GLUCOSE METABOLISM IN THE ANTERIOR PITUITARY, POSTERIOR PITUITARY, PINEAL BODY AND BRAIN AND ITS SIGNIFICANCE IN THE RELEASE OF THYROTROPHIN IN VITRO

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by

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ABSTRACT

The conversion of C-1 of glucose to CO_2 in vitro by bovine posterior pituitary slices was 13-20 times as great as that of C-6; this finding indicates an active hexosemonophosphate shunt as reported for other endocrine tissues. Nerve endings isolated by centrifugation from the posterior pituitary gave a similar ratio. Tissue obtained from the paraventricular nucleus of bovine hypothalamus gave a C-1:C-6 oxidation ratio of 1.1, similar to ratios found in other areas of the brain including the hypothalamus and indicative of glucose oxidation primarily through the Embden-Meyerhof and Krebs cycles. Anterior pituitary slices gave C-1:C-6 oxidation ratios similar to those from posterior lobe, but total glucose oxidation was only 5% to 10% as great. Total glucose oxidation by posterior pituitary tissue was significantly higher than that of brain tissue. Epinephrine, 10^{-4} M, stimulated C-1, but not C-6, oxidation in paraventricular nucleus and in anterior and posterior pituitary. The percentage increase in C-1 conversion to CO_2 produced by epinephrine was similar for posterior pituitary slices and for isolated nerve endings.

The conversion of C-1 label from glucose to CO_2 <u>in vitro</u> by bovine pineal bodies was 7-24 times as great as that of C-6. These values for C-1:C-6 oxidation ratios are similar to those found for all known

endocrine tissues and in contrast to those for brain which range from 1.0 to 1.4. Total glucose oxidation, both C-1 and C-6, and C-1:C-6 ratios were lower in pineal bodies from adult (3-8 years) than from young (5-10 months) animals. Total glucose oxidation by the posterior pituitary was lower in the adult than in the young, generally lower in the anterior pituitary of the adult, and higher in the brain of the adult. Epinephrine, 10^{-4}M, increased the oxidation by pineal tissue of C-1 by 170% and C-6 by 46%. The relatively high C-1:C-6 ratios found for pineal tissue are indicative of an operative hexosemonophosphate pathway. These findings provide biochemical support for the hypothesis that the pineal body has an endocrine function in mammals.

The activity of the hexosemonophosphate shunt was estimated quantitatively in pineal body, anterior pituitary, posterior pituitary, and brain cortex, extending the above observations which indicated the presence of the hexosemonophosphate pathway in these organs. The contribution of the hexosemonophosphate shunt to total glucose catabolism was determined from the specific yield of CO_2 from C-6 labelled glucose. Total glucose utilization was measured by summing ^{14}C found in CO_2, lactate, fatty acids, and glycerol and by measuring the depletion of non-labelled glucose from the medium. In the anterior pituitary, posterior pituitary and pineal body, 5%, 4%, and 3% of the utilized glucose, respectively is catabolized via the hexosemonophosphate shunt, whereas in brain the activity of the pathway is negligible. The rate of glucose utilization is fairly constant over the incubation periods in all four tissues, being highest in brain, and least in anterior pituitary, with posterior pituitary, and pineal body giving intermediate values.

The effects of vasopressin, oxytocin and epinephrine on thyrotrophin (TSH) release and ^{14}C-glucose oxidation by bovine anterior pituitary tissue in vitro were examined, in attempts to assess the role of cell metabolism in the secretory process. The dose-response curves for TSH release by all three hormones were bell-shaped, vasopressin and oxytocin maximally enhancing TSH release at 10^{-10} or 10^{-9}M, and epinephrine at 10^{-7} or 10^{-6}M. Phenoxybenzamine, 10^{-8}M, blocked the stimulatory effect of epinephrine, but not of vasopressin, on TSH release. ^{14}C-1 or ^{14}C-6 glucose oxidation to CO_2 was unaffected by concentrations of all three agents which were maximally effective in enhancing TSH release. Incubations carried out in the cold resulted in a slight depression of the stimulated release of TSH by vasopressin. Under N_2 atmosphere, neither oxytocin nor vasopressin had a significant effect on TSH release. Glucose oxidation was consistently reduced by 85-90% in the cold compared to 37°, and by 80% in N_2 atmosphere compared to O_2. Spontaneous release of TSH was generally greater in N_2 than in O_2 atmosphere at 37°. It was concluded that there is no direct coupling between glucose oxidation and the spontaneous or stimulated release of TSH from cytoplasmic stores.

TABLE OF CONTENTS

List of Tables

List of Figures

List of Abbreviations

ACTH	Adrenocorticotrophic hormone
C	Centigrade
CNS	Central nervous system
Cyclic-3'5'-AMP	Cyclic-3'5'-adenosine monophosphate
CRF	Corticotrophin-releasing factor
EM	Embden-Myerhoff
g	Grams
hr	Hour
HMP	Hexosemonophosphate
M	Molar
mg	Milligram
ml	Milliliter
mU	Milliunits
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
N	Normal
PB	Pineal body
POB	Phenoxybenzamine
POPOP	1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene
PP	Posterior pituitary
PPO	2,5-dipheny-oxazole
PTU	Propylthiouracil
PVN	Paraventricular nucleus
T_4	Thyroxine
TRF	Thyrotrophin-releasing factor
TSH	Thyroid stimulating hormone (thyrotrophin)

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TO MY WIFE

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I. STATEMENT OF THE PROBLEM

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The anterior pituitary (AP), posterior pituitary (PP), and the pineal body (PB) are three organs which are anatomically, embryologically, and functionally interrelated. The PP and PB are both derived from neural tissue and thus share similar embryological organs. The AP and PP are believed to be functionally related, since the neurohypophysial hormones, oxytocin and vasopressin, have been implicated in the regulation of AP trophic hormone secretion (reviewed by Martini, 1966). Some recent evidence suggests that the PB is an endocrine gland and that it may exert an influence over gonadotrophic hormone secretion from the AP (Zweens, 1963; 1965). In addition, the AP and PP are closely related to the brain both anatomically and functionally. Chemical mediators elaborated in the hypothalamus, in conjunction with products of peripheral glands, modify AP trophic hormone secretion. The neurohypophysial hormones are synthesized in the paraventricular and supraoptic nuclei of the hypothalamus, and secretion of these peptide hormones from the PP is regulated by nervous pathways to these nuclei. The AP, PP, PB and brain thus comprise a group of organs whose function, origin, and anatomy are in many respects interrelated.

The role of secretory cell metabolism in secretion is a major question concerned with the fundamental nature of the secretory process. It has been demonstrated that the phosphorylase system (Haynes and Berthet, 1957; Bdolah and Schramm, 1965) and phospholipid metabolism (Hokin and Hokin, 1965) are in some way related to the process of secretion. In addition, the hexosemonophosphate (HMP) shunt has been implicated in the secretory process (Haynes and Berthet, 1957, Field et al., 1960a; Dumont, 1965).

The HMP shunt is a direct oxidative pathway for glucose and provides substrates for nucleic acid and lipid metabolism. This pathway is present in every endocrine gland examined but has not been previously demonstrated in the PP and PB. Also, the possible role of this pathway in the synthesis, storage or secretion of hormones is not definitely known. In secretory cells, the HMP shunt may be related to hormone synthesis, since it provides substrate for nucleic acid synthesis, or possibly to secretion-linked membrane turnover dependent on lipid metabolism.

The present studies were designed:

1. to compare and contrast the activity of the HMP shunt and other aspects of glucose metabolism in the AP, PP, PB with those in brain, since similarities in the pattern of glucose metabolism within these endocrine organs would support the hypothesis that the HMP shunt has a fundamental role in the secretory process.

2. to elucidate the possible role of the HMP shunt in the secretory cycle by examining the pattern of glucose catabolism in the mammalian neurohypophysis, since in the cells of this organ the components which synthesize the neurohypophysial hormones are anatomically distinct from those which store and secrete the hormones. Therefore, glucose oxidation was compared in the PP and in nerve endings isolated from the PP (where the peptide hormones are stored) and in the paraventricular nucleus of the hypothalamus (where the hormones are synthesized).

3. to determine whether the release of trophic hormone (TSH) from the AP induced by neurohypophysial hormones is directly coupled to glucose metabolism. $^{14}CO_2$ production from ^{14}C-1- and ^{14}C-6-labelled glucose and TSH release were compared at $37^{o}/O_2$, $37^{o}/N_2$, $0^{o}/O_2$ and $0^{o}/N_2$. The effect of epinephrine on TSH release and glucose oxidation in the AP was also

examined, since epinephrine has been implicated in AP trophic hormone secretion (Harris, 1955). In addition, the effect of phenoxybenzamine (a classical adrenergic blocking agent) on the stimulation of TSH release promoted by epinephrine and vasopressin was examined to determine the nature of the "receptors" in the AP concerned with TSH release.

II. INTRODUCTION

ANATOMICAL RELATIONS OF THE ANTERIOR PITUITARY, POSTERIOR PITUITARY AND PINEAL BODY

The hypophysis cerebri (pituitary gland) is a small, ovoid body projecting down from the base of the brain. It lies on the midline, within the pituitary fossa of the sphenoid bone, posterior to the optic chiasma and anterior to the mammillary bodies. It is overlain on its superior aspect by a tough membrane, the diaphragma sellae, through which the infundibular stalk travels to connect the pituitary with the hypothalamus. The pituitary gland is divided into four parts, the anterior lobe (anterior pituitary, pars distalis, adenohypophysis), posterior lobe (posterior pituitary, pars nervosa, neurohypophysis), intermediate lobe (pars intermedia) and the pars tuberalis. The anterior pituitary (AP) and posterior pituitary (PP), which form the main substance of the pituitary gland, have diverse embryological origins. The AP arises as an upward evagination (Rathke's pouch) of the ectoderm of the primitive buccal cavity. The PP arises as a diverticulum of the floor of the third ventricle. The craniopharyngial canal, connecting Rathke's pouch with the buccal cavity eventually disappears, but the diverticulum of the third ventricle persists as the infundibular stalk, connecting the PP with the hypothalamus.

