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Amino Acid Uptake in the Developing Chick Embryo Heart

THE EFFECT OF INSULIN ON GLYCINE AND LEUCINE ACCUMULATION

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1. The accumulation of [1-^{14}C]glycine and the uptake, accumulation, incorporation (into protein, lipid, glycogen) and oxidation of L-[1-^{14}C]leucine in 5-day-old chick embryo hearts were investigated *in vitro*, and the effects of insulin, puromycin and 4-methyl-2-oxopentanoic acid on these processes were studied. 2. With glycine, the ratio of concentration of the labelled amino acid in the cell water to that in medium markedly exceeded unity. Insulin significantly increased this ratio. Puromycin did not prevent the insulin effect. 3. With leucine, the concentration ratio of the labelled amino acid between intracellular and extracellular water approached unity in the absence of puromycin and was doubled by its presence. In neither case did insulin substantially alter this ratio. The addition of 4-methyl-2-oxopentanoic acid had no effect in the absence of insulin, but produced a significant increase of the concentration ratio in the presence of the hormone. 4. Leucine uptake was increased slightly by insulin in all experimental conditions except in the presence of puromycin, where a more pronounced stimulation was observed. The hormone had no effect on the incorporation of the labelled amino acid into protein, but accelerated its oxidation to carbon dioxide; the latter effect was particularly evident in the presence of puromycin and disappeared after the addition of 4-methyl-2-oxopentanoic acid.

In the preceding paper (Guidotti *et al.* 1968) evidence was presented demonstrating that insulin stimulated the intracellular accumulation of α-aminoisobutyric acid by chick embryo hearts under a variety of experimental conditions. In rat diaphragm and heart, in which a similar effect of the hormone on non-utilizable amino acids had been reported (Kipnis & Noall, 1958; Manchester & Young, 1960; Manchester & Wool, 1963), insulin was found to enhance the intracellular concentration of only a few naturally occurring amino acids, e.g. glycine, methionine, proline, hydroxyproline, serine and threonine (Manchester & Young, 1960; Akedo & Christensen, 1962; Wool, 1964; Scharff & Wool, 1965). However, in the presence of sufficient puromycin to abolish protein synthesis, insulin increased the accumulation of most, if not all, natural amino acids in isolated rat diaphragm (Wool, Castles & Moyer, 1965) and perfused rat heart (Scharff & Wool, 1965).

The purpose of the present work was to investigate the effect of insulin on the transfer and accumulation of natural amino acids by 5-day-old chick embryo hearts and to define the experimental conditions under which this effect could be demonstrated. Glycine and L-leucine were selected for this study because they possess opposite properties with regard to their accumulation in muscle tissues: the accumulation of the former is stimulated by insulin (Manchester & Young, 1960; Manchester & Wool, 1963), whereas the accumulation of the latter is either unaffected or depressed by the hormone (Akedo & Christensen, 1962; Wool, 1964; Scharff & Wool, 1965).

Under appropriate conditions, insulin increased the accumulation of both labelled amino acids by the chick embryo hearts.

MATERIALS AND METHODS

Chemicals. The sources for most of the materials used are listed by Guidotti *et al.* (1968). The [1-^{14}C]glycine and the L-[1-^{14}C]leucine were obtained from The Radiochemical Centre (Amersham, Bucks.); unlabelled glycine and L-leucine were from British Drug Houses Ltd. (Poole, Dorset). 4-Methyl-2-oxopentanoic acid (α-ketoisocaproic acid) was purchased from Sigma Chemical Co. (St Louis, Mo., U.S.A.). Chromatographic-grade butan-1-ol, acetic acid, chloroform, methanol, ethanol and ninhydrin were

from E. Merck A.-G. (Darmstadt, Germany); Hyamine hydroxide 10-X and thixotropic gel powder (Cab-O-Sil) were from Packard Instrument Co. Inc. (Downers Grove, Ill., U.S.A.).

