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THYROID FUNCTION, PLASMA INSULIN, GLUCOSE AND KETONES AND IN VITRO HEPATIC GLUCONEOGENESIS IN RATS UNDER CHRONIC LOW IODINE INTAKE

By

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ABSTRACT

Weaning female rats were fed for 13–14 months with a low iodine diet (LID) and compared with age matched controls on KIO_3 supplemented LID (C). At sacrifice the LID rats had large goitres, low plasma PBI and thyroidal ^{127}I content, a rapid thyroidal-^{131}I (*I) turnover and increased *MIT/*DIT and $*T_3/*T_4$ ratios. Body weights were slightly higher in LID and pituitary growth hormone like protein bands were the same in both groups. There were no differences between LID and C, whether fed or fasted for 48 h with regard to plasma insulin, glucose and ketone bodies. *In vitro* disposition of trace or 10^{-2} M alanine-U-^{14}C by liver slices were the same as measured by ^{14}C incorporation into CO_2, glucose and lactic acid. The response of these parameters to fasting was normal: plasma insulin and glucose decreased and ketone bodies and *in vitro* gluconeogenesis increased. The lack of alteration in the intermediary metabolism of rats, sufficiently iodine deficient to develop goitre, has been interpreted as indicating either that: 1) thyroid hormones have no direct effect on the parameters of intermediary metabolism studied, or 2) there is enough hormone available to the tissues to keep a normal metabolism but not to suppress TSH secretion, thus suggesting different sensitivities to the hormone.

Experimental hypothyroidism produced by thyroidectomy and/or propylthiouracil (PTU) administration has been used to study the effect of thyroid hormone deficiency on intermediary metabolism (*Redd & Tepperman* 1968:

Young 1968; *Menahan & Wieland* 1969; *Böttger et al.* 1970). These situations are accompanied by alterations in other endocrine sites. Both PTU and thyroidectomy decrease or arrest the body weight gain of growing rats (*Salmon* 1938; *Evans et al.* 1960) as a consequence of decreased growth hormone (GH) content in the pituitary (*Griesbach & Purves* 1945; *Daughaday et al.* 1968). Thyroidectomy produces a decrease in the *in vitro* output of pancreatic insulin (*Malaisse et al.* 1967). Recent findings show that thyroidectomy and PTU treatment also produce alterations in the circulating levels of insulin (*Jolin et al.* 1970). Because of these mixed situations it is difficult to decide which effects are due directly to the decrease in the amounts of the thyroid hormones available to the animal and which to the other alterations known to affect intermediary metabolism. These considerations prompted us to look for an experimental design in which low plasma PBI levels were at least not accompanied by changes in the growth of the animals. This was obtained by the chronic intake of a low iodine diet (LID) in rats. In these animals we have evaluated the thyroid function as well as a few aspects of their intermediary metabolism.

MATERIALS AND METHODS

Animals

Wistar male rats were fed from the time of weaning on a medium-residue low-iodine diet of the Remington type (LID). The iodine content of different batches of diet varied between 0.05 to 0.09 μg/g. Distilled water was used. The animals were compared with age and sex matched controls (C) fed with the same diet supplemented with potassium iodate (1.7 μg/g). Both groups of animals were maintained with their respective diets for 13 to 14 months, after which they were sacrificed as indicated below.

Evaluation of thyroid function

24 h before being killed the animals were injected intraperitoneally with 10 μCi of ^{131}I-iodide (*I) (Junta de Energía Nuclear, Madrid, Spain) and the changes in thyroidal *I content were followed by *in vivo* counting of the radioactivity in the neck under light ether narcosis. The rats were killed under ether anaesthesia and blood collected from the inferior vena cava into heparinized test tubes. The thyroid glands were rapidly dissected and weighed, after which they were homogenized in 0.5 ml of HCl-tris buffer, pH 8.6 containing 10^{-3} M PTU. Aliquots of the homogenates were used for ^{127}I evaluation and for digestion at 37°C with pronase (Sigma Chem. Co.) for 7–8 h (*Tong et al.* 1963). Aliquots were used for separation of iodoaminoacids by paper chromatography. Individual aliquots of the digested mixture were spotted on Whatman 3 chromatography strips along with carrier of T_4, T_3, DIT, MIT, I and 10^{-3} M PTU, to avoid artifactual deiodination (*Morreale de Escobar et al.* 1963). Ascending chromatography in n-butanol-ethanol-1 M ammonia (5:1:2, by volume) separated both the iodothyrosines and iodothyronines. Localization of the radio-