The AP receives no specific innervation. Sympathetic fibres have been traced to blood vessels, but these have not been associated with the glandular cells. The PP is supplied by neurons whose perikarya are located in the supraoptic and paraventricular nuclei of the hypothalamus, and whose axonal terminations form the main substance of the PP. The axons of these cells traverse the infundibular stalk to form the hypothalamo-neurohypophysial tract.

The vascular supply of the pituitary gland is derived from branches

of the internal carotid artery, the superior and inferior hypophysial arteries. Vascular anastomoses between the AP and PP have been described (Holmes and Zuckerman, 1959; Sheehan and Stanfield, 1961). The main blood supply of the AP is apparently through a portal system of veins which were observed by Green and Harris (1949) to flow from the median eminence as a thick plexus, and empty into the numerous sinusoids of the AP. Neuronal terminations of hypothalamus neurons have been described (Harris, 1955) to come into close contact with the capillaries of the portal system, providing a functional link between the hypothalamus and the AP. The vascular supply thus arises from three sources, the arterial supply which directly supplies the gland, the vascular anastomoses with the PP, and the hypophysial portal system. This complex blood supply puts the AP cells in contact with agents entering the general circulation, with agents directly from the PP, and with agents directly from the hypothalamus.

The pineal body (epiphysis cerebri) is a small, pine-cone shaped organ lying between the superior colliculi in the quadrigeminate groove. It is attached by a stalk to the posterior wall of the third ventricle. Embryologically it is formed from an evagination of the roof of the third ventricle. Thus both the PP and pineal body (PB) have a similar developmental origin. The vascular supply of the PB is derived from the posterior cerebral artery.

The secretory cells of the PB are innervated by sympathetic nerve fibres. According to the most recent evidence (Moore et al., 1967) the effect of environmental light on the PB is initiated in the retina, and transmitted to the superior cervical ganglion via the inferior accessory optic tract. These preganglionic sympathetic nerves synapse at the superior cervical ganglia with post-ganglionic fibres which reach the PB by the medial

forebrain bundle. PB activity is thus regulated by an afferent supply originating in the retina.

REGULATION OF ANTERIOR PITUITARY SECRETION

a. The negative feedback system.

A negative feedback system has been shown to operate between the AP and the thyroid, the AP and the adrenal cortex, and the AP and the gonads. In the following discussion, only literature concerning the thyroid-hypophysial system will be reviewed, although similar information is available for all of these systems. The earliest report (Niepce, 1851) pointing to a negative feedback system was a description of hypertrophy of the AP in goitrous cretins. Early classical experiments on amphibian metamorphosis, showing first, that destruction of the tadpole hypophysis prevents metamorphosis and produces thyroid atrophy, and second, that metamorphosis can be induced by feeding the tadpoles thyroid extract, suggested some mutual regulatory influence of the hypophysis and thyroid. Similarly, in hypophysectomized mammals, the thyroid gland is not responsive to stimuli normally producing an increase in activity, such as cold or iodine deficiency (Goldberg et al., 1953; Wolf and Greep, 1937). Decreasing the level of thyroxine (T_4) by goitrogens (D'Angelo, 1955) or partial thyroidectomy (Logethetopoulos and Doniach, 1955) causes an increase in hypophysial secretion of TSH. The depletion of I^{131} from the rabbit thyroid gland (Brown-Grant, 1957), which is an indirect measure of circulating TSH, diminishes when T_4 is injected, suggesting inhibition of TSH release.

These experiments support the concept of a servo-mechanistic relationship between the thyroid and the pituitary. According to this concept, decreased levels of target gland hormone (T_4) enhance, and increased blood levels inhibit, the secretion of the pituitary trophic

hormone (eg. TSH). The sensitivity of the pituitary to the target gland hormone is set by a so-called "thyrostat" mechanism. This negative feedback mechanism thus acts to maintain a constant level of circulating T_4.

Historically, a major problem has been to determine whether T_4 directly acts on the pituitary to control TSH secretion, or whether regulation is indirect via the central nervous system. Microinjections of T_4 directly into the AP generally produce inhibition of TSH secretion (Von Euler and Holmgren, 1956; Harrison, 1961). While in some experiments T_4 injected into the hypothalamic areas had no effect on AP function (Von Euler and Holmgren, 1956; Harrison, 1961), Yamada (1959) and Yamada and Greer (1959), found that T_4 injected into the "hypothalamic thyrotrophin" areas produced inhibition of TSH release, suggesting that T_4 might act at both sites. Bogdanove and Crabill (1961), found that thyroid homografts in the pituitary, but not in the hypothalamus, inhibited TSH release. In rats with lesions in the median eminence TSH secretion was reduced, and T_4 inhibited and goitrogens enhanced the release of TSH, demonstrating clearly that while the hypothalamus has a tonic influence, T_4 can act directly on the AP (D'Angelo and Traum, 1958). Lesions of the AP prevent the goiter formation usually seen after goitrogen administration (D'Angelo and Traum, 1956). In stalk-sectioned rabbits, in which waxed-paper plates were inserted between the cut ends of the stalk, systemic administration of T_4 still produced inhibition of TSH release (Brown-Grant et al., 1957). Thus, the pituitary is responsive to changes in the internal environment, even when hypothalamo-hypophysial connections are partially or totally lacking.

Another very useful approach has been to graft pituitaries to other parts of the body to completely remove the pituitary from any direct

CNS control. Hypophysectomized mice with intraocular pituitary homografts were quite responsive to changes in T_4 levels, and increased concentrations of T_4 were inhibited and goitrogens enhanced the release of TSH from the homografts (Scow and Greer, 1955).

Christensen (1960) has provided evidence that it is the distribution of free, non-protein-bound T_4 which results in inhibition of TSH. He found that the administration of both 2,4-dinitrophenol (DNP) and salicylates produced a decrease in thyroidal activity and a decrease in protein-bound-iodide (PBI). Christensen reported that both these agents increased the level of non-protein-bound T_4 by a thyroxine-releasing effect as measured in human serum in vitro. The observation that DNP and salicylates decrease thyroidal activity and PBI seems contradictory to the negative feedback theory. However, these facts become consistent with this concept in the light of Christensen's suggestion that it is the "free" T_4 which regulates the AP function.

b. Evidence for Hypothalamic Control.

Well documented deviations are known to occur from the classical negative feedback theory of regulation (Brown-Grant, et al., 1957). The anatomy of the vascular connections between the pituitary and the hypothalamus clearly is favorable to some neural control of the AP, and a great deal of evidence has been amassed to show that in fact the CNS, mainly through the hypothalamus, does influence AP function.

In the premetamorphic frog, isolation of the pituitary from the hypothalamus by stalk section prevents metamorphosis. The pituitary during this transformation, is no longer sensitive to high doses of T_4 illustrating the importance of central control (Kaye, 1961). After stalk section there is generally a decrease in secretory activity, and some

decrease in weight, of the pituitary (Brown-grant et al., 1957; Barnett and Greep, 1951). It was discovered quite early that even though the stalk-sectioned pituitary responded to T_4 (Brown-Grant et al., 1957; Barnett and Greep, 1951), cold stress in the rat no longer caused increased TSH secretion (Brolin, 1947).

Transplanting of the pituitary to another site usually results in some decrease in pituitary weight (Goldberg and Knobil, 1957), and a decrease in thyroid weight (Greer et al., 1953). Morphological changes in the pituitary are similar to those that occur in stalk-sectioned animals (Von Euler and Holmgren, 1956; Martini et al., 1959). I^{131} uptake, and thyroid/serum iodide ratios were reduced to about 2/3 normal (Greer et al., 1953), and the pituitary still retained TSH activity (Martini et al., 1959). Goitrogen (propylthiouracil) treatment in rats with intraocular implants did not produce stimulation of TSH in the transplanted pituitary (Von Euler and Holmgren, 1956).

Hypothalamic lesions, by destroying areas that influence TSH secretion by the AP, have been useful in studying hypothalamic control of the pituitary. Lesions, particularly in the supraoptic and median eminence regions of the hypothalamus, were effective in reducing TSH release, thus reducing or completely inhibiting various indices of thyroidal function (e.g., I^{131} uptake, thyroid/serum iodide ratio, response to goitrogens and cold stress) (Ganong et al., 1955; D'Angelo, 1958; Bogdanove and D'Angelo, 1959). Effective hypothalamic lesions generally prevent any increase in, but not inhibition of, TSH secretion from the pituitary (Reichlin, 1960). It is significant also that electrical stimulation of the hypothalamus increased thyroid activity. In a classic paper by Harris and Woods (1958), electrical stimulation of the supraoptic region of the

hypothalamus and the AP resulted in increased thyroid activity. Stimulation in the median eminence region resulted in an increase in ACTH release and a decrease in TSH release, which was apparently due to increased blood steroid levels, since after adrenalectomy stimulation of both TSH release and ACTH release occurred. Overlapping of TSH and ACTH controlling areas was suggested by the authors to explain this phenomenon.

