DEPRESSED RNA SYNTHESIS IN THE BRAINS AND LIVERS OF THYROIDECTOMIZED, NORMAL AND HORMONE INJECTED RATS

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INTRODUCTION

Neonatally thyroidectomized rats incorporate *in vivo* injected amino acids into brain protein at lower rates than do normal control animals[2,7,12] Activities of a number of brain enzymes are also reduced in such hypothyroid rats[10,11,17,23] Early administration of thyroxine is often effective in restoring brain enzyme activity and protein synthesis to normal levels[12,17,23] Excellent reviews of the above aspects have appeared[6,14,23]

Two studies have recently appeared comparing brain RNA synthesis in hypothyroid and normal rats judged by rates of incorporation of *in vivo* injected orotic acid, a pyrimidine precursor, into brain RNA In one, Balázs *et al* [3] found no differences in the normal and hypothyroid animals, but did report a 10% reduction in rate of liver RNA synthesis in the latter, suggesting for brain, an effect of thyroid hormones on protein synthesis rather than at the transcriptional level Geel and Timiras[13] reported partial agreement with Balázs, but in contrast demonstrated an increased turnover of pyrimidine nucleotides in the cortices of hypothyroid rats

It seems unlikely to us that the effects of thyroid hormones are confined to brain cortex translational processes, since cortices of hypothyroid rats have lower RNA/DNA ratios than normal control animals[3,13,23] Moreover, recent reports[10,25] suggest a probable effect at the transcriptional level in 10-day-old hypothyroid rat brain A depressed RNA synthesis was also shown in rat brain stem following hypophysectomy[15] Thus neural tissue can respond to some hormonal alterations, by changed rates of RNA synthesis, in qualitatively the same fashion as peripheral organs such as liver[29] It is known that neonatal thyroidectomy results in altered brain vascular patterns[9] The entry of amino acids into brain tissue is also significantly changed[10,28] as is the turnover of RNA[13] in the hypothyroid rat brain The use of *in vivo* injected precursor substances to determine synthetic capacity, in response to hormonal alteration, when subject to such complications may assess the sum of all processes rather than specifically estimate synthetic capacity Such difficulties have been encountered by others and discussed[1,4,18] The present study compares RNA and nuclear protein

synthetic capacity in normal and thyroidectomized rats using *in vitro* techniques which allow control of the specific activity of the immediate precursor substances at the reaction site and assure saturation of the enzymatic apparatus with substrate The results obtained here do show depressed brain RNA synthesis and a larger depression of liver RNA synthesis than seen by others[3] using *in vivo* injection of orotic acid

MATERIALS AND METHODS

Chemicals

ATP, CTP, GTP, UTP, yeast RNA, salmon sperm DNA, 2-phosphoenol pyruvic acid, pyruvate kinase (rabbit muscle), ribonuclease 5× cryst (bovine pancreas), spermidine trihydrochloride, sucrose, and Tris-(hydroxymethyl)-aminomethane (all A grade) were obtained from the California Biochemical Corp (Los Angeles, Calif) KCl and $MgCl_2$ from the Sigma Chemical Company Deoxyribonuclease I (bovine pancreas) electrophoretically pure was from the Worthington Biochemical Corporation (Freehold, N J) ^{3}H-GTP (spec act 100 mCi/mmole) was purchased from the Schwartz/Mann Biochem Co [4,5-^{3}H]leucine (spec act of 5 Ci/mmole) was purchased from New England Nuclear Corp (Boston, Mass) Sodium L-thyroxine was supplied by the Smith, Kline and French Laboratories

Experimental hypothyroidism

Long–Evans rats born to mothers maintained on a low iodine diet (0 17 μg I_2/g diet) were radiothyroidectomized one day after birth by injection of 100 μCi of carrier-free ^{131}I in accordance with the procedure of Goldberg and Chaikoff[16]

Replacement therapy

Commencing on the 6th day after birth, groups of hypothyroid rats received daily replacement therapy of either sodium L-thyroxine (10 μg/100 g body weight) or of bovine growth hormone[24] (100 μg per rat) Other hypothyroid and normal control groups were injected intraperitoneally with a similar volume of the alkaline vehicle used to dissolve the hormones

Symbols used for various animal groups

Normal — intact offspring of pregnant mothers fed a low iodine diet

$\bar{T}$ (thyroidectomized) — offspring of mothers on low iodine diet injected with 100 μCi of ^{131}I/rat at one day of age

$\bar{T} + T_4$ (thyroidectomized, thyroxine injected) — animals of the thyroidectomized group injected intraperitoneally with 10 μg of sodium L-thyroxine/100 g body weight daily from 6th day of age

$\bar{T}$ + GH (thyroidectomized, growth hormone injected) — animals of the

thyroidectomized group injected intraperitoneally with growth hormone 100 μg/rat daily from 6th day of age