active spots was determined by radioautography. The appropriate spots were counted in a well-type Na-thallium-activated scintillation counter. Plasma was kept frozen for total ^{127}I and PBI estimation. All ^{127}I measurements were carried out by a modified Zak procedure (*Benotti & Benotti* 1963).

Body weight and pituitary content of growth hormone-like protein

Homogenates of pituitary were prepared in saline buffered with potassium phosphate 0.01 M pH 7.8 and submitted to polyacrylamide gel electrophoresis following the procedure described by *Escobar del Rey et al.* (1968). The protein bands associated with GH and albumin were identified by microdensitometry of the polyacrylamide gels, on the basis of *Lewis et al.* (1965) and *Jones et al.* (1965) in the rat. The adequacy of this technique for rough estimation of pituitary content of a GH-like protein was based on the findings of *Lewis et al.* (1969) showing good correlation between the GH biological potency and the protein of staining the polyacrylamide gels.

Intermediary metabolism

For these studies the rats were sacrificed by decapitation, without anaesthesia.

Analysis of plasma. – The blood was obtained from the neck of the animals and put into heparinized tubes, and protein-free filtrates of plasma were prepared with $Ba(OH)_2$-$ZnSO_4$ (*Somogyi* 1945) and analysed for glucose (*Huggett & Nixon* 1957) and total ketones (*Bessman & Anderson* 1957). Plasma radioimmunoassayable insulin was evaluated with the radioinsulin kit provided by Radiochemical Centre, Amersham, England, rat insulin being used as standard.

Alanine-U-^{14}C utilization by liver slices. – Liver slices,, 0.5 mm in thickness, were cut freehand and floated in Krebs-Ringer bicarbonate (KRB) buffer pH 7.4. Slices were briefly blotted, weighed on a torsion balance, and 90–120 mg portions were put into vessel filled with 1 ml of KRB containing alanine-U-^{14}C (1 μCi/ml) (Radiochemical Centre, Amersham, England). The vessels were covered with rubber caps from which a small polyethylene cup was suspended. They were gassed with O_2/CO_2 (95/5) through needles for 5 min in a Dubnoff metabolic shaker at 37°C and the incubation was processed for 90 min at 100 cycles/min. $^{14}CO_2$ was evolved at the end of the incubation by the introduction of 0.1 ml N H_2SO_4 into the media and the $^{14}CO_2$ was »trapped« in hydroxide of hyamine (250 μl) (Packard, Zürich), placed in the polyethylene cup, by shaking gently for 90 min at room temperature. The media were centrifuged to remove any insoluble debris. 50 μl aliquots of each supernatant were chromatographed in two media: a) pyridine, acetic acid, water, isopropyl alcohol (8:1:4:8, by volume) and b) the upper phase of butanol, water, methanol and formic acid (320:320:80:1, by volume). Ascending chromatography was done in both media on one-inch-wide strips of Whatman No. 3 paper, with carrier of cold glucose, lactic acid, pyruvic acid, aspartic acid and glutamic acid and alanine (5 μg of each in 10 μl) and the spots were identified by means of radioactive guide samples by autoradiography.

Radioactive assay. – Hyamine cups ($^{14}CO_2$), appropriate spots of chromatograms and aliquots of the medium were counted in liquid scintillation mixture containing 15 g 2,5-diphenyloxazole (PPO), 150 mg p-bis(2-(5-phenyloxazolyl)) benzene (POPOP) and 240 g naphthalene in 3000 ml of xylene: dioxane: 95 % ethanol (5:5:3, by volume). Radioactive measurements were expressed as a function of the appropriate counting standards (i. e. as percentage of the total alanine-U-^{14}C added to each vessel) and were related to the initial wet weight of the slices.