Tissue preparation and incubations. Hearts from 5-day-old chick embryos were prepared and preincubated in Krebs–Henseleit bicarbonate buffer as described by Guidotti *et al.* (1968). Fifteen hearts were then blotted on acid-hardened Whatman filter paper (no. 50), quickly weighed on a Cahn electrobalance and incubated for either 30 or 90 min. in 2 ml. of Krebs–Henseleit bicarbonate buffer, pH 7·4, containing 8 mM-glucose and the amino acid under study: 0·25 mM- or 2 mM-[1-^{14}C]glycine, 0·2 mM- or 2 mM-L-[1-^{14}C]leucine. The specific activity of the amino acid was always adjusted to maintain the radioactivity of the medium between 1·5 and 2·8 μC/ml. Incubation was at 37·5° in an atmosphere of $O_2 + CO_2$ (95:5). When indicated, insulin (bovine) was added at a concentration of 0·2 unit/ml.; puromycin concentration was 100 μg./ml.; 4-methyl-2-oxopentanoic acid concentration was 2 mM; all these substances were also present in preincubation medium.

Measurement of amino acid accumulation. At the end of the incubation, the hearts were quickly blotted on acid-hardened Whatman filter paper and transferred into open long-necked ampoules containing 2 ml. of aqueous 4 mM solution of unlabelled glycine or L-leucine, preheated at 95°. The ampoules were placed in a boiling-water bath and refluxed for 10 min. Extraction fluids and media (the latter diluted fivefold with aqueous 5 mM solution of unlabelled glycine or leucine) were frozen until chromatographic analysis could be carried out. After thawing and centrifugation, ten 5 μl. samples of each extraction fluid and of (initial and final) medium were spotted on silica-gel-coated polyethylene terephthalate Eastman chromatogram sheets (type K301R2). Chromatograms were developed for 2 hr. at 20° with water-saturated butan-1-ol–acetic acid (4:1, v/v) in the Eastman chromatogram developing apparatus (Eastman Kodak Co., Rochester, N.Y., U.S.A.). Labelled glycine and leucine spots were located either with the aid of marker amino acids run concurrently and sprayed with ninhydrin (0·1%, w/v, in water-saturated butan-1-ol) or directly by means of radioactivity measurements made with a scanner fitted with an adapter for thin-layer chromatograms (model 7201, Packard Instrument Co. Inc.). Zones containing the amino acid under study were cut out with scissors and transferred to counting vials subsequently filled with 10 ml. of a scintillation mixture composed of toluene–methylCellosolve (7:3, v/v), 2,5-diphenyloxazole (0·4%, w/v) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (0·01%, w/v). Within a few minutes the greater part of the radioactive compounds (approx. 60% of leucine and more than 80% of glycine) was eluted into the scintillation fluid and their even distribution was secured by gentle agitation. Moreover, contact with the scintillation mixture rendered the chromatographic-sheet fragments completely transparent, so that their quenching effect was negligible. Radioactivity measurements were performed as described by Guidotti *et al.* (1968). The absolute radioactivities in disintegrations/min. were calculated by the use of internal standards.

The method described above has proved to be as accurate as more conventional procedures of chemical assay, which involve pipetting errors (Guidotti, Borghetti & Loreti, 1966). In a series of measurements on 50 spots from the same sample of labelled glycine the standard deviation from the mean was 1·2% (with a counting error of 1%). Further, the chromatographic separation of the amino acid from tissue extracts and incubation media gave assurance that the calculated intracellular/extracellular concentration ratios for radioactivity reflected the true ratios of distribution of the labelled amino acid between the two compartments.

The means for calculating the extracellular space, the intracellular water volume and the intracellular/extracellular concentration ratios of labelled amino acids were as described by Guidotti *et al.* (1968).