Isolation of brain and liver nuclei

Rats were sacrificed by decapitation, brains and livers were removed and dissected free of the larger blood vessels and connective tissue Brain cortices and diencephalons were pooled, minced and then homogenized manually in a Dounce homogenizer (pestle A) Livers were minced and thereafter homogenized in an electrically driven homogenizer Ice-cold 0 25 *M* sucrose containing 0 05 *M* Tris buffer pH 6 9, 0 025 *M* KCl and 0 005 *M* $MgCl_2$ (referred to as TKM) was used for homogenization 1/5 (w/v) A crude nuclear fraction was isolated by centrifugation (2000 $\times$ *g* for 15 min) through a two layer discontinuous sucrose gradient of 0 25 *M* and 0 40 *M* sucrose containing TKM The supernate containing mitochondria, microsomes and polysomes was discarded The crude nuclear pellet was resuspended in 0 25 *M* sucrose TKM Hypertonic sucrose 2 3 *M* (TKM) was added to a final sucrose concentration of 1 65 *M* and samples were mixed by repeated inversion Ten ml of 1 8 *M* sucrose TKM or 2 0 *M* sucrose TKM were added as an underlay in brain and liver samples respectively and centrifugation of these two layer discontinuous sucrose gradients at 37,000 $\times$ *g* for 45 min was carried out yielding a purified nuclear pellet These nuclear pellets were suspended in 0 25 *M* sucrose and observed by oil immersion light microscopy subsequent to methyl green staining The nuclei appeared intact and the brain nuclear preparations were similar to those described by Sporn *et al*[27] Both neuronal and glial nuclei were evident, exhibiting the morphological characteristics described by Rappoport *et al*[26] Aliquots of these nuclear suspensions in 0 25 *M* sucrose were subsequently used to study nuclear RNA and protein synthesis

Determination of nucleic acid concentration

RNA and DNA were extracted from the nuclear suspensions by procedures described by Munro[22] RNA concentration was determined from the spectral absorbance at 260 nm after alkaline hydrolysis and acid reprecipitation Yeast RNA was treated similarly and used as a standard DNA concentration was determined using the diphenylamine reaction as modified by Burton[5] Salmon sperm DNA was used as a standard

Incubation and determination of radioactivity

Aliquots of the nuclear suspensions were added to chilled tubes containing incubation medium [^{3}H]Leucine or ^{3}H-GTP was added and the tubes were incubated at 37°C for 10 min (RNA assay) or 20 min (nuclear protein synthesis) RNA assays were done in triplicate using the complete assay mixture Duplicate tubes containing no exogenously added nucleoside triphosphates other than the one radioactive triphosphate were incubated along with the three samples of the complete mixture Any ra-

dioactivity observed in the incomplete system was subtracted from that seen in the complete system RNA synthesis was terminated by adding 5 ml of ice-cold 3 5% perchloric acid containing 0 1 *M* sodium pyrophosphate These acid-precipitated samples were left in an ice bath for 15 min and then collected on glass fiber filters (GF/C) Assays of nuclear protein synthesis were conducted in a total of 5 tubes containing the specified incubation medium Two tubes were used as blanks, acid being added at zero time (5 ml of 0 5 *N* perchloric acid containing 1 mg/ml of non-radioactive L-leucine) Reactions were terminated by addition of similar acid solutions used for the blank tubes These acid-precipitated samples containing radioactive protein were left in an ice bath for 30 min and then heated at 90°C for 30 min The heated samples were cooled and collected on glass fiber filters These filters containing radioactive RNA or protein were now washed 5 times with cold 1 *M* HCl, twice with cold 95% ethanol and once with ether Radioactivity was counted (after hyamine solubilization) by liquid scintillation at 37% efficiency in a toluene system containing PPO (2,5 diphenyloxazole) and dimethyl POPOP (1,4-bis[2-(4-methyl-5-phenyloxazole)]-benzene)

Assay concentrations

In vitro synthesis of rat brain RNA (after Dutton and Mahler[8], with slight modification) μMoles/ml (total volume 1 0 ml) Tris buffer pH 8 5 (100), spermidine 3 HCl (2), phosphoenol pyruvate (2), pyruvic kinase 2 5 E U Mg^{2+} (8), sucrose (100), ATP, CTP, UTP (0 2) and ^{3}H-GTP (0 02), 1 μCi

In vitro liver RNA synthesis was determined using an assay system described by McGregor and Mahler[20]

μMoles/ml (total volume 1 0 ml) ATP, UTP, GTP and CTP (0 05) 1 μCi of ^{3}H-GTP Tris (50) pH 8 5, Mg^{2+} (1 5) NaF (8), phosphoenol pyruvate (2 5), pyruvic kinase 2 5 E U and spermidine (5 6)