RESULTS

Thyroid function

Plasma and thyroid ^{127}I content. – A chronic intake of low iodine diet (LID) produces a decrease in the total plasma ^{127}I content (Table 1). Practicaly 100 % of the iodine in the plasma of the LID group is represented by PBI (92.9 %), while in the C animals it is only 62.2 %. These differences are accompanied by alterations at the thyroid level. The thyroid weight either expressed as mg or as mg/100 g body weight, is approximately four times greater in the LID than in the C group and its total ^{127}I content per gland is 25 times lower in LID than in C, and 100 times lower when expressed per mg of gland (Table 2).

*Thyroid turnover of *I.* – 3 h after iodide-*I injection, the radioactivity in the thyroid was 4.6 times higher in the LID than in the C animals (Table 2). From this time onwards up to 24 h after the injection of the tracer the radioactivity in the thyroid of LID declines, while that of the C group continues to rise for 9 h and mantains this level until the 24th h, suggesting a plateau. Despite these opposite changes, the radioactivity remaining at 24 h after *I in the gland of the LID animals is still higher than for the C group. By this time, the percentage distribution of radioactive iodoaminoacids in the thyroid was quite different from one group to another (Table 2). While *DIT and $^{*}T_4$ were lower in the LID than in the C animals, *MIT and $^{*}T_3$ were higher, so that the *MIT/*DIT and $^{*}T_3/^{*}T_4$ ratios were 4.4 and 13.9 times

Table 1.
^{127}I and ^{131}I-containing fractions in plasma C and LID rats[1].

	Mean ± SEM		*P*
	C	LID	
^{127}I (μg/100 ml)	4.5 ± 0.7	1.41 ± 0.00	< 0.001
PB^{127}I (μg/100 ml)	2.8 ± 0.9	1.30 ± 0.04	< 0.02
*I[2]	2.4 ± 0.2	19.2 ± 0.8	< 0.001
PB*I	1.20 ± 0.08	18.2 ± 0.8	< 0.001

1) *P* indicates the level of significance of the difference between C and LID. The number of animals per group was 6.

2) *I determinations were performed 24 hours after injection of the tracer and expressed as % of injected counts/100 ml of plasma.

Table 2.
Effect of low iodine diet on parameters of thyroid function in the rat[1].

	Mean ± SEM		*P*[2]
	C	LID	
Weight (mg)	21.3 ± 0.9	91.7 ± 14.3	< 0.001
Weight (mg/100 g body wt.)	9.3 ± 0.4	35.2 ± 5.3	< 0.001
^{127}I (μg I/mg gland)	1.3 ± 0.2	0.014 ± 0.002	< 0.001
^{131}I Uptake after 3 hours[3]	19.1 ± 1.0	88.2 ± 2.5	< 0.001
„ „ „ 6 „	23.8 ± 1.5	76.6 ± 5.9	< 0.001
„ „ „ 9 „	25.9 ± 1.7	62.0 ± 7.6	< 0.001
„ „ „ 24 „	24.8 ± 1.7	46.4 ± 7.0	< 0.02
Intrathyroidal *I distribution[4]			
Origin	3.9 ± 0.2	8.2 ± 1.3	< 0.01
MIT	21.7 ± 1.1	41.4 ± 2.5	< 0.001
DIT	49.5 ± 1.1	21.2 ± 1.2	< 0.001
I^-	3.5 ± 0.4	3.9 ± 0.2	< N. S.
T_3	1.5 ± 0.1	11.8 ± 1.5	< 0.001
T_4	18.9 ± 0.8	10.6 ± 1.1	< 0.001
MIT/DIT	0.47 ± 0.04	2.0 ± 0.1	< 0.001
T_3/T_4	0.08 ± 0.00	1.11 ± 0.07	< 0.001

[1] Same animals as those in Table 1.
[2] Statistical analysis as in Table 1; N. S. = not significant: $P > 0.05$.
[3] Calculated as % of injected radioactivity.
[4] Determined 24 h after administration of the tracer and calculated as % of *I present on the chromatogram in the corresponding spots.

higher in the LID than in the C group. These alterations in the thyroidal *I distribution are accompanied by changes in the *I distribution in the plasma. Total *I and *I-PBI in the plasma are 8 and 15.2 times higher in the LID than in C groups respectively (Table 1), as would be expected with a faster *I turnover in the gland of the LID animals (Table 2). One interesting finding was that, as in the case of the cold iodine content in the plasma, practically 100 % of the total plasma *I of the LID group is in the form of *I-PBI (94.8 %), while in the C group, it is only 50 % (Table 1).