Incorporation of ^{14}C-labelled leucine into protein, lipid and glycogen. At the end of the incubation period: (i) the hearts were removed, blotted and ground in a mortar with 5% (w/v) trichloroacetic acid containing 3 mM-L-leucine (unlabelled) for subsequent protein purification, which was performed by the method of Rabinovitz, Olson & Greenberg (1954) in the presence of bovine plasma albumin as carrier protein; purified protein was dissolved in Hyamine hydroxide; (ii) the hearts were removed, quickly washed in ice-cold Krebs–Henseleit bicarbonate buffer and homogenized in ice-cold chloroform–methanol (2:1, v/v) for subsequent lipid extraction by the procedure of Folch, Lees & Sloane-Stanley (1957); purified lipid was dissolved in the chloroform–methanol mixture; (iii) the hearts were removed, washed in chilled bicarbonate buffer and homogenized at room temperature in 5% trichloroacetic acid containing 2 mg. of unlabelled glycogen (prepared from rat liver)/ml. for subsequent glycogen precipitation and purification by the procedure of Carroll, Longley & Roe (1956); the purified glycogen was dissolved in water. In all of the above procedures care was taken to avoid loss and radioactive contamination of the material to be purified. Samples of protein in Hyamine hydroxide and of lipid in chloroform–methanol were transferred to counting vials containing 10 ml. of a scintillation mixture composed of 0·4% (w/v) 2,5-diphenyloxazole and 0·01% (w/v) 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene in toluene; aqueous samples of glycogen were transferred to vials and dispersed by agitation in 10 ml. of the same mixture supplemented with 4% (w/v) Cab-O-Sil (Gordon & Wolfe, 1960). Radioactivity measurements were performed with a Tri-Carb spectrometer as described by Guidotti *et al.* (1968). Absolute radioactivities in disintegrations/min. were calculated by the use of internal standards.

Conversion of L-[1-^{14}C]leucine into $^{14}CO_2$. In these experiments the hearts were incubated in reaction flasks sealed with a rubber cap through which a small glass ladle was inserted so as to form an elevated centre well (Kontes Glass Co., Vineland, N.J., U.S.A.). Each flask contained 2 ml. of Krebs–Henseleit bicarbonate buffer supplemented with 8 mM-glucose, 0·2 mM-L-[1-^{14}C]leucine and, when indicated, insulin (0·2 unit/ml.), puromycin (100 μg./ml.) and 4-methyl-2-oxopentanoic acid (2 μmoles/ml.). Incubation was at 37·5° in an atmosphere of $O_2 + CO_2$ (95:5) After 90 min., 0·3 ml. of M-Hyamine hydroxide in methanol was injected through the rubber cap into the centre well and 2 ml. of 2 N-HCl into the incubation medium. The incubation was continued for an additional 80 min. and then the Hyamine hydroxide solution was transferred directly to counting vials containing 10 ml. of a scintillation mixture composed of 0·4% (w/v) 2,5-diphenyloxazole and 0·01% (w/v) 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene in

toluene. Radioactivity measurements were performed with a Tri-Carb spectrometer, and absolute disintegrations were calculated by the use of internal standards.

RESULTS

Chick embryo hearts incubated for 90min. in the presence of labelled glycine (0·25 and 2mM) were found to accumulate this amino acid in the intracellular water against a concentration gradient (Table 1). The distribution ratio between intracellular and extracellular water was higher when the initial concentration of the amino acid in medium was lower, as expected for a process subject to saturation. Insulin increased the ratio with either 0·25mM- or 2mM-glycine; however, its effect was more pronounced at the higher than at the lower external concentration of amino acid. Puromycin, added to the medium at a concentration that suppressed protein synthesis almost completely (Guidotti *et al.* 1968), did not appreciably alter the distribution ratio of the labelled amino acid at an initial concentration of 0·25mM, but tended to decrease this ratio at higher glycine concentrations (i.e. under conditions closer to the saturation velocity of the transport system). The latter result and the partial inhibition of the intracellular accumulation of α-aminoisobutyric acid by chick embryo hearts incubated for 90min. in the presence of puromycin (Guidotti *et al.* 1968) are in accord with the observations reported by Elsas, Albrecht, Koehne & Rosenberg (1967) on the effect of this antibiotic on the accumulation of α-aminoisobutyric acid by rat diaphragm; this agreement is further substantiated by the fact that in both rat diaphragm and chick embryo heart the stimulation by insulin of the intracellular amino acid accumulation, though not prevented, was definitely decreased by puromycin (Table 1).