The literature reviewed above, in general, supports the concept that the hypothalamus is important in maintaining the normal rate of secretion of TSH and in modifying this secretion in response to various stimuli which act through the CNS. Evidence has accumulated that this CNS control is operative through the hypophysial portal system. Some of the evidence has been summarized above, and the results of Nikitovitch-Winer and Everett (1958) reinforce these findings. In their experiments autotransplants of the pituitary were made to the kidney of rats. The pituitary was later retransplanted to either the temporal lobe or the median eminence. In the animals with retransplants in the temporal lobe, the pituitary no longer secreted certain hormones and released only small amounts of others. In rats with the pituitary homograft retransplanted onto the median eminence, the pituitary regained cellular differentiation and significantly improved in function. It thus seems clear that, to function normally, the pituitary requires a blood supply specifically derived from hypophysial portal vessels. Florsheim et al. (1963) found that, by joining in parabiotic union a rat with an anterior hypothalamic lesion to a hypophysectomized partner, "goiter" block induced in the lesioned rat administered propylthiouracil (PTU) could be reversed across the parabiotic union. The material having this effect was not TSH, and it was postulated that this substance was a neurohumoral TSH-releasing

factor (TRF) of hypothalamic origin.

c. Evidence for Hypothalamic Releasing Factors.

The concept of a specific humoral mediator for TSH, elaborated in the hypothalamus, and reaching the adenohypophysis via the hypophysial portal system, is obviously a reasonable and attractive explanation for the mechanism of neural control over the secretion of TSH. It has been suggested numerous times that the products of neurosecretion, i.e. oxytocin and/or vasopressin, are involved in the regulation of adenohypophysial function. Attempts to localize a TSH-controlling area by electrolytic lesions of the supraoptic and paraventricular nuclei have proved inconclusive. D'Angelo and Traum (1958) found that large antero-tuberal lesions in rats, which did not include either the supraoptic or paraventricular nuclei, decreased TSH secretion. These lesions may, however, have interfered with the hypothalamo-hypophysial tracts. Olivecrona's careful study (1957) indicates that localized destruction of the paraventricular nuclei does not affect any parameters of thyroid activity. Also, Greer and Erwin (1954) found that in rats localized destruction of the paraventricular nuclei did not affect any parameters of thyroid activity. However, Greer and Erwin did find that while lesions in the supraoptic nuclei had no effect, destruction of the paraventricular nuclei prevented the typical thyroid hypertrophy usually seen after goitrogen administration. Since none of these experiments measured quite the same parameters, and the techniques used were very different, it is difficult even to say whether these experiments are incompatible with one another. However, it is generally accepted that the lesions most effective in reducing adenohypophysial function are those in the region of the median eminence (Pollock et al., 1966). The effect of dehydration on the I^{131} release rate was studied by Reichlin, (1957). He

found that dehydration of rats, which produces increased rates of vasopressin synthesis and release, resulted in decreased thyroid activity. There also seems to be little relationship between neurosecretion and AP function under conditions of hypo or hyperthyroidism.

A number of studies have been done in which vasopressin or oxytocin were injected *in vivo* or added *in vitro*. Most of the studies suffer from (1) the lack of proper dose response curves, (2) inadequate methodology in TSH determinations, (3) in some cases, lack of proper controls. A large number of experiments have been published indicating that vasopressin and/or oxytocin stimulate thyroidal activity. However, Harris, (1963) and Arimura *et al.*, (1964), observed that the neurohypophysial hormones also had this effect in hypophysectomized animals, indicative of a direct effect of the thyroid gland. Other investigators have failed to demonstrate any affect of the neurohypophysial hormones on thyroid activity (Crossin *et al.*, 1960; Reichlin, 1957) or TSH release measured directly (Rose *et al.*, 1960). On the other hand, vasopressin given to humans (Rosner *et al.*, 1962; Peterson et al., 1960) produced an increase in thyroid activity. Woods and Bard (1961) found vasopressin and oxytocin stimulates TSH release in cats, but had no effect when the animals were hypophysectomized. Kovács and Vértes, (1962) found oxytocin stimulated I^{131} uptake in rats, and this was abolished in hypophysectomized animals. Neurohypophysial hormones have been found to stimulate TSH release *in vitro* (Woods and Bard, 1961; Kovács and Vértes, 1962). LaBella (1964a; 1964b) reported that oxytocin and vasopressin were capable of stimulating TSH release both *in vitro* from bovine AP and *in vivo*, measuring the depletion of I^{131} from PTU treated, day-old chicks, as an index of TSH levels. Due to the obvious inconsistencies in the literature, the possible physiological

role of the neurohypophysial hormones in AP function, in general, and TSH secretion in particular, is highly controversial.

It is known that biogenic amines such as epinephrine influence the secretion of a number of AP hormones, and the role of such agents in AP regulation has long been a subject under investigation (Harris, 1955). The pituitary can rapidly concentrate an i.v. dose of epinephrine up to ten times plasma levels (Axelrod et al., 1959) indicating that the pituitary may be exposed to high concentrations of epinephrine from the systemic circulation. Brown-Grant (1960) has described a reciprocal relation between TSH and ACTH during stressful stimuli and after administration of epinephrine. He found that TSH secretion was inhibited, and ACTH secretion stimulated, by both treatments. Fraja and Martini, (1952) observed that epinephrine injected suboccipitally into dogs produced increased levels of circulating TSH. Contradictory results were obtained by Harrison (1961) who found that microinjection of epinephrine directly into the hypothalamus causes inhibition of TSH release. Soffer et al. (1949) in a paper often overlooked found that epinephrine stimulated the secretion of both TSH and ACTH in adrenalectomized animals. The authors suggested that the inhibition of TSH by epinephrine was an indirect result of increased levels of steroid produced by ACTH, since they found steroids inhibited TSH release, and concluded that epinephrine in the absence of inhibitory influences can stimulate TSH directly.

There has thus been an active search, using a variety of in vivo and in vitro approaches, for a TRF. Biochemical isolation of hypothalamic peptides has also been attempted, since hypothalamic extracts have been reported to stimulate the release of TSH (Harris, 1960). Peptides have been isolated from these extracts which differ from oxytocin and vasopressin

and which appear to contain some TRF activity. Shibusawa and coworkers (1959a; 1959b) published a series of papers in which they reported isolation of a TRF. This material was claimed to have been found in the urine, cerebral spinal fluid, anterior hypothalamus, and the neurohypophysis, and was active in vitro and in vivo. However, their work has been criticized strongly on methodological and statistical gounds (Reichlin, 1963). Schreiber et al., (1961, 1962) have isolated a hypothalamic peptide which they reported releases TSH from AP in vitro. The specificity of this peptide was not tested in vitro, but it was found not to affect thyroid activity in hypophysectomized rats. Guillemin, et al., (1962, 1963) have also isolated a peptide from the hypothalamus which stimulates TSH release in vitro and in vivo. On the basis of their chemical analysis of this substance, (Guillemin, et al., 1965), it was tentatively proposed that the TRF is an octadecapeptide. Recently, Guillemin, et al., (1966) has provided evidence that TRF may not be entirely, or typically, peptidic in structure.

BIOASSAYS FOR TSH

It has been estimated that there are close to 100 different bioassays procedures used for TSH determinations. The large number of assays indicates that at present no assay is generally accepted by workers in the field to be sensitive, precise, or convenient enough for all purposes.

Junkmann and Schoeller (1932) reported what was likely the first truly quantitative assay for TSH. They based their assay on histological changes produced by TSH on guinea pig thyroids. For many years the activity of TSH was reported in terms of Junkmann-Schoeller units. Since TSH increases thyroid weight as a linear function of the

dose, and due to simplicity and ease of measurement, this assay has been widely used. The guinea pig (Rowlands and Parkes, 1943) and the chick (Albert et al., 1946) have been frequently used as test preparations, but the chick was considered to be the more sensitive (Cope, 1938). Generally, histological examination of the thyroids was combined with the gravimetric assay, since alone this assay is quite insensitive (Rawson and Salter, 1940). TSH administration also causes an increase in acinar cell height which can be easily measured. This increased cell height is proportional to the dose of TSH. The method is quite sensitive, but its objectivity can be criticized, since the "typical" acinar cell must be chosen from a population of cells; also, some distortion may arise in the fixing and staining of the tissue.

D'Angelo and Gordon (1950) designed an assay for TSH around the observation that TSH stimulates metamorphosis in the tadpole. They improved the sensitivity of the assay by starving the tadpoles, thus inducing thyroid atrophy and metamorphic stasis. Generally, both measurements of the hind limb and histologic examination of the thyroid were combined. This method is quite specific and appears to be the most sensitive TSH assay designed, capable of measuring accurately the concentration of TSH in body fluids.

Biochemical methods have proved to be popular in assaying TSH. Radioisotopes (especially I^{131}) have been exceedingly useful. The thyroidal uptake of I^{131} *in vitro* has been used widely. Ghosh et al. (1951) proposed an assay based on this parameter using hypophysectomized rats. The accumulation of I^{131} *in vitro* by the thyroid has also been used as an assay for TSH (Bakke and Lawrence, 1956). It is generally considered, however, that the I^{131} uptake is not as sensitive or satisfactory an index

of thyrotrophic activity as the discharge of I^{131} from the thyroid. Stable iodine (I^{127}) depletion was used quite early for TSH assays (Foster et al., 1933) but not until the advent of radioisotopic iodine (I^{131}) did the method reach a level of sensitivity that was acceptable. An assay employing I^{131} depletion from guinea pig thyroid glands was designed by Adams and Purves (1955). They found that the sensitivity of the assay was much improved if thyroxine was administered, since this treatment inhibited the endogenous secretion of TSH. Adams and Purves' assay was later modified by McKenzie (1958a) using mice instead of guinea pigs. This depletion assay is of particular interest, since it enabled these two groups to find a thyrotrophic substance with a much longer duration of action than TSH in the serum of hyperthyroid patients (Adams, 1958; McKenzie, 1958b). A further modification of this assay by Yamazaki et al. (1963) has also made it useful in the determination of TRF. An in vitro assay was designed by Bottari and Donovan (1958) using I^{131} depletion from guinea pig thyroids. Addition of increasing doses of TSH to the incubation medium produces a linear dose-response curve, measured by an increase in radioactivity in the medium.