Growth and growth hormone in the pituitary gland

The profound alterations in thyroid function in the LID rats is not accompanied by a cessation of body weight increase, the final body weight values

being 263 ± 9 g for the LID and 234 ± 5 g for the C group ($P < 0.01$)[1]. The area of the microdensitometer tracing of the pituitary polyacrylamide electrophoresis gels corresponding to the GH-like protein (expressed as % of the area corresponding to the albumin band as 100 %) was 348 ± 43 and 363 ± 31 (N.S.) for LID and C rats respectively. These results agree with those obtained by electromicroscopic studies of the pituitaries of these animals (*Gonzalez et al.*, unpublished observations) which show that in both groups there is a normal number and size of granules in the somatotrophic cells.

Intermediary metabolism

Circulating fuels and insulin. – Separate experiments were performed with rats that were fed and with rats fasted for 48 h. Plasma glucose, total ketone bodies and insulin were not significantly different in the LID and C groups, whether fed or fasted (Table 3). The reaction to fasting was normal in both groups: plasma glucose and insulin fall with fasting while the level of ketone bodies rises.

In vitro alanine-U-^{14}C utilization by liver slices. – Steady state levels of circulating fuels could be normal even if synthesis was altered, as changes could be masked in parallel by concomitant changes in degradation and synthesis. To check whether or not such alterations occur, we have studied the *in vitro* handling of alanine-U-^{14}C by liver slices. Two concentrations of the radioactive compound were used: 70×10^{-6} µM (»tracer«) and 10^{-2} M (»substrate«). These two different concentrations were used to detect and/or correct any differences in the endogenous alanine pools of both groups. The results are summarized in Table 4. As described by other investigators (*Freinkel et al.* 1965), the uptake of alanine-U-^{14}C by the tissue was lower when we use »substrate« concentration of the precursor than when tracers are used. In either case no differences were found between the uptake of the LID and the C animals. The percentage to initial radioactivity (both »tracer« and »substrate«) converted to glucose-^{14}C, lactic acid-^{14}C and $^{14}CO_2$ was not different in the LID and C rats, either fed or fasted. The absence of any difference between the groups is unlikely to be due to the lack of any sensitivity of the technique used, since the response of both groups to fasting was as expected i. e. increased glucose-^{14}C and $^{14}CO_2$ formation and decreased incorporation of the radioactivity into lactic acid-^{14}C.

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[1] As rats had not been weighed initially, but only divided at random into LID or C animals, we do not know whether the slight *increase* in body weight of the LID rats has any significance.

Table 3.
Plasma insulin. glucose and ketones from fed and fasted C and LID rats. Mean ± SEM.

	Fed		*P*	Fasted		*P*[1]
	C	LID		C	LID	
Insulin (μU/ml)	49.0 ± 2.0	46.3 ± 3.6	N. S.	24.2 ± 1.3 ***	27.1 ± 2.9 **	N. S.[2]
Glucose (mg/100 ml)	128.1 ± 5.4	142.0 ± 4.7	N. S.	99.3 ± 5.1 **	96.4 ± 3.6 ***	N. S.
Ketone bodies (μM/l)	203 ± 29	306 ± 116	N. S.	1427 ± 136 ***	1583 ± 186 ***	N. S.

[1] *P* indicates probability that the difference between the C and LID mean values is due to chance. The *P* value corresponding to the difference between fed and fasted groups is shown by asterisks: * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.
[2] N. S. = not significant: $P > 0.05$. Number of animals is 6–8 per group.

Table 4.