When chick embryo hearts were incubated in the presence of labelled leucine (Table 1), the concentration ratio of the amino acid between intracellular and extracellular water approached unity whether the initial amino acid concentration in the medium was 2mM and the incubation lasted for 30min. or whether the initial concentration was 0·2mM and the incubation lasted for 30 or 90min. These findings indicated that no intracellular accumulation of the labelled amino acid occurred under these experimental conditions. The addition of insulin did not raise the concentration ratio above unity when the concentration of leucine in the medium was 2mM and decreased this ratio at lower concentrations; in 30min. experiments this effect of the hormone was statistically significant ($P < 0·01$). A decrease of the intracellular concentration of leucine in rat hearts on the addition of insulin to the perfusion medium has been reported

Table 1. *Effects of insulin, puromycin and 4-methyl-2-oxopentanoic acid* in vitro *on the accumulation of* [1-^{14}C]*glycine and* L-[1-^{14}C]*leucine by 5-day-old chick embryo hearts*

Fifteen hearts preincubated for 15min. in Krebs–Henseleit bicarbonate buffer containing 8mM-glucose (and the additions described for the incubation medium) were incubated in 2ml. of the same medium supplemented with labelled glycine or leucine in the absence or in the presence of the following additions: insulin, 0·2unit/ml.; puromycin, 100μg./ml.; 4-methyl-2-oxopentanoic acid, 2μmoles/ml. Incubation was at 37·5° in an atmosphere of $O_2 + CO_2$ (95:5). The procedures for amino acid purification and radioactivity measurements are described in the text. The values are means ± S.E.M. with numbers of observations in parentheses.

Amino acid added	Initial concn. (mM)	Incubation time (min.)	Ratio: Concn. of free labelled amino acid in cell water / Concn. of free labelled amino acid in medium					
			Control		Puromycin		4-Methyl-2-oxopentanoic acid	
			No insulin	Insulin	No insulin	Insulin	No insulin	Insulin
[1-^{14}C]Glycine	0·25	90	12·13 ± 0·76 (3)	15·35 ± 0·13 (3)*	13·19 ± 0·43 (3)	15·58 ± 0·67 (3)*	—	—
[1-^{14}C]Glycine	2·0	90	7·33 ± 0·21 (6)	11·98 ± 0·38 (6)‡	5·97 ± 0·35 (3)	8·54 ± 0·47 (3)*	—	—
L-[1-^{14}C]Leucine	0·2	30	1·04 ± 0·03 (4)	0·83 ± 0·02 (4)†	—	—	—	—
L-[1-^{14}C]Leucine	0·2	90	1·17 ± 0·08 (6)	1·09 ± 0·08 (6)	2·34 ± 0·14 (4)	2·32 ± 0·14 (4)	1·19 ± 0·01 (8)	1·71 ± 0·08 (8)‡
L-[1-^{14}C]Leucine	2·0	30	0·99 ± 0·03 (9)	0·99 ± 0·04 (9)	1·66 ± 0·03 (3)	1·64 ± 0·01 (3)	—	—

* $P < 0·05$ as compared with 'no insulin'.
† $P < 0·01$ as compared with 'no insulin'.
‡ $P < 0·001$ as compared with 'no insulin'.

by Scharff & Wool (1965). In the presence of puromycin the concentration ratio of the labelled amino acid rose above unity under both of the experimental conditions investigated (0·2mM-leucine, 90min. incubation; 2mM-leucine, 30min. incubation), but in neither case was an effect of insulin detectable. The addition of 2mM-4-methyl-2-oxopentanoic acid to the medium did not alter the values of leucine concentration ratio in the absence of insulin, but disclosed a significant effect of the hormone on this ratio, which increased well above unity ($P < 0·001$ versus control values).

With the intention of investigating the effects of insulin and of other additions on the metabolism of labelled leucine taken up by chick embryo hearts, we made attempts to evaluate the amount of radioactivity that disappeared from medium and then became incorporated into protein, lipid and glycogen or that was converted into carbon dioxide and other metabolic products under the same experimental conditions as those used to study the intracellular accumulation of the free amino acid (Table 2). The disappearance of labelled amino acid from the medium during the incubation period was estimated as the difference between the radioactivity due to leucine (after chromatographic separation) present in the medium at the beginning and at the end of the incubation. For 90min. incubations of the hearts in the presence of L-[1-^{14}C]leucine (initial concentration 0·2mM) the actual amount of labelled amino acid that disappeared from the medium varied with the different experimental conditions. It decreased markedly in the presence of puromycin and moderately in the presence of 4-methyl-2-oxopentanoic acid (as compared with controls). Insulin slightly increased leucine disappearance from control or 4-methyl-2-oxopentanoic acid-supplemented media and definitely enhanced its disappearance in the presence of puromycin.