I^{131} depletion from the day-old chick has proved to be one of the more sensitive and convenient assays for TSH. It was first used by Gilliland and Strudwick (1953) and later modified by Bates and Cornfield (1957). Gilliland and Strudwick pretreated their chicks with thyroxine to decrease endogenous TSH, and Bates and Cornfield improved their assay by also pretreating with (PTU) to inhibit reincorporation of label. Success with this method has been limited in the past, according to Bates and Cornfield, mainly by the difficulty in consistently obtaining chicks which have a high uptake of I^{131}.

POSTERIOR PITUITARY SECRETION

The PP is composed of secretory cells whose perikaryi form the paraventricular and supraoptic nuclei of the hypothalamus. The active principles of these cells, oxytocin and vasopressin, are synthesized in the perikaryon, and are transported down, bound to a carrier protein by axoplasmic flow (Sachs, 1960) to the neuronal terminations residing in the PP. The neurohypophysial peptide hormones are stored in association with this carrier protein in these nerve endings and secreted in response to an appropriate stimulus. Release of the neurohypophysial hormones is effected when a wave of excitation reaches the nerve terminal. Douglas and Poisner (1964) found that in the absence of calcium, release of the neurohypophysial hormones could not be elicited, and they suggested that excitation and secretion in the PP are calcium-coupled.

The chief stimulus for the secretion of vasopressin is dehydration (Ortmann, 1951), but hemorrhage is also a potent stimulus for vasopressin release. Oxytocin is apparently important during parturition, and during this event the uterus becomes much more sensitive to oxytocin (Van Dyke, 1961) and oxytocin secretion is greatly stimulated (Fitzpatrick, 1961). Oxytocin is also involved in the milk-ejection reflex, and suckling results in a stimulation of oxytocin secretion (Sawyer, 1961). The fact that oxytocin and vasopressin are secreted concomitantly in a number of situations (Cross, 1961) has aroused speculation concerning the neural regulation of neurohypophysial secretion. Bindler et al. (1967) have resolved separate oxytocin and vasopressin containing nerve-endings by density gradient centrifugation, suggesting that each neurosecretory cell synthesizes only a single peptide hormone.

Vasopressin is an extremely potent antidiuretic agent. It acts to increase the permeability to water of the distal tubules and collecting ducts of the kidney, the urinary toad bladder, and the frog skin, thus effecting the reabsorption of water (Sawyer, 1961). Ussing (1960) suggested that vasopressin increases the permeability to water of these structures by increasing the diameter of the pores of the cell membrane. Ginetzinsky (1958) proposed that vasopressin increased the permeability to water by stimulating hyaluronidase activity, which would have the effect of breaking down hyaluronic acid, the so-called interstitial "cement". This theory, however, has not been widely accepted. More recently, Orloff and Handler (1961) suggested that vasopressin's effect on water permeability is mediated through the formation of adenosine-3'5'-AMP, since vasopressin stimulates the formation of cyclic AMP, and other agents known to increase the concentration of cyclic AMP increase water permeability. Cyclic AMP has also been implicated as an intermediate in the effect of epinephrine in stimulating glycogenolysis and ACTH in stimulating steroidogenesis (Sutherland and Rall, 1961). Vasopressin is also a potent vasopressor agent, but whether this effect has physiological significance is not known at present. Oxytocin is highly potent in stimulating uterine contractility and contractions of the myoepithelial cells of the mammary gland (Sawyer, 1961). In general, oxytocin and vasopressin have overlapping activities, with oxytocin approximately 20 times as effective as vasopressin on the uterus and mammary gland, and vasopressin about 20 times as effective as oxytocin on the kidney and blood pressure.

Some metabolic effects of the neurohypophysial hormones may be related to their mechanism of action. Oxytocin and vasopressin

stimulated oxidative metabolism of the toad urinary bladder (Goodfriend and Kirkpatrick, 1963) and the production of $^{14}CO_2$ from both ^{14}C-1- and ^{14}C-6-labelled glucose from the rat mammary gland (Goodfriend and Topper, 1961). Vaughan (1964) reported that vasopressin stimulated lipolysis and also phosphorylase activity in rat adipose tissue, and Orloff and Handler (1961) found that vasopressin stimulated the formation of cyclic AMP in rat kidney slices. It is interesting that these effects of the neurohypophysial hormones on several target organs are similar to some of the metabolic actions of epinephrine (Hagen and Hagen, 1964).

No clearly defined role for oxytocin in the male has been described. However, the possibility that the neurohypophysial hormones act as releasing factors for AP trophic hormones may explain the presence of oxytocin in the male PP. LaBella (1964b; 1967) has suggested that oxytocin and vasopressin may interact with the target gland hormone to modify trophin secretion. This hypothesis was based on the observation that in vitro, T_4 enhanced the stimulatory effect of vasopressin on TSH release, while T_4, in combination with oxytocin, reversed the stimulation of TSH usually seen with oxytocin alone.

PINEAL BODY SECRETION

The PB in many lower vertebrates is a light receptor organ, as indicated by the presence of retinal pigments, ganglion cells similar to the rods and cones of the retina, nervous connections with the brain, and in some cases, a lens (Oksche, 1965). In certain lizards, in addition to this retina-like appearance, there are also present cells which are closely similar to the epithelial cells of glands. In turtles and snakes the PB develops a more characteristic glandular histology. In birds and mammals the retinal pigments are absent and the PB is entirely glandular

in appearance. The occurrence of these changes is thought to coincide with a shift in control of the PB by light directly to indirect control by a sympathetic nerve pathway originating in the retina (Moore et al., 1967). These workers found that superior cervical ganglionectomy or lesions of the medial forebrain bundle (through which travels the inferior accessory optic tract) abolishes the variations in melatonin synthesis initiated by changes in the photoperiod. Owman (1960), Wurtman et al. (1964b) and Axelrod et al. (1965) have confirmed that this nervous pathway regulating PB function is derived from the sympathetic nervous system, since the nerve terminals contain noradrenaline and serotonin.

Two early concepts concerning the function of the PB were, first, that this organ is a sphincter, and second, that it is the seat of the soul. A more modern concept, based on substantial experimental evidence, is that the PB is an endocrine gland. The chief methods for investigating PB function are pinealectomy, implantation and injection of PB extracts. The majority of such studies have been concerned with the possible role of the PB in gonadal regulation and in the regulation of body growth (Kitay and Altschule, 1954). Considerable controversy concerning these hypotheses existed and interest in this field waned when it appeared that no new insight into the function of the PB was imminent. However, Lerner et al. in 1959 isolated an active principle from the PB, N-acetyl-5-methoxy tryptamine or melatonin. This methoxyindole compound is a potent skin-lightening agent in amphibians (Lerner et al., 1959) and the fact that low concentrations of this compound blanches frog skin in vitro was used in the design of a bioassay for melatonin. Wurtman et al. (1964a; 1966) and Axelrod et al. (1964; 1966) found that the synthesis of melatonin varied with the daily light-dark cycle; in the rat, synthesis of melatonin was greater at night, and in the hen,

synthesis was greater in the light. They found that the effects of melatonin on the gonads were generally inhibitory; low concentrations of melatonin injected into rats depressed ovarian weight and decreased the percentage of vaginal smears showing estrous phases (Wurtman et al., 1963). Enhanced endogenous secretion of melatonin had similar effects on gonadal weights and the interval between estrous. There is some evidence that melatonin secretion and gonadotrophin secretion may be interrelated (Zweens, 1965). However, Wurtman et al. (1964b) found that the PB response to light occurred even in hypophysectomized rats. This concept is complicated by the finding (Pavel and Petrescu, 1966) that the bovine PB contains a peptide, argenine vasotocin, which has an antigonadotrophic effect on rat ovary and uterus. However, changes in synthesis or secretion of this peptide with light or other environmental conditions has not been demonstrated.

It is thus becoming apparent that the PB functions to translate diurnal lighting changes into neuroendocrine information, which is utilized is some way to regulate reproductive, and possibly other important functions.

MECHANISMS OF SECRETION

The secretory process can be defined as the process by which substances are synthesized, stored, and finally extruded from cells upon the appropriate stimulus, i.e., cells whose function is directed primarily toward the extrusion of intracellular materials. Morphologically, secretory cells can be divided into those cells containing and those cells devoid of secretory granules. While most secretory cells contain storage granules surrounded by a membrane, some important exceptions are the gastric parietal cell, and cells which secrete water and electrolytes. Thus, the accumulation and storage of secretory material is a common, but not essential, feature of secretory cells. In steroid producing cells, however, lipid droplets, rather than typical secretory granules are seen. Secretory cells have also been categorized as to the method of extrusion. In the holocrine type of secretion, such as occurs in sebaceous glands, the entire cell is desquamated into a central lumen. In the apocrine type, such as in the axillary sweat glands, the cells shed their apical region, which includes secretory material as well as cytoplasm. The most common and widely studied secretory process is the merocrine type, in which the secretory material is extruded across the plasma membrane.