In vitro utilization of L-alanine-U-^{14}C by liver slices from fed and fasted C and LID rats. Data are % of initial counts/100 mg of tissue (Means ± SEM).

	Fed		*P*	Fasted		*P*
	C	LID		C	LID	
»Tracer« alanine-U-^{14}C						
Alanine uptake	70.4 ± 4.0	68.9 ± 2.6	N. S.	72.5 ± 5.9 N. S.	59.7 ± 6.9 N. S.	N. S.
CO_2	12.1 ± 1.0	12.0 ± 1.2	N. S.	24.7 ± 3.3 **	18.1 ± 2.6 *	N. S.
Glucose	2.7 ± 0.5	1.7 ± 0.7	N. S.	8.0 ± 0.4 ***	4.9 ± 1.5 N. S.	N. S.
Lactic acid	31.2 ± 4.1	28.3 ± 1.5	N. S.	12.6 ± 1.6 ***	11.6 ± 2.4 ***	N. S.
»Substrate« alanine-U-^{14}C						
Alanine uptake	30.4 ± 4.1	21.7 ± 3.5	N. S.	33.6 ± 4.3 N. S.	33.8 ± 3.5 *	N. S.
CO_2	4.9 ± 0.9	2.9 ± 0.4	N. S.	5.2 ± 0.6 N. S.	4.9 ± 0.6 *	N. S.
Glucose	1.3 ± 0.4	1.2 ± 0.4	N. S.	3.9 ± 0.4 **	3.9 ± 0.6 **	N. S.
Lactic acid	6.8 ± 1.0	6.0 ± 0.9	N. S.	3.3 ± 0.5 *	3.0 ± 0.3 *	N. S.

The significance of the differences between C and LID groups and Fed and Fasted are expressed as in Table 3. Number of animals is 5–7 per group. The alanine uptake represents the % of alanine captured by 100 mg of tissue. CO_2, glucose and lactic acid values represent the incorporation of alanine-U-^{14}C in the media to those parameters.

DISCUSSION

Chronic feeding of the rats with LID resulted in alterations of intrathyroidal and extrathyroidal iodine economy typical of iodine deficiency. Thus, the high proportion of total plasma ^{127}I and *I in the circulation and the high thyroidal *I uptake, followed by a rapid disappearance of the label from the gland, all point to an attempt of the gland to make the most of the inadequate iodine supply. Despite this, normal circulating levels of thyroid hormones are not maintained, as shown by the low plasma PBI. This is accompained by a considerably enlargement of the gland. The very high intrathyroidal *MIT/*DIT and $*T_3/*T_4$ ratios described here are consistent with the very low ^{127}I content of the glands, in agreement with previous findings from this laboratory (*Herrera et al.* 1968; *Lamas & Morreale de Escobar* 1969) and other laboratories (*Lachiver & Leloup* 1955; *Querido et al.* 1957; *Studer & Greer* 1965; *Heninger & Albright* 1966; *Abbassi & McKenzie* 1970). Though not directly measured, it is likely that circulating levels of TSH were increased in these animals: thus it has been found by other investigators (*Studer & Greer* 1965; *Abbassi & McKenzie* 1970) that plasma TSH levels were high in animals which had been on LID and which showed alterations of the intra and extrathyroidal iodine economy similar to those reported here. Thus it can be concluded that chronically inadequate iodine intake in these animals results in an increase in the activity and size of the thyroid representing a compensatory effort to provide the organism with a sufficient supply of the thyroid hormones. But the question arises, has this aim actually been achieved? The supply of thyroid hormones is evidently inadequate to maintain a normal plasma PBI and to avoid thyroid enlargement. Presumably, the latter change has been due to a decrease in the inhibitory effect which normal supplies of thyroid hormones exert on TSH release. However, this does not necessarily mean that the supply of thyroid hormones is too low to maintain other biological parameters in a normal condition, as stressed by the work of *Evans et al.* (1964). In rats which are intensely »hypothyroid«, that is, deprived intensely of thyroid hormone from a very early age, striking alterations in growth and development occur, many of which are attributable to an inadequate production and release of most of the adenohypophyseal hormones, TSH excepted (*Griesbach & Purves* 1945; *Contopoulos & Koneff* 1963; *Lazo-Wasen* 1960; *Schooley et al.* 1966) and of other hormones such as insulin (*Malaisse et al.* 1967; *Jolin et al.* 1968; *Escobar del Rey et al.* 1968). The doses of $L\text{-}T_4$ needed to avoid the effect of severe thyroid hormone deprivation (*Evans et al.* 1964) are about 10 times lower than those needed to depress high TSH release rates to normal values (*Purves* 1964). The present rats, fed chronically on LID, did not show the typical effects of severe thyroid deprivation: 1) they appear to have a normal content of a pituitary GH-like protein, as determined by microdensitometry of poly-