In control experiments approx. 60% of the radioactivity of leucine that disappeared from medium was recovered in the protein fraction and no more than 6% as free intracellular amino acid; approx. 20% was recovered in carbon dioxide. Negligible quantities of radioactivity were found incorporated into lipids; glycogen, as expected, was essentially free of radioactivity; approx. 10% of the leucine radioactivity that disappeared from medium could not be accounted for. The conversion into carbon dioxide was the sole metabolic reaction stimulated by insulin under these experimental conditions. In fact, the hormone slightly decreased both the incorporation of radioactivity into protein and the amount of radioactivity recovered as intracellular free leucine. When puromycin was added to the incubation medium the radioactivity incorporated into protein decreased to very low levels; conversely, the radioactivity recovered as free intracellular leucine, carbon dioxide and lipid increased markedly. Insulin in the presence of puromycin further enhanced the recovery of radioactivity in carbon dioxide and lipids, but was without effect on the intracellular accumulation of free labelled leucine. In the presence of puromycin

Table 2. *Disappearance from the medium and recovery of radioactivity after incubation of 5-day-old chick embryo hearts with* L-[1-^{14}C]*leucine*

Fifteen hearts (30±1mg. wet wt.) preincubated for 15min. in Krebs–Henseleit bicarbonate buffer containing 8mM-glucose (and the additions described for the incubation medium) were incubated for 90min. in 2ml. of the same medium supplemented with 0·2mM-L-[1-^{14}C]leucine (equal to 6660000disintegrations/min.) in the absence and in the presence of insulin (0·2unit/ml.), puromycin (100μg./ml.) or 4-methyl-2-oxopentanoic acid (2μmoles/ml.). Incubation was at 37·5° in an atmosphere of O_2+CO_2 (95:5). The procedure of purification and radioactivity measurements of the various compounds are described in the text. Values, corrected to a weight of 30mg. of heart tissue, are means of three to eight experiments. Numbers in parentheses in the first line of results represent values of leucine radioactivity that disappeared from the medium expressed as percentages of the total initial radioactivity. The numbers in parentheses after all the other results represent the values of radioactivity recovered or metabolized intracellularly expressed as percentages of the leucine radioactivity that disappeared from the medium in each experimental condition.

	Radioactivity (disintegrations/min.)					
	Control		Puromycin		4-Methyl-2-oxopentanoic acid	
	No insulin	Insulin	No insulin	Insulin	No insulin	Insulin
Leucine disappeared from medium	899100 (13·5)	939060 (14·1)	372960 (5·6)	499500 (7·5)	666000 (10·0)	705960 (10·6)
Intracellular recovery						
Free leucine	50872 (5·6)	46174 (4·9)	104201 (27·9)	101632 (20·4)	53762 (8·1)	77660 (11·0)
Proteins	563573 (62·7)	519113 (55·3)	4057 (1·1)	8077 (1·6)	160874 (24·2)	164508 (23·3)
Lipids	901 (0·1)	1129 (0·1)	2131 (0·6)	2890 (0·6)	770 (0·1)	934 (0·1)
Glycogen	42 (0)	87 (0)	11 (0)	25 (0)	5 (0)	15 (0)
Oxidation to CO_2	185845 (20·7)	283064 (30·2)	245056 (65·7)	358315 (71·7)	259189 (38·9)	263378 (37·3)
Radioactivity not accounted for (% of initial radioactivity that disappeared from the medium)	10·9	9·5	4·7	5·7	28·7	28·3

the fraction of leucine radioactivity that could not be accounted for decreased sharply, suggesting that it might be represented, at least in part, by peptide radioactivity. The addition of 4-methyl-2-oxopentanoic acid to the incubation medium enhanced the conversion of labelled leucine into carbon dioxide and into other non-identified products (possibly labelled 4-methyl-2-oxopentanoic acid), but it markedly depressed the labelling of proteins. Under such circumstances insulin stimulated the intracellular accumulation of free labelled leucine, but did not appreciably affect its conversion into carbon dioxide or its incorporation into protein.

DISCUSSION

The present study was undertaken in an effort to demonstrate that insulin affects both the transfer and accumulation of natural amino acids by chick embryo heart tissue.