Elucidating the cell mechanisms involved in the secretory process is a problem which has concerned physiologists for many years. In an early study dealing with morphological aspects of secretion, Herring (1908) suggested that the large granular cells of the AP empty their contents directly into the blood stream, supporting the theory that the secretory process was basically a protoplasmic degeneration of the secretory cell. In a later review, Bowen (1929), reflecting a more modern concept of secretion, stated, "Secretory activity seems rather

to be a function in which the whole cell system is somehow involved, presumably in the sense that it cooperates in providing the raw or finished materials which are deposited in the secretory vacuoles." Bowen suggested that the so-called Golgi apparatus is the focal point of the secretory process, since the secretory granules appear from this region of the cell. Farquhar and Rinehart (1954) observed that after thyroidectomy, the number of secretory granules in the AP decreased, and the remaining granules congregated in the Golgi region. They suggested that the Golgi complex participates in the synthesis and release of secretory material. Palade (1960) has proposed a general theory of secretion divided into four steps, based on electromicroscopic studies of secretory cells in various stages of the secretory cycle. He suggested that the first step is the synthesis of secretory material on the ribosome, followed by the transport of this material into the lumen of the endoplasmic reticulum. During the third step, zymogen granules containing the secretory material are formed by a budding off of endoplasmic reticulum in the Golgi region of the cell. In Palade's fourth step, the zymogen granules migrate to the apex of the cell, where they are stored until extruded by a process of "reverse pinocytosis" (De Robertis and Vaz Ferreira, 1957) or "emiocytosis" (Lacy, 1959). The most clearly definitive work on the origin and fate of the secretory granule has been performed by Siekevitz and Palade (1958a; 1958b; 1960). These workers were able to follow the progress and movements of newly synthesized pancreatic enzymes by labelling the enzymes with ^{14}C-labelled amino acids and isolating various subcellular fractions. They observed that the enzymes move from the ribosome where they are synthesized into the cisternae of the endoplasmic reticulum. The secretory material then becomes segregated into droplets, and these

droplets condense and subsequently are enclosed by membrane apparently derived from the endoplasmic reticulum to form the typical zymogen granule.

Two mechanisms have been proposed to account for the extrusion of secretory material. One is that the secretory granule simply becomes discontinuous, and the secretory material released within the cell is transported or diffuses across the plasma membrane. For example, Lever and Findlay (1966) observed a discontinuity in the secretory granules of adrenomedullary and β-pancreatic cells, and similar "scroll-shaped" granule membranes have also been observed by Bindler et al. (1967). Daniel and Lederis (1963) have suggested, on the basis of increased diffusability of neurohypophysial hormones after ether anaesthesia and other drug treatments, that these peptide hormones are released inside the nerve endings from their secretory granule binding sites. There is considerable evidence, however, that secretory material is extruded, at least in some cells, by the process of reverse pinocytosis or emiocytosis, whereby the secretory granule fuses with the plasma membrane and the secretory material released from the cell through a breakdown of the surface at the point of contact. This process has been visualized, using electronmicroscopy, in the AP (Farquhar, 1961), adrenomedullary (De Robertis and Sabatini, 1960), and pancreatic exocrine and β-islet cells (Palade, 1960; Lacy and Hartcroft, 1959).

Recently it has been observed that nucleotides and a specific protein contained within the chromaffin granules of adrenomedullary cells, are released along with catecholamines when these cells are stimulated either electrically or with cholinergic drugs (Douglas and Poisner, 1966;

Kirshner et al., 1966). Since neither nucleotide or protein could be expected to diffuse across the plasma membrane, these findings support the concept of emiocytosis as the operative mechanism, as least in adrenomedullary, and probably other cells containing secretory granules.

METABOLISM IN SECRETORY CELLS

Certain metabolic changes occur in secretory cells during the secretory cycle. Such changes have led to the suggestion that the phosphorylase system, phospholipid metabolism, and the HMP shunt play some fundamental role in the secretory process.

a. Phosphorylase and cyclic-3'5'-AMP

The presence of the phosphorylase system and cyclic-3'5'-AMP has been confirmed in nearly every cell where it has been examined, and its role in smooth, skeletal, and cardiac muscle contractility has been extensively studied (Sutherland & Rall, 1960). Epinephrine is very potent in many different cells in stimulating the activation of phosphorylase via adenyl cyclase which catalyzes the formation of cyclic-3'5'-AMP. Some experimentation has also been carried out on the role of this system in the adrenal cortex. Haynes and Berthet (1957) originally postulated that the stimulation of steroid levels were closely correlated with the concentration of NADPH. According to their hypothesis, the increased levels of NADPH are generated from the catabolism, via the HMP shunt, of hexose-phosphate derived from glycogenolysis. This theory is consistent with the concept that a high reducing capacity in the adrenal cortical cell is essential for the hydroxylation steps in steroid synthesis. Direct evidence that this system is not directly involved in steroidogenesis was obtained by Yago et al. (1963), who found ACTH actually inhibited active phosphorylase

levels, and by Harding and Nelson (1964a; 1964b), who found that there was no correlation between steroid secretion and the concentration of NADPH-generating systems. A site of action of ACTH alternative to the stimulation of phosphorylase was suggested by Riley and Haynes (1963), since they found that cyclic AMP inhibited the inactivation of phosphorylase but had no apparent effect on activation. Even in this model, however, higher levels of phosphorylase and NADPH would still be expected. Roberts and coworkers (1964) found cyclic AMP stimulated 11-β-hydroxylase activity, indicating cyclic AMP might act without necessarily activating phosphorylase.

Recently, Bdolah and Schramm (1965) and Babad et al. (1967) have investigated the role of cyclic AMP as an intermediate in the stimulation of secretion of amylase from rat parotid gland in vitro. They found that caffeine and theophylline, agents which inhibit the inactivation of cyclic AMP, were as effective as epinephrine in stimulating the secretion of amylase. While cyclic AMP had little effect on amylase secretion, presumably because of low permeability, cyclic AMP derivatives which are able to permeate the cell membranes more readily were effective in stimulating secretion. This approach is an interesting one, but much more work is needed to clarify the role of this compound in secretion.

The general importance of the phosphorylase system in providing hexosephosphates for cell metabolism is well established, but the role of this system in specialized processes such as steroidogenesis, muscle contraction, and secretion is unknown. The possible importance of cyclic AMP as an intermediate, unrelated to phosphorylase activation, in some cell responses may also be an important consideration in elucidating the role of this system.

b. Phospholipid metabolism

Hokin and Hokin have demonstrated in a number of different tissues that there is a correlation between phospholipid turnover and secretion. For example, acetylcholine, noradrenaline, and adrenaline, which stimulate mucin and amylase secretion in salivary glands, also stimulate the incorporation of P^{32} into phospholipid (Hokin and Sherwin, 1957). Similar results were obtained with acetylcholine and pancreozymin on pancreas slices (Hokin and Hokin, 1951), acetylcholine on the adrenal medulla and nervous tissue (Hokin and Hokin, 1955), and in the adenohypophysis using corticotrophin-releasing factor (Hokin et al., 1958). In all cases, atropine blocked the phospholipid and secretory effects of acetylcholine and other cholinergic drugs, and dibenamine blocked the secretory but not the phospholipid effect of catecholamines (Hokin and Sherwin, 1957). Hokin and Hokin suggested that the phospholipid effect may be concerned with some step prior to the final step in the secretory process, i.e., prior to emiocytosis. This was based on the findings in pancreatic slices that omission of calcium from the medium prevented the secretion of amylase by acetylcholine, but not the increased incorporation of P^{32} into phospholipid (Hokin, 1966). Since ACTH does not enhance phospholipid metabolism in the adrenal cortex, which differs from the above tissues in that it does not store hormone in secretory granules (Hokin et al., 1958), the phospholipid effect in secreting cells may be concerned with some aspect of hormone storage.

c. Hexosemonophosphate shunt

The observations that the HMP shunt is present in all endocrine glands and can be modified by agents which enhance secretion has aroused interest in the possible role of the pathway in secretion. The HMP shunt

is a direct oxidative pathway for glucose, serving primarily to supply NADPH, a cofactor used in reductive synthesis, and ribose sugars, which are precursors for nucleic acid synthesis. Through evolution, the HMP shunt has become important as a biosynthetic pathway, supplying through NADPH, hydrogen for fatty acid synthesis, steroid synthesis, and in aromatic hydroxylation reactions such as in the formation of tyrosine (Horecker, 1967). In the erythrocyte this pathway is of considerable importance in providing NADPH for the reduction of methemoglobin (Marks, 1967).

The presence or absence of the HMP shunt has been evaluated using a number of different techniques, such as chemical analysis of glucose-6-dehydrogenase or 6-phosphogluconate dehydrogenase, and also by measuring certain parameters of ^{14}C-glucose oxidation, such as the production of $^{14}CO_2$ from ^{14}C-1 or ^{14}C-6-glucose. High C-1:C-6 $^{14}CO_2$ ratios, providing qualitative evidence for the presence of the HMP shunt, have been noted in a number of different organs (Bloom, 1955) and a high C-1:C-6 oxidation ratio is a common feature of all endocrine glands studied (Field et al., 1960a).

Methods for quantitating the relative activity of this pathway have been presented by Katz and Wood (1963). The contribution of this pathway to total glucose catabolism has been measured in a number of different organs. It is highly active in the mammary gland (Wood et al., 1965) and in adipose tissue (Katz et al., 1966), both having a high rate of lipid synthesis. The relative activity of the HMP shunt has also been determined in the adrenal cortex (Weaver and Landau, 1963), the pancreatic islet cells of the goosefish (Hostetler et al., 1966), and the thyroid gland (Dumont and Elroy, 1966a; 1966b). The presence of an active HMP

shunt is thus apparently a feature of all endocrine glands, and possibly is related in some way to the secretory potential of gland cells. Since this pathway is important in supplying NADPH for lipid synthesis (Katz and Rognstad, 1966), it is perhaps at least in part concerned with the active membrane changes which occur during secretion.

The effect of some agents on the activity of the HMP shunt has been examined in a number of endocrine glands. In the thyroid, acetylcholine (Pastan et al., 1961), catecholamines (Pastan et al., 1962), and TSH (Field et al., 1960b), stimulate the oxidation of ^{14}C-1-glucose to a greater extent than ^{14}C-6-glucose, suggesting increased HMP shunt activity. Since these agents stimulate secretory activity in the thyroid, this group suggested a possible relationship between this pathway and secretion. Similarly, Dumont and Elroy (1966a) found in thyroid slices in vitro that TSH markedly stimulated the HMP shunt but did not modify the activity of the EM and Krebs pathways. Barondes et al. (1961) reported that catecholamines and serotonin stimulate ^{14}C-1 oxidation in AP. Keen et al. (1965) reported increased glucose concentration specifically stimulated the HMP shunt in rat pancreatic islet cells, although Hostetler et al. (1966) found that glucose concentration had no effect on the activity of this pathway in goosefish islet cells. ACTH was reported by Weaver and Landau (1963) to stimulate all pathways for glucose oxidation to approximately the same extent in the adrenal cortex. These results indicate that the HMP shunt can be modified by agents which influence secretion. However, the specificity of such an effect has in most cases not been adequately studied, and its relationship to secretion is speculative at present.