acrylamide electrophoresis gels. Although this method might not be sensitive enough to detect small differences, it does clearly show differences between normal and intensely hypothyroid rats (*Jolin et al.* 1968; *Escobar del Rey et al.* 1968; *Nicoll et al.* 1969; *Jolin et al.* 1970). The absence of a major difference in the pituitary content of a GH-like protein between normal rats and those on LID is consistent with their normal growth and normal number and size of granules in the somatotrophic cells observed in electromicroscopical studies. (*Gonzales et al.*, unpublished observations). 2) The rats on LID show normal levels of plasma insulin, glucose and ketone bodies in both fed and 48 hours fasted conditions. Although we have not studied conditions of maximal stimulation, such as glucuse infusion, we believe that the metabolic challenge provided by 48 hours of fasting should be sufficient to unmask any differences in these parameters, as it does in other situations (*Herrera et al.* 1969). 3) The *in vitro* utilization of alanine-U-^{14}C by liver slices from the rats on LID was similar to that from the controls, both fed and fasted. These parameters are, however, greatly altered (*Castro & Herrera,* unpublished observations) in thyroidectomized rats fed LID and made profoundly hypothyroid. It is possible that maintenance of normal intermediary metabolism requires extremely small amounts of thyroid hormones, as does the maintenance of adenohypophyseal function. Indeed, alterations in the intermediary metabolism of intensely hypothyroid rats may well be secondary to the inadequate functioning of the adenohypophysis and the pituitary-dependent glands of such animals. With regard to the possibility that thyroid hormones do not have a direct effect on intermediary metabolism, it appears relevant to recall the lack of success of the many attempts carried out in different laboratories, our own included (*Castro & Herrera,* unpublished observations) to demonstrate any direct *in vitro* effects of thyroid hormones on parameters of intermediary metabolism.

On the other hand, it is possible that, despite the very low plasma PBI of the rats chronically fed on LID, the amounts of T_3 available to the peripheral tissues are higher than in C rats (*Heninger & Albright* 1966), compensating for the possible metabolic disturbances due to the low circulating levels of T_4 and thus the »lack of thyroid hormones« in these animals is actually not very severe.

In conclusion, the present results show that a prolonged iodine deficient intake results in an intense alteration in iodine economy, accompanied by compensatory efforts by the thyroid to provide the body with as much thyroid hormone as possible in the face of the low availability of iodine. No signs of severe thyroid hormones deprivation were observed in these animals. In this situation several parameters of intermediary metabolism were found to be unaltered, as compared to rats on an adequate iodine supply. It would appear that the maintenance of a normal intermediary metabolism requires smaller amounts of thyroid hormones than needed for the suppression of TSH

release by the pituitary. Whether or not the amounts required for maintenance of normal intermediary metabolism are only slightly lower, or as low as those needed for the maintenance of adenohypophyseal functions is currently being studied. Present results support the concept that low PBI levels and a markedly increased thyroid size and iodine turnover should not necessarily be equated with »hypothyroidism« when induced by iodine deficiency, if this term is used to indicate that total thyroid hormonal production does not meet most body requeriments. They once more draw attention to the variations in sensitivities to the thyroid hormones of several biological responses (*Evans et al.* 1960), intermediary metabolism included. A parallel to the present experimental situation may be found in human subjects adapted to situations of extreme iodine-deficiency, but who show no clinical signs of »hypothyroidism«, despite elevated plasma TSH levels (*Adams et al.* 1968).

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