In vitro nuclear protein synthesis brain and liver μMoles/ml (total volume 1 0 ml) L-[^{3}H]leucine (0 011), 1 0 μCi, sodium phosphate pH 6 (100), phosphoenol pyruvate (2), pyruvic kinase 2 5 E U , $MgCl_2$ (2), and sucrose (100)

RESULTS

Body and organ weights

Table I presents representative body, liver and brain weights of 45-day-old female rats subjected to hormonal manipulations (see Methods) Body, liver and brain weights were significantly reduced in the thyroidectomized and in the thyroidectomized + growth hormone group ($P < 0\ 005$) relative to the normal or the thyroidectomized + thyroxine group Although the weights of the thyroidectomized group receiving daily growth hormone (100 μg) were significantly greater than the thyroidectomized group, they were much below the normal or the thyroxine supplemented animals The minimal effective dose of this growth hormone preparation was reported as 10 μg with respect to the Tibia test[13] There were no significant dif-

TABLE I

BODY AND ORGAN WEIGHTS OF HORMONALLY ALTERED AND NORMAL RATS

The thyroidectomized group receiving thyroxin injections and the group of normal controls were not significantly different from one another All other experimental groups were significantly different from one another in terms of body, liver and brain weight ($P < 0.005$) based upon comparison by Student's t-test Values are means ± S E M Numbers between parentheses indicate number of animals

*Female rats*** *(45-day-old)*	*Experimental groups**			
	Normal	$\bar{T}$	$\bar{T} + T_4$	$\bar{T} + GH$
Body weight	118 2 ± 2 6 (48)	37 4 ± 0 92 (37)	110 9 ± 6 7 (8)	57 8 ± 1 9 (18)
Liver weight	5 39 ± 0 32 (8)	1 09 ± 0 05 (8)	5 58 ± 0 41 (8)	2 10 ± 0 13 (15)
Brain weight	1 642 ± 0 047 (8)	1 285 ± 0 027 (9)	1 674 ± 0 068 (8)	1 442 ± 0 021 (15)

* Experimental groups are as defined in the Methods section
** Weight in grams

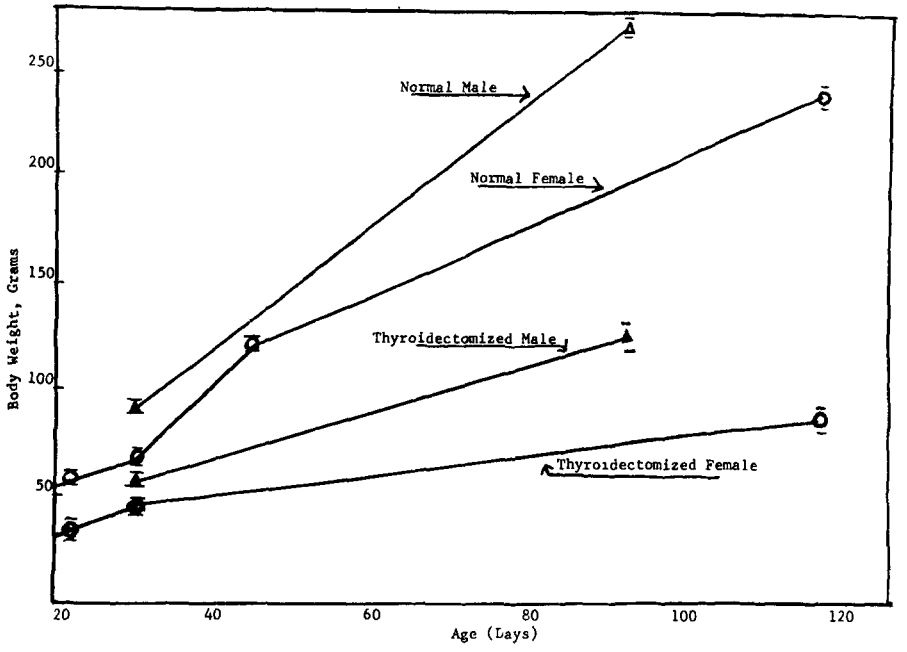

Fig 1 Effect of neonatal thyroidectomy on body weight increase of male and female rats Means ± S E M are shown for at least 15 animals at each time interval

ferences between normal controls and the thyroidectomized thyroxine supplemented animals, thus indicating that the effects attributed to thyroxine lack were not due to radiation damage The effects of thyroid deprivation on body weight appeared generally independent of and were of similar magnitude in rats of either sex (Fig 1) Body weight differences between normal and the thyroidectomized groups intensified as the animals became older Thyroidectomized animals receiving thyroxine re-