III. QUANTITATIVE ESTIMATION OF THE HEXOSEMONOPHOSPHATE SHUNT IN BOVINE ANTERIOR PITUITARY, POSTERIOR PITUITARY, PINEAL BODY, AND BRAIN.

INTRODUCTION

An important route for glucose catabolism in endocrine glands is apparently the HMP shunt, as indicated by the observation that carbon of the 1-position (C-1) is more readily converted to CO_2 in these tissues than is carbon of the 6-position (C-6). This is a result of the fact that in the HMP shunt, the C-1 of glucose is split off as CO_2 in the first cycle, while in the EM pathway, due to isotopic equilibrium of the triose-phosphates, both the 1- and 6-C-atoms end up in the methyl group of pyruvate, and consequently equal amounts of CO_2 are produced via the Krebs cycle from glucose labelled either in the 1- or 6-position. Thus if the HMP shunt is operative as well as the EM-Krebs pathways, glucose labelled in the 1-position would yield more $^{14}CO_2$ than glucose labelled in the 6-position. C-1:C-6 oxidation ratios ranging from 20 to 50 have been reported, for example, for thyroid (Field et al., 1960b) as well as for testes, ovary, AP, parathyroid, and adrenal (Field et al., 1960a). On the other hand, ratios close to unity have been reported for kidney and skeletal muscle (Bloom and Stetten, 1953) and brain (Bloom, 1955; Hotta, 1962). Haynes et al. (1959) have implicated the HMP shunt in the regulation of steroidogenesis in the adrenal, and Field et al. (1960a) have suggested that this pathway may be concerned with hormone synthesis by endocrines in general. Redman and Hokin (1959) demonstrated that phospholipid turnover is stimulated in actively secreting cells and that this effect occurs in the membrane fraction of tissue homogenates. Since NADPH, generated via the HMP shunt is an important cofactor in fatty acid synthesis, the activity of this pathway may be related to the enhanced lipid turnover of membranes which occurs during secretion.

The neurosecretory cells of the neurohypophysis are functional

nerve cells embryologically related to brain neurons, but in addition they serve an endocrine function. In these cells the components which synthesize the neurohypophysial peptide hormones are anatomically distinct from those portions which store the neurosecretory products to release them upon appropriate stimulation. This organ would thus appear to be ideal for examining glucose metabolism in each of the functionally distinct parts of the same cell, i.e., the perikaryon within specific hypothalamic nuclei, and the axon terminations within the posterior lobe of the pituitary gland. The C-1:C-6 oxidation ratio has been reported for anterior (Field et al., 1960a; Barondes et al., 1961; Dumont, 1960) but not posterior pituitary tissue.

In our laboratory we have been investigating storage and release of AP and PP hormones, as well as the enzymic and metabolic properties of the respective glands. The conversion of ^{14}C-1-labelled and ^{14}C-6-labelled glucose to $^{14}CO_2$ has been examined in vitro in tissue slices from both lobes of bovine pituitary gland, in isolated neurohypophysial nerve terminals, and in slices from various areas of bovine brain including the region of the paraventricular nuclei.

While the physiological role of the PB in higher vertebrates has not yet been clearly established, a great deal of evidence has been amassed (Kitay and Altschule, 1954) which points to an endocrine function for this organ. In more primitive vertebrates this structure served, and still does to a considerable extent in sub-mammalian species, as a light-sensitive organ. In mammals, the PB apparently no longer subserves this direct sensory role, and its histological appearance supports the suggestion that it has evolved into a secretory organ (Owman, 1960; de Robertis and Pellegrino de Iraldi, 1961). Although the PB is known to contain

high concentrations of 5-hydroxytryptamine (serotonin) and melatonin, an endocrine function for this organ has not been unequivocally demonstrated. The effects of pinealectomy on gonadal function have been equivocal, but recent studies (Wurtman et al., 1963; Zweens 1963) indicate that there is a relationship between the PB and the sex glands. Because there is no known exception to the rule that endocrine tissues have high C-1:C-6 glucose oxidation ratios, the proposal of an endocrine function for the PB would be strengthened by the finding of a high ratio in that organ. Furthermore, because the presumed glandular activity of the PB is believed to be more prominent in the young (Owman, 1960), ^{14}C-1- and ^{14}C-6-glucose oxidation was measured in this organ, as well as in AP, PP, and brain of both young and old animals.

Although complications such as recycling of hexose-phosphate into the HMP shunt, and the existence of alternative pathways for glucose-6-phosphate metabolism limit the interpretation of data obtained from the use of ^{14}C-1- and ^{14}C-6-glucose, it is generally accepted that a C-1:C-6 oxidation ratio greater than unity provides qualitative evidence for the presence of the HMP shunt (Katz and Wood, 1960; Freinkel, 1964). Recently, Katz and coworkers (1963; 1966) have proposed a method for estimating the contribution of the HMP shunt to total glucose catabolism, based on the specific activity of $^{14}CO_2$ from ^{14}C-1- and ^{14}C-6-labelled glucose. Using this method we have also made quantitative estimations of the activity of the HMP shunt in the bovine AP, PP, PB, and brain cortex.

METHODS AND MATERIALS

Tissues.

Bovine PB's, pituitary glands and brains were obtained at a nearby slaughterhouse approximately 30 minutes after death of the animals. The tissues were transported to the laboratory in vessels surrounded by ice, the trip taking about 20 min. Each lobe of the pituitary was isolated, freed of connective tissue and fat, and blotted on filter paper to remove blood. PB's were cleaned in a similar fashion. The brain was dissected to reveal the ventricle, and the two adjacent paraventricular nuclei, visibly demarcated in the fresh brain, were carefully dissected out with a razor blade as free of surrounding tissue as possible. Tissues were not separated according to age or sex except where indicated. "Young" animals were estimated to be 5-10 months old and "adult" animals, 3-8 years old. For a given experiment "young" or "adult" tissue was pooled from several animals. Isolated nerve endings were prepared by centrifuging homogenates of posterior pituitary at 4200 g/15 min as previously described (LaBella and Sanwal, 1965).

^{14}C-labelled glucose.

Crystalline glucose - ^{14}C-1 and glucose - ^{14}C-6 (14 μC/mg) were obtained from New England Nuclear Corp. The glucose was dissolved in 0.9% NaCl and frozen in a concentration of 2.5 μC/ml. The immediate addition of H_2SO_4, the reagent employed to terminate the tissue incubation reaction and drive off $^{14}CO_2$, to a solution containing ^{14}C-glucose but no tissue resulted in the evolution of a labile ^{14}C-labelled substance. The absolute amount of radioactivity which contaminates $^{14}CO_2$ evolved during incubation with tissue varies in different experiments from 200 to 1000 dpm/0.5 μC. To determine whether a radioactive impurity was present

in the glucose, thin layer chromatography in two different solvent systems resolved only a single detectable radioactive spot corresponding to glucose. The labile ^{14}C-labelled contaminant has been found in every batch of ^{14}C-1 or ^{14}C-6-glucose purchased from the above supplier. This background activity can be substracted and does not interfere insofar as counts due to $^{14}CO_2$ produced by tissue slices are significantly higher. This labile contaminant has been noted also by Hoskin (1960) and could seriously interfere with studies in which $^{14}CO_2$ activity is of a low order.

Collection of $^{14}CO_2$ produced during incubation of tissue slices or subcellular fractions.

Tissues were sliced with a razor blade and diced into 0.2 mm squares with a McIlwain Tissue Chopper. The slices were preincubated at 37°C for 15 min under an atmosphere of 95% O_2/5% CO_2 in a single large flask with Krebs-bicarbonate medium pH 7.4 containing 2 mg glucose/100 ml. The medium was decanted and 50 mg aliquots of the blotted tissue placed into 25 ml serum bottles containing medium and test substances to a final volume of 5.0 ml. One-half μC ^{14}C-glucose in 0.2 ml was added to each bottle. Subcellular fractions were suspended in Krebs-bicarbonate so that the desired amount of fraction was contained in 2.0 ml. Trapping and counting of $^{14}CO_2$ was carried out in the apparatus described by Cuppy and Crevasse (1963). The bottles were gassed with 95% O_2/5% CO_2 and quickly sealed with rubber stoppers from which were suspended stainless-steel vial mounts containing glass center-wells. The bottles were incubated at 37°C for 30 min in a Dubnoff Shaker, 0.3 ml 6N H_2SO_4 injected through the stopper to stop the reaction and evolve $^{14}CO_2$, and 0.3 ml hyamine hydroxide injected through the stopper into the center-well.

The bottles were shaken for 60 min at room temperature, the center wells removed and placed in counting vials containing 0.4% PPO and 0.01% POPOP in toluene.

Determination of glucose utilization

Bovine PB's, pituitaries, and brain cortex were obtained and prepared as outlined above. Tissue aliquots (100-300 mg) were placed in 3 ml of Krebs-Henseleit buffer, pH 7.4, containing 50 mg % glucose. One to two μC of ^{14}C-1 of ^{14}C-6 glucose was added to each flask. Incubations were carried out at 37°C under 95% O_2 - 5% CO_2.