With regard to the two amino acids selected for this investigation, [1-^{14}C]glycine and L-[1-^{14}C]-leucine, the former responded to the hormone with a significant increase of its intracellular concentration, whereas the latter did not. It should be pointed out that, when natural amino acids are used, the amount recovered as free intracellular amino acid may represent a small fraction of that which enters the cell because of intracellular utilization. A complete balance accounting for all the amino acid taken up from the medium therefore provides more meaningful information when the intracellular utilization is larger than accumulation. This was not the case with labelled glycine, since the low rate of its oxidation (Manchester, 1965) and the relatively high value of the ratio of its intracellular accumulation to its incorporation into protein (Wool *et al.* 1965) permitted the formation of pronounced concentration gradients between intracellular and extracellular water. Under these conditions the effect of insulin on accumulation, and thereby on amino acid transport, was readily discernible even in the presence of active protein synthesis (Table 1). On the other hand, with leucine the amount of labelled amino acid taken up by the cardiac tissue was much greater than that accumulated free intracellularly (Table 2). Under these circumstances insulin increased slightly the uptake (measured as disappearance from the medium) of labelled leucine by the heart tissue, but was without effect on its intracellular accumulation. The failure of the hormone to enhance leucine accumulation and therefore its concentration ratio between intracellular and extracellular water may have been connected with the simultaneous acceleration of the intracellular oxidation of the amino acid (Table 2). Similar results reported for rat diaphragm and heart (Manchester & Young, 1960; Akedo & Christensen, 1962; Scharff & Wool, 1965) were ascribed to a concurrent hormonal stimulation of leucine incorporation into protein; in these muscle tissues the suppression of protein synthesis by puromycin revealed the effects of the hormone on amino acid transport and accumulation (Wool *et al.* 1965; Scharff & Wool, 1965). In the chick embryo heart, the incorporation of amino acids into protein was not accelerated by insulin at early stages of embryological development (Loreti, Gaja, Ragnotti, Borghetti & Guidotti, 1965); accordingly, when puromycin was added to inhibit protein synthesis, the intracellular accumulation of labelled leucine, though greater than in the absence of the antibiotic, was not increased by the hormone. Under these conditions, however, the effect of insulin on total uptake of the amino acid became more manifest, as expected from the suppression of the quantitatively more important route of leucine utilization at this stage of embryological development (Table 2).

As discussed above, a possible effect of insulin on the intracellular accumulation of labelled leucine may have been obscured by the concurrent stimulation of its intracellular oxidation by the hormone. The addition of 4-methyl-2-oxopentanoic acid, the first intermediate of leucine catabolism, which suppressed this stimulation almost completely, unmasked a definite effect of insulin on the intracellular accumulation of the labelled amino acid, whose concentration ratio rose well above unity (Table 1). Why 4-methyl-2-oxopentanoic acid abolished the effect of the hormone on leucine oxidation was not clearly established. Models based on a functionally homogeneous intracellular leucine pool failed to provide an adequate explanation for all the results presented in Table 2 (particularly for the decreased labelling of proteins and the high rate of labelled leucine oxidation in the presence of 4-methyl-2-oxopentanoic acid). At present, we are considering the possible occurrence in the heart cell of two intracellular leucine pools (one relevant to protein synthesis, fed by the amino acid entering the cell or synthesized via transamination from 4-methyl-2-oxopentanoic acid, and one concerned with leucine catabolism) connected by insulin-sensitive transfer sites. Although such a model would rationalize all the experimental observations, conclusive evidence for its biological counterpart must await further investigation.

The results presented, indicating that insulin stimulates the accumulation of labelled glycine and, under proper conditions, the uptake and accumulation of labelled leucine, support the conclusion that, in the 5-day-old chick embryo heart, the hormone promotes the transfer of at least two natural amino acids from the extracellular to the intracellular space, though with leucine it is

difficult to decide whether this effect is primary on transport, or secondary to the intracellular action of insulin. The finding that insulin stimulates amino acid uptake by hearts at stages of embryological development earlier than those in which insulin is known to accelerate amino acid incorporation into protein (Loreti *et al.* 1965) and glucose transfer across the cell membrane (Guidotti & Foà, 1961; Guidotti, Loreti, Gaja & Foà, 1966) may provide ontogenetic evidence for the independence of these hormonal effects.

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