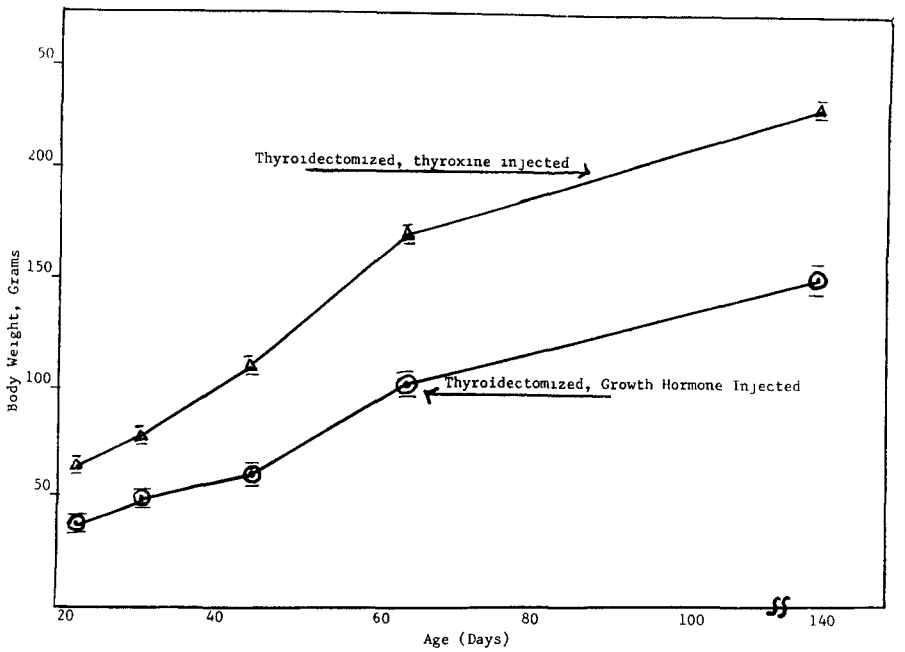

Fig 2 Effect of neonatal thyroidectomy and replacement therapy on body weight increase of female rats Means ± S E M are shown for at least 10 animals at each time interval

TABLE II

EFFECTS OF DIFFERENT COMPONENTS AND INHIBITORS ON INCORPORATION

The complete system contains Tris buffer (pH 8 5) 100 m*M*, spermidine 2 m*M*, 2-phosphoenol pyruvic acid 2 m*M*, pyruvate kinase 2 5 E U , $MgCl_2$ 8 m*M*, sucrose 100 m*M*, ATP, CTP, UTP 0 2 m*M*, and ^{3}H-GTP 0 5 μCi (spec act 100 mCi/mmole) Nuclear DNA 100 μg all in a total volume of 1 0 ml Eliminated components were replaced with an equal volume of glass distilled water Values are the means of triplicate determinations of a typical experiment Further details are given in the Methods section

Incubation mixture (additions or deletions)	*Specific radioactivity (disint /min/100 μg DNA)*	*Per cent of complete system*
Complete system	1061	100
Minus ATP, UTP and CTP	76	7
Minus phosphoenol pyruvate and pyruvate kinase	517	49
Minus spermidine	494	47
Minus Mg^{2+}	625	59
Plus (8 m*M*) NaF	1125	106
Plus (8 m*M*) NaF and (70 m*M*) KCl	1617	152
Plus (8 m*M* NaF, 70 m*M* KCl) and 100 μg salmon sperm DNA	1618	152
Plus 60 μg actinomycin C	111	11
Plus (20 m*M*) sodium pyrophosphate	66	6
Plus 300 μg Pan RNAse*	50	5
Plus 300 μg DNAse*	58	5
Complete system*	1100	104