$^{14}CO_2$ was collected as described above. The procedures used for estimating glycerol, fatty acids, and lactate were similar to those outlined previously by Katz et al. (1966). After the $^{14}CO_2$ was collected, the incubation medium and tissue were decanted and filtered with gentle suction to separate the tissue fragments from the medium. The tissue was washed with water several times, and the filtrate made up to a volume of 10 ml (aqueous fraction).

For lipid extraction the tissue was transferred to a 10 ml chloroform-methanol (2:1) solution. The tissue was extracted for 12 hours at 40°C, the solvent decanted, and the tissue re-extracted with fresh solvent overnight. The combined lipid extract was then evaporated to dryness, and the residue taken up in 2 ml water and 10 ml heptane. The water phase was removed and the heptane washed several more times with water to remove any water-soluble contaminants. The heptane, containing the lipid extract, was dried with anhydrous sodium sulfate to remove water, and evaporated to dryness. The lipid extract was then saponified overnight in ethanolic 0.5 N NaOH at 60°C, the ethanol driven off, the solution acidified to pH 2-3 with H_2SO_4, and water and heptane

added. After the phases separated, aliquots of the water phase (containing glycerol) and of the heptane phase (containing the fatty acids) were removed for counting.

Total ^{14}C in the tissue residue (containing glycogen and supporting tissue) was determined by counting and aliquot of the residue dissolved in 1 N KOH.

Lactate was estimated by taking a 1 ml aliquot of the original aqueous fraction, diluting it with water to 10 ml, and precipitating the glucose with 2 ml 10% copper sulfate and excess calcium hydroxide. An aliquot of the supernatant (containing mainly lactate) was removed for counting. ^{14}C-glucose controls were run in order to correct for contamination.

Total glucose (non-labelled) in the medium was measured according to the method described by Hoffman (1937).

Counting of radioactive samples.

Radioactive aliquots of the various fractions were added to vials containing 0.4% PPO and 0.01% POPOP in toluene and counted in a Packard Liquid Scintillation Spectrometer. Aqueous solutions were added to a mixture of the scintillation fluid and ethylene glycol monomethyl ether, and the counts were corrected for quenching by external standardization.

RESULTS

a. Oxidation of ^{14}C-1 and ^{14}C-6-glucose by the hormone synthesizing and hormone secreting portions of neurohypophysial neurons.

On a weight basis, PP slices metabolized C-1 of glucose to CO_2 more readily than did tissue from AP (Table 1, Expt. 1), cerebral cortex (Table 1, Expt. 1), and hypothalamus (Table 1, Expt. 3). High oxidation ratios of C-1:C-6 occurred for both anterior and posterior pituitary tissue, whereas ratios close to 1 were found for brain. Thus, brain oxidized C-6 of glucose to CO_2 much more actively than either lobe of the pituitary. Tissue from the paraventricular nucleus gave almost identical ratios as did cortex and other areas of the hypothalamus (Table 1, Expt. 4 and 5), although metabolizing exogenous glucose at a significantly higher rate. Hypothalamic tissue was obtained several cm from the paraventricular nucleus (Table 1, Expt. 3) and in two experiments (Expt. 4 and 5) only a few mm away. The C-1:C-6 ratios were very similar irrespective of the area of the hypothalamus chosen.

Isolated nerve endings from the posterior pituitary gave C-1:C-6 ratios similar to those obtained with slices of this gland (Table 2). The progressively decreasing C-1:C-6 oxidation ratios determined in the more slowly sedimenting fractions (Table 2, Expt. 2) apparently reflects the greater proportion of free mitochondria to nerve endings, the former particles being much more active with respect to C-6 oxidation. Considerable day-to-day variability in the metabolic activity of the tissues was encountered, but this has been noted previously by others (Barondes et al., 1961).

Epinephrine stimulated the metabolism of C-1 of glucose, but not of C-6, in all three tissues (Table 3). The percentage stimulation of C-1 oxidation by epinephrine was similar for PP slices (Table 1,

TABLE 1

$^{14}CO_2$ PRODUCTION FROM ^{14}C-1- AND ^{14}C-6-GLUCOSE IN VITRO BY ANTERIOR PITUITARY, POSTERIOR PITUITARY AND BRAIN

		$^{14}CO_2$ CPM/GM/30'		
EXPT.	TISSUE	^{14}C-1	^{14}C-6	^{14}C-1/^{14}C-6
1	A.P.	6,500	600	11
	P.P.	91,900	6,600	14
	Cortex	67,700	53,000	1.3
2	A.P.	12,700	620	20
	A.P. +Epi.	35,900 (183%)	---	
3	P.P.	290,000	13,500	21
	P.P. +Epi.	413,000 (42%)	---	
	Hypothal.	95,400	81,900	1.2
	Hypothal. + Epi.	321,000 (236%)	---	
4	PVN	316,000	279,000	1.1
	Hypothal.	253,000	235,000	1.1
5	PVN	165,000	152,000	1.1
	Hypothal.	143,000	104,000	1.4

Each value is the mean of duplicates.
A.P. - anterior pituitary, P.P. - posterior pituitary
Hypothal. - hypothalamus, PVN - paraventricular nucleus
Epi. - epinephrine, 10^{-4} M.
Values in parentheses represent % increase in counts with epinephrine.

TABLE 2

$^{14}CO_2$ PRODUCTION FROM ^{14}C-1- AND ^{14}C-6-GLUCOSE IN VITRO BY SUBCELLULAR FRACTIONS FROM POSTERIOR PITUITARY GLAND.

		$^{14}CO_2$ CPM/MG PROTEIN/30'		
EXPT.	FRACTION	^{14}C-1	^{14}C-6	^{14}C-1/^{14}C-6
1	Nerve endings	47,900	3,500	14
2	Nerve endings	13,300	1,100	12
	Nerve endings + Epi.	17,600 (32%)	---	
	Heavy mito.	8,100	1,100	7
	Light mito.	1,900	312	6

Epi. - epinephrine, 10^{-4}M
Value in parentheses represents % increase in counts with epinephrine.

Each value is the mean of duplicates.

TABLE 3

EFFECT OF EPINEPHRINE ON $^{14}CO_2$ PRODUCTION
FROM ^{14}C-1- AND ^{14}C-6-GLUCOSE IN VITRO
BY ANTERIOR PITUITARY, POSTERIOR PITUITARY, AND HYPOTHALAMUS

		$^{14}CO_2$ CPM/GM/30'		
TISSUE	EPINEPHRINE [M]	^{14}C-1	^{14}C-6	^{14}C-1/^{14}C-6
A.P.	0	15,800	860	18
	10^{-6}	15,700	820	19
	10^{-5}	19,000	690	28
	10^{-4}	27,800 (76%)	860	32
P.P.	0	381,000	9,780	39
	10^{-4}	777,000 (104%)	10,600	74
PVN	0	219,000	209,000	1.1
	10^{-4}	379,000 (73%)	205,000	1.8

Each value is the mean of duplicates.
Values in parenthesis represent % increase
in counts with 10^{-4} M epinephrine.

Expt. 3) and for isolated nerve endings from that gland (Table 2, Expt. 2), when these two preparations were tested at the same time. In another experiment, however, oxidation of C-1 by PP slices was stimulated 104% by the same concentration of epinephrine (Table 3).

b. Oxidation of ^{14}C-1 and ^{14}C-6-glucose by pineal body, pituitary, and brain from young and adult animals.

C-1:C-6 ratios for PB's ranged from 6.9 to 23.9 and were always higher for young as compared to adult beef (Table 4). Total glucose oxidation (both labels) per unit weight of pineal tissue was greater for young animals. In the only experiment (Expt. 5) in which PB's were separated according to sex, there was no sex difference in oxidation of either ^{14}C-1- or ^{14}C-6-glucose.

Epinephrine stimulated the oxidation of both ^{14}C-1 and ^{14}C-6 by pineal tissue, although the former label was much more affected (Table 5). There did not appear to be any marked age effect with respect to the action of epinephrine.

Oxidation of labelled glucose by brain, AP, and PP was compared to that by pineal tissue (Table 6). Brain tissue taken from the region immediately adjacent to the pineal body gave a C-1:C-6 of 1.0 (Expt. 10, Dienc.), similar to the ratio for cortex and other regions of the brain. Glucose oxidation (both labels) by PP was consistently lower in adult animals (Expts. 8,9, 11-14), similar to findings with the PB. However, in the former tissue C-6 oxidation was reduced proportionately more than was C-1 oxidation (increased ratio) in all but one experiment, whereas in the latter the reverse was true (decreased ratio). Glucose oxidation by brain cortex was higher in the adult than in the young animal (Expts. 8 and 9). AP glands from young animals oxidized ^{14}C-1 to a greater

TABLE 4

OXIDATION OF ^{14}C-1- AND ^{14}C-6-GLUCOSE IN VITRO BY PINEAL BODIES FROM YOUNG AND ADULT ANIMALS.

EXPT.	PINEAL	$^{14}CO_2$ CPM/GM/30'		
		^{14}C-1	^{14}C-6	^{14}C-1/^{14}C-6
1	Young	56,800	4,610	12.3
	Adult	41,800	4,560	9.2
2	Young	59,100	7,140	8.3
	Adult	29,600	4,300	6.9
3	Young	101,000	8,450	12.0
	Adult	47,600	4,760	10.0
4	Young	73,800	7,640	9.7
	Adult	35,100	4,160	8.4
5	Young male	208,000	9,450	22.0
	Young female	208,000	8,700	23.9

Each value is the average of triplicates.

TABLE 5

EFFECT OF EPINEPHRINE ON THE OXIDATION OF ^{14}C-1- AND ^{14}C-6-GLUCOSE IN VITRO BY PINEAL BODIES FROM YOUNG AND ADULT ANIMALS.

