinoxidase activity of rat liver and the adenosine triphosphatase activity to a lesser extent; the choline oxidase activity was unaltered. Either acute or prolonged administration of dinitrophenol did not give rise to any significant change in the liver succinoxidase activity.

4. No significant influence of the *in vitro* addition of thyroxine on the activity of hexokinase or succinoxidase could be demonstrated under a wide variety of experimental conditions. Thyroglobulin and iodinated casein were similarly ineffective.

RÉSUMÉ

1. L'administration de thyroxine pendant une période de huit jours résultait en une augmentation marquée de l'activité hexokinasique d'extraits de muscle strié de Rat, mais non d'homogénats de cerveau.

2. L'activité hexokinasique de muscle strié était inchangée quelques heures après l'administration de doses importantes de thyroxine ou de 2.4-dinitrophénol.

3. Un traitement prolongé à la thyroxine augmentait l'activité succinoxydasique de foie de Rat, et, à un degré moindre, l'activité adénosinetriphosphatasique; l'activité choline-oxydasique était inaltérée. Une administration forte ou prolongée de dinitrophénol ne donnait lieu à aucun changement significatif de l'activité succinoxydasique du foie.

4. Nous n'avons pu démontrer aucune influence significative de l'adjonction *in vitro* de thyroxine sur l'activité hexokinasique ou succinoxydasique sous des conditions expérimentales très variées. La thyréoglobuline et la caséine iodée étaient semblablement inactives.

ZUSAMMENFASSUNG

1. Verabreichung von Thyroxin während acht Tagen hatte eine bedeutende Zunahme der Hexokinaseaktivität von Extrakten von gestreiftem Rattenmuskel, nicht aber von Hirnhomogenaten zur Folge.

2. Die Hexokinaseaktivität von gestreiftem Muskel war ein paar Stunden nach Verabreichung von grosser Dosen Thyroxin oder 2.4-Dinitrophenol unverändert.

3. Längere Thyroxinverabreichung steigerte die Succinoxidase-Aktivität von Rattenleber und in geringerem Masse die Adenosin-triphosphatase-Aktivität; die Cholinoxydase-Aktivität war unverändert. Starke oder längere Verabreichung von Dinitrophenol hatte keine bedeutende Veränderung der Lebersuccinoxydase-Aktivität zur Folge.

4. Nach *in vitro* Zugabe von Thyroxin unter sehr verschiedenen Versuchsbedingungen konnte kein Einfluss von Bedeutung auf die Hexokinase- oder Succinoxydase-Aktivität nachgewiesen werden. Thyreoglobulin und jodiertes Kasein waren ähnlich unwirksam.

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