* Ten min preincubation at 37°C

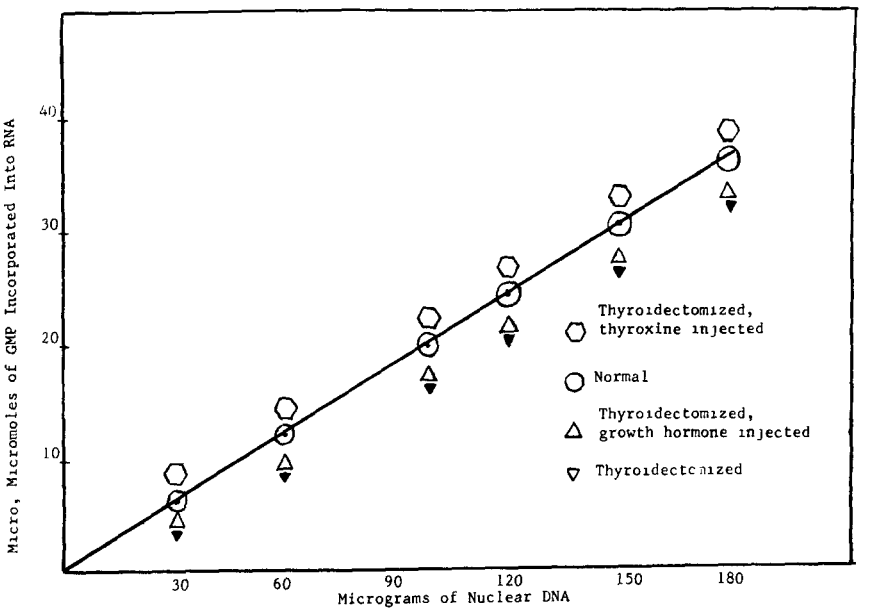

Fig 3 Incorporation dependence upon concentration of brain nuclei of normal and hormonally altered rats Each point represents the mean of single assays in triplicate

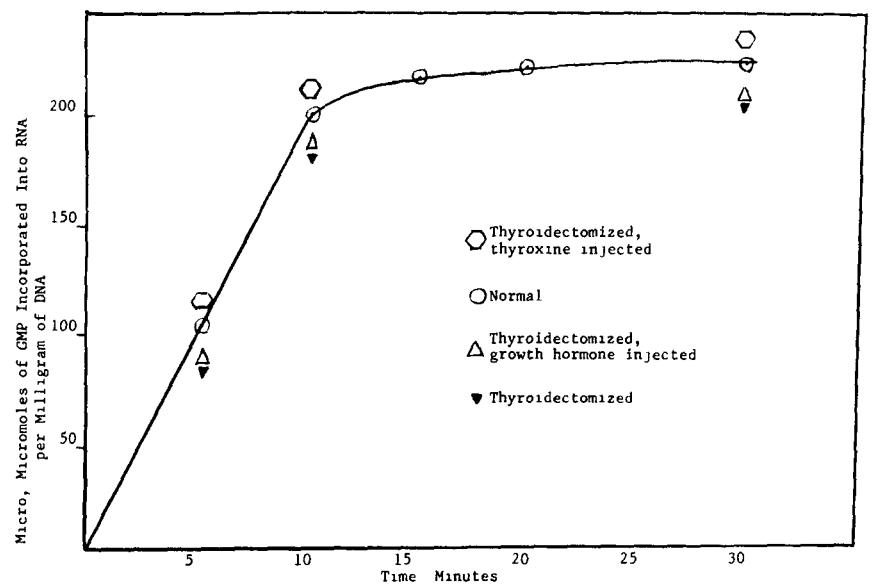

Fig 4 Time dependence of incorporation using brain nuclei from normal or hormonally altered rats Each point shown represents the mean of a single assay in triplicate

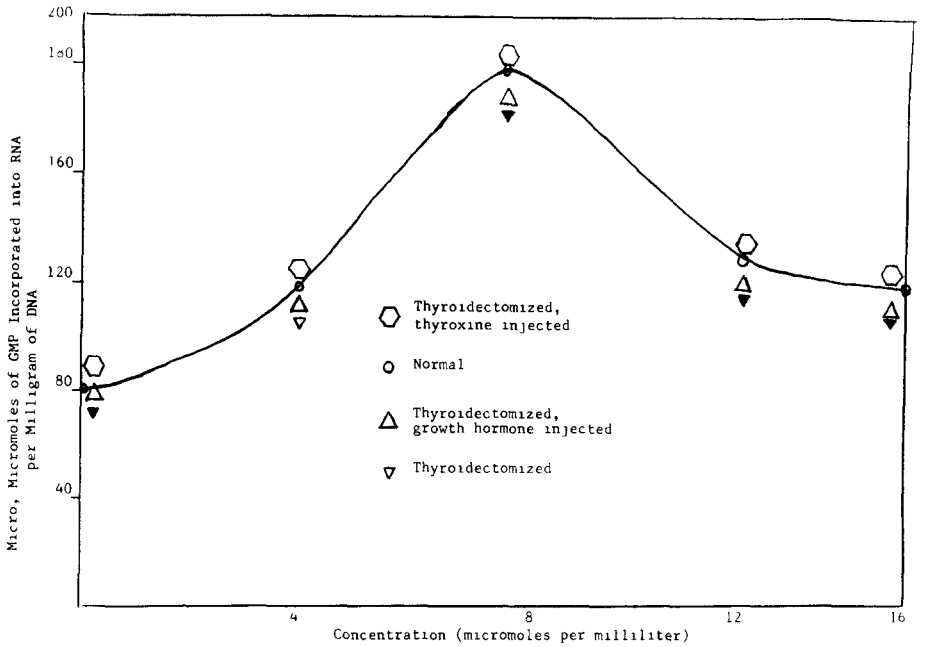

Fig 5 Effect of Mg^{2+} concentration on *in vitro* incorporation of ^{3}H-GMP into RNA Points represent means of single assays in triplicate

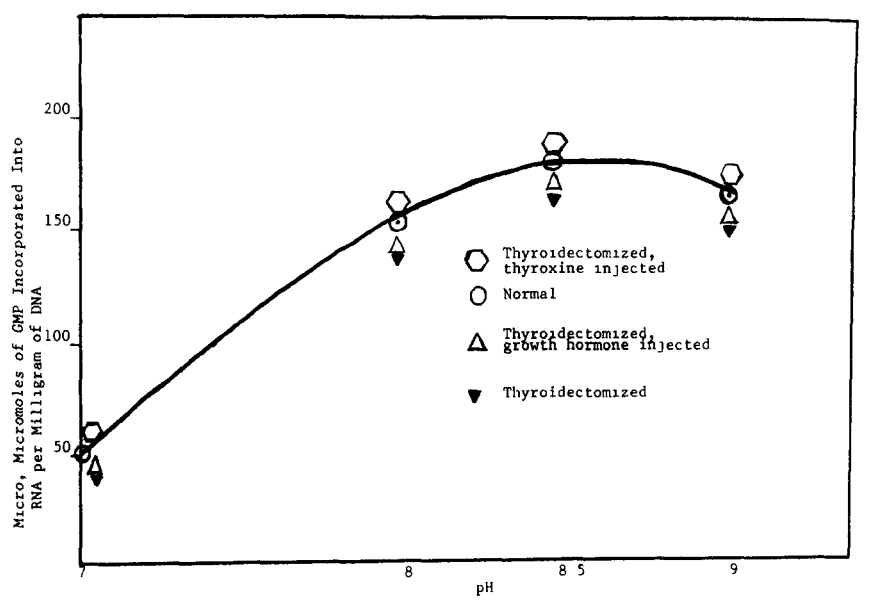

Fig 6 Effect of pH on *in vitro* incorporation of ^{3}H-GMP into RNA for 10 min at 37°C Incubation assay and conditions are described in the Methods section Points represent means of single assays in triplicate

placement therapy were much larger than a similar group receiving growth hormone at all ages shown (Fig 2)

Table II shows the effects of assay components and inhibitors upon the extent of incorporation of ^{3}H-GTP into RNA using rat brain cortex and diencephalic cell nuclei of normal rats No significant changes in the patterns of response to these chemical components or inhibitors were seen using nuclei derived from any of the experimental groups

Brain nuclei isolated from groups of thyroidectomized and normal rats had identical RNA/DNA ratios of 0 41 (unpublished observations) Maximal *in vitro* synthesis of RNA (Table II) required the presence of all 4 nucleoside triphosphates, a divalent cation, DNA template, spermidine and an ATP generating system The synthesized product was acid precipitable and hydrolyzable by either RNAse or by exposure to alkaline conditions (0 3 *N* KOH, 37°C, 60 min) These characteristics are typical of this DNA-dependent RNA polymerase system[8]

Rates of *in vitro* brain RNA synthesis were a linear function of nuclear DNA concentrations for all of the nuclear preparations studied (Fig 3) RNA synthesis proceeded linearly for 10 min, after which time no further significant synthesis occurred (Fig 4) Maximal synthesis was obtained at a Mg^{2+} concentration of 8 m*M* (Fig 5) and at a pH close to 8 5 (Fig 6)

Increasing brain RNA synthesis was seen with increasing GTP concentration (constant nuclear DNA, 100 μg) up to 0 01 m*M* GTP (unpublished observations) Maximal synthesis of RNA (Fig 7) was obtained at far lower nucleoside triphosphate concentrations when the incorporation system contained the ATP generating

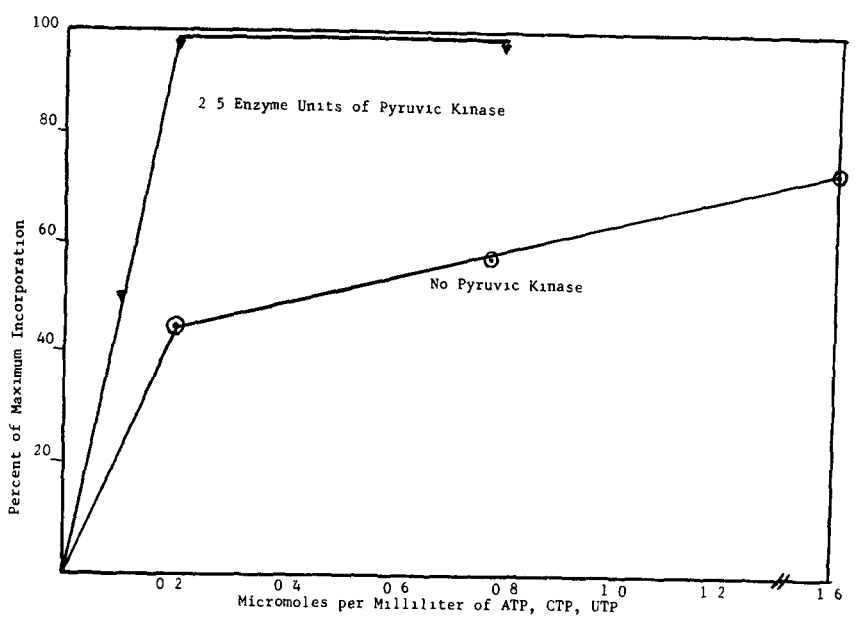

Fig 7 Effect of increasing nucleoside triphosphate concentration with constant ^{3}H-GTP (0 3 μCi and 3 9 10^{-4} m*M*) on incorporation into RNA in the presence and absence of an ATP generating system Normal brain nuclei 100 μg of DNA were used, each point is the mean of a single assay in triplicate

TABLE III

In vitro RNA SYNTHESIS USING ISOLATED BRAIN NUCLEI OF NORMAL AND HORMONALLY ALTERED RATS

The components of the assay mixture are Tris buffer (pH 8 5) 100 m*M*, spermidine 2 m*M*, 2-phosphoenol pyruvic acid 2 m*M*, pyruvate kinase 2 5 E U , $MgCl_2$ 8 m*M*, sucrose 100 m*M*, ATP, CTP, UTP 0 2 m*M* and ^{3}H-GTP 0 02 m*M*, 1 0 μCi Total volume 1 0 ml All probability values given represent the results of a *t*-test for paired samples, data are means ± S E M Numbers in parentheses indicate number of assays Each assay was done in triplicate using two brains

Group	*pMoles of GMP incorporated into RNA/mg of DNA (incubated 10 min at 37°C)*			
Female rats (22–25 days old)	Normal female	192 3 ± 13 4 (10)	$\bar{T} + T_4$*	179 5 ± 7 1 (4)
		$P < 0\ 001$		$P < 0\ 01$
	$\bar{T}$* female	162 ± 12 6 (10)	$\bar{T}$ + GH*	149 8 + 12 3 (4)
Male rats (90 days old)	Normal male	190 8 ± 13 (5)		
		$P < 0\ 01$		
	$\bar{T}$* male	162 2 ± 10 2 (5)		

* Symbols defined in Methods

system These characteristics were similar in all of the brain nuclear preparations studied

The amounts of RNA synthesized per unit DNA in each nuclear preparation are presented in Table III Cerebral and diencephalic nuclei of thyroidectomized rats (males and females) exhibited a 16% depression of *in vitro* RNA synthesis compared to nuclei of intact control rats ($P < 0\ 001$ and $P < 0\ 01$) respectively (Table III) Older male rather than female rats were studied to avoid the possible complications resulting from cyclic activity (in female rats) of hypothalamic releasing factors and anterior pituitary hormones which might also influence the parameters studied here Nuclei from thyroidectomized rats injected i p with thyroxine (10 μg of sodium L-thyroxine/100 g body weight) show greater RNA synthesis (17%, $P < 0\ 01$) than a paired thyroidectomized group injected with massive levels of bovine growth hormone (100 μg/day) Brain RNA synthesis in the group of thyroidectomized rats receiving thyroxine was not significantly different from that seen in normal intact females but the thyroidectomized group receiving growth hormone exhibited 22% ($P < 0\ 01$) less RNA synthesis than the normal female group (Table III) Statistical comparisons between the normal female, $\bar{T} + T_4$ and the $\bar{T}$ + GH group were made using Student's *t*-test, these were not treated as paired samples since nuclei were not simultaneously prepared and assayed

No significant changes in brain nuclear protein synthesis were observed in any of the groups shown in Table III Brain nuclei derived from all groups showed an average incorporation of 40 pmoles of leucine/mg of nuclear DNA

Liver nuclei derived from 90-day-old male rats were used to study *in vitro* synthesis of RNA and protein (Table IV) This liver RNA assay system was used by McGregor and Mahler[20] to study RNA synthesis in liver nuclei following hormonal treatments Incorporation into hepatic RNA (Table IV) was here strictly linear for

TABLE IV

In vitro RNA AND PROTEIN SYNTHESIS IN ISOLATED RAT LIVER NUCLEI

The components of the liver RNA assay mixture are ATP, UTP, CTP and GTP 0 05 m*M* with 1 Ci of ^{3}H-GTP, Tris buffer (pH 8 5) 50 m*M*, $MgCl_2$ 1 5 m*M*, NaF 8 m*M*, 2-phosphoenol pyruvate 2 5 m*M*, pyruvate kinase 2 5 E U and spermidine 5 6 m*M* Total volume 1 0 ml The components of the liver nuclear protein synthesis assay mixture are L-[^{3}H]leucine 0 011 m*M* (1 μCi), sodium phosphate (pH 6) 100 m*M*, 2-phosphoenol pyruvate 2 m*M*, pyruvate kinase 2 5 E U , $MgCl_2$ 2 m*M* and sucrose 100 m*M* Probability values represent the results of a *t*-test for paired samples Values are means ± S E M Numbers in parentheses indicate number of assays, each in triplicate

*Group**	*RNA synthesis (pmoles of GMP into RNA in 5 min 37°C, per mg of DNA)*	*Protein synthesis (pmoles of leucine into protein in 20 min 37°C, per mg of DNA)*
Normal	252 8 ± 33 5 (4)	19 3 ± 4 6 (7)
	$P < 0$ 01	not significantly different
T	142 3 ± 39 3 (4)	25 2 ± 3 8 (7)

* 90-day-old male rats were used, symbols defined in Methods section

5 min (data not shown) for both normal and thyroidectomized rat liver nuclei Nuclei isolated from livers of hypothyroid rats showed 43% less RNA synthesis per unit DNA ($P < 0$ 01) than normal control rat liver nuclei, again no significant detectable changes in hepatic nuclear protein synthesis were observed

DISCUSSION

These studies demonstrate depressed levels of brain and liver RNA synthesis subsequent to neonatal radiothyroidectomy Chronic administration of physiological levels of thyroxine restored brain RNA synthesis to normal while similar administration of massive levels of growth hormone was ineffective, suggesting a degree of hormonal specificity of responsiveness Krawiec *et al* [19], in contrast, found growth hormone at least as effective as triiodothyronine in restoring the brain weight, the DNA and RNA of neonatally thyroidectomized rats to normal levels Geel and Timiras[13], however, reported growth hormone ineffective in the latter regards Nuclear protein synthesis (both brain and liver) appeared unaffected by thyroidectomy as studied here Since only total nuclear RNA and total acid-precipitable nuclear protein were studied, rather than specific classes of RNA and nuclear protein, it may be that changes in specific classes of RNA or nuclear protein went undetected because the altered component represented a very small portion of the total *in vitro* synthesis

These reductions of *in vitro* synthesis of brain or liver RNA could conceivably be the result of an altered permeability to RNA precursors at the nuclear membrane or at intranuclear synthetic sites This is deemed unlikely since nuclei from both normal and hormonally altered rat brain showed no significant differences in the extent of stimulation of RNA synthesis when all 4 nucleoside triphosphates were added to the incomplete assay system containing one of the four (GTP) These results can also be caused by an enhanced nuclease content or the presence of inhibitory factors in the hypothyroid rat brain nuclear fraction However, mixing experiments (unpublished

studies) did not suggest this to be the case since mixing normal brain nuclei and nuclei from thyroidectomized rats always resulted in an incorporation which was equal to the sum of each, when studied separately This was true even when the intact nuclei were totally disrupted allowing for the mixing of possible nuclease or inhibitory factors Alterations of template efficiency or a reduced activity of the RNA polymerase enzyme do appear likely explanations Although exact mechanisms remain undefined the developmental responses mediated by thyroxine in both rat brain and liver do involve the synthesis of RNA Gispen *et al* [15] showed depressed levels of all classes of RNA in rat brain stem following hypophysectomy Hormones may then act as metabolic regulators in brain as in peripheral organs by changing RNA synthesis These hormonal controls would appear to apply to postulates discussed by McIlwain[21] suggesting brain metabolic adaptation in response to inducing agents requiring a changed synthesis of RNA These results also suggest that thyroid hormone fulfills a role in developing mammalian brain as a differentiating agent and which does have an influence on RNA synthesis This influence upon RNA synthesis may in turn serve a regulatory role in brain protein synthesis and the development of a mature pattern of brain enzyme synthesis

SUMMARY

Depression of rat brain and liver RNA synthesis was seen at 22 and 90 days subsequent to neonatal radiothyroidectomy Cerebral RNA synthesis (per unit DNA) was depressed by 16% ($P < 0.001$) while hepatic RNA synthesis was reduced by 43% ($P < 0.01$) Nuclear protein synthesis was not detectably altered RNA and protein synthesis determinations were conducted, using isolated nuclei in standardized *in vitro* assay systems which circumvented complications (often seen *in vivo* approaches) such as pool size changes, cell membrane transport alterations and mediation of blood–brain barrier related effects

Chronic daily administration *in vivo* of L-thyroxine (10 μg/100 g body weight) restored the depressed cerebral synthesis of RNA to normal levels while daily administration of bovine growth hormone (100 μg per rat) did not

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