			$^{14}CO_2$ CPM/GM/30'		% Increase by Epinephrine		^{14}C-1/^{14}C-6	
EXPT.	PINEAL	EPI.	^{14}C-1	^{14}C-6	^{14}C-1	^{14}C-6	Contr.	Epi.
6	Young	-	55,300	7,650	171	41	7.2	13.9
	Young	+	150,000	10,800				
	Adult	-	32,200	5,160	157	33	6.2	12.0
	Adult	+	82,600	6,870				
7	Adult	-	144,000	24,600	183	63	5.9	10.1
	Adult	+	408,000	40,200				

Each value is the average of triplicates.

Contr, - control.

EPI. - epinephrine, 10^{-4} M.

TABLE 6

OXIDATION OF ^{14}C-1- AND ^{14}C-6-GLUCOSE IN VITRO BY ANTERIOR PITUITARY AND POSTERIOR PITUITARY FROM YOUNG AND ADULT ANIMALS

EXPT.	TISSUE	$^{14}CO_2$ CPM/GM/30' ^{14}C-1	^{14}C-6	^{14}C-1/^{14}C-6
8	AP - Young	15,500		
	AP - Adult	15,600		
	PP - Young	433,000		
	PP - Adult	290,000		
	Cortex - Young	140,000		
	Cortex - Adult	215,000		
9	PP - Young	278,000	46,400	6.0
	PP - Adult	171,000	17,100	10.0
	Cortex - Young	95,500	70,500	1.4
	Cortex - Adult	153,000	139,000	1.1
10	Dienc. - Adult	225,000	223,000	1.0
	Cortex - Adult	127,000	88,000	1.4
11	PP - Young	296,000	49,600	6.0
	PP - Adult	192,000	28,700	6.7
12	AP - Young	10,600		
	AP - Adult	4,900		
	PP - Young	175,000	15,700	11.1
	PP - Adult	146,000	15,500	9.4
13	AP - Young	15,700		
	AP - Adult	12,000		
	PP - Young	231,000	26,000	8.9
	PP - Adult	198,000	13,500	14.7
14	AP - Young	14,100		
	AP - Adult	8,900		
	PP - Young	216,000	47,500	4.6
	PP - Adult	115,000	15,400	7.5
15	AP - Young	21,400	1,690	12.7
	AP - Adult	13,100	783	16.7

Each value is the average of triplicates.
AP - anterior pituitary. PP - posterior pituitary.
Dienc. - diecephalon.

TABLE 7

WEIGHTS OF PINEAL BODIES, ANTERIOR PITUITARIES, AND POSTERIOR PITUITARIES FROM YOUNG AND ADULT ANIMALS

Organ	Weight*	
	Young	Adult
	range (mg)	
Pineal	65-130	190-350
AP	540-650	1900-4100
PP	120-190	270-430

* Ten organs per group.

extent than those from adults in 4 of 5 (Expts. 12 - 15) experiments; in the other experiment (Expt. 8) there was little difference in ^{14}C-1 oxidation between young and adult. In experiments 12, 13, and 14 (Table 6) counts of radioactivity from C-6 oxidation by AP were too low to permit the calculation of accurate, meaningful C-1:C-6 ratios, but in all instances ^{14}C-6 conversion to $^{14}CO_2$ was lower in AP from the adult than in that from the young. In the last experiment (Expt. 15), twice as much tissue (200 mg) as usual was employed and incubation was extended to 45 min in order to increase ^{14}C-6 conversion to $^{14}CO_2$ and thereby permit the determination of more accurate C-1:C-6 ratios. In this instance C-6 oxidation was apparently proportionately lower than C-1 oxidation in the adult, as evidenced by the higher C-1:C-6 ratio. Organ weights for young and adult animals are presented in Table 7. PB's, AP's and PP's were all significantly larger in the adult.

c. Quantitative estimation of HMP shunt in anterior pituitary, posterior pituitary, pineal body, and brain.

The distribution of ^{14}C in CO_2, fatty acids, glycerol and lactate resulting from the metabolism of ^{14}C-glucose is shown in Table 8. Specific yield refers to the fraction of the total glucose utilized which is recovered in a given product. While the replicate samples agreed quite closely within experiments, there were some discrepancies between experiments 1 and 2, particularly in the yields of lactate and glycerol. ^{14}C-glucose contamination of the supernatant containing lactate may account for some of the variability of lactate, but we have no explanation for the variability of the glycerol yields. It is not likely due to water soluble contaminants, since the lipid washings contained little radioactivity.

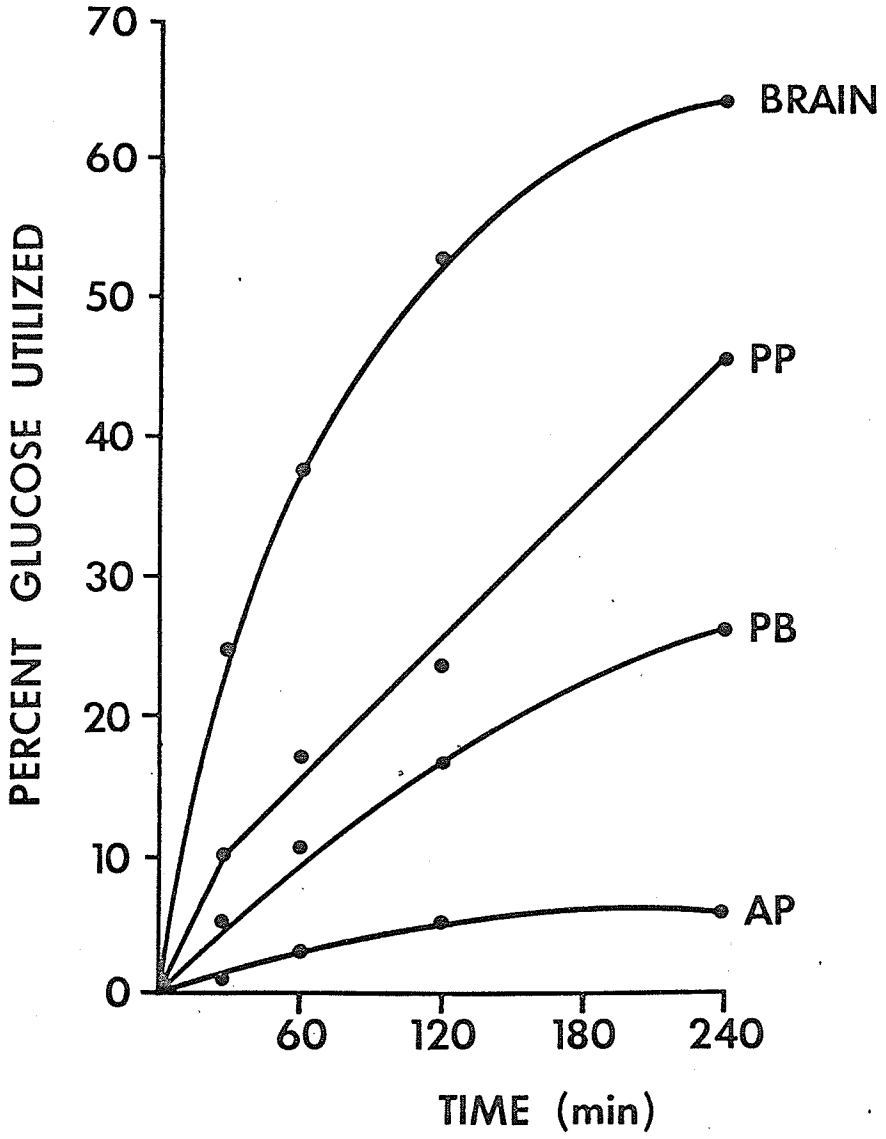


Figure 1. Rate of glucose utilization by AP, PP, PB and brain cortex. The medium contained 50 mg % glucose. The results are the averages of duplicate determinations expressed as % of the total glucose utilized.

TABLE 8

RELATIVE INCORPORATION OF C^{14} OF GLUCOSE INTO CO_2 LACTATE, FATTY ACIDS AND GLYCEROL

		GLUCOSE UTILIZATION (CPM)					SPECIFIC YIELD			
EXPT. 1										
Tissue	Glucose Label	CO_2	Lactate	Fatty Acids	Glycerol	Total	CO_2	Lactate	Fatty Acids	Glycerol
AP	1	17,400	87,600	1,100	44,600	151,000	.120	.58	.007	.30
	6	2,900	57,300	1,400	73,900	136,000	.021	.42	.010	.55
PP	1	92,200	600,000	1,600	82,100	776,000	.12	.77	.002	.11
	6	17,900	835,000	3,300	33,200	889,000	.02	.94	.004	.04
PB	1	31,700	866,000	3,200	31,500	932,000	.034	.93	.003	.034
	6	8,200	1,250,000	5,400	45,500	1,310,000	.005	.95	.004	.035
Brain	1	150,000	1,500,000	6,000	14,100	1,700,000	.090	.90	.004	.008
	6	140,400	1,530,000	4,700	55,800	1,731,000	.082	.88	.003	.032
EXPT. 2										
AP	1	22,400	102,000	2,100	35,400	162,000	.140	.63	.013	.22
	6	3,200	128,000	2,900	62,900	197,000	.016	.65	.015	.32
PP	1	93,200	535,000	3,400	57,200	694,000	.140	.77	.004	.082
	6	19,000	527,000	3,800	44,300	594,000	.032	.89	.006	.075
PB	1	40,200	888,000	4,000	43,300	976,000	.041	.90	.004	.043
	6	8,600	891,000	6,700	18,200	924,000	.009	.96	.007	.020
Brain	1	159,000	1,410,000	4,300	52,900	1,630,000	.098	.87	.003	.032
	6	148,000	1,630,000	4,100	78,900	1,860,000	.080	.88	.002	.042

200 mg tissue in 3 ml was incubated for 2 hours in the presence of either 2 μC of ^{14}C-1 or ^{14}C-6 glucose
Each value is the mean of triplicates

In AP, PP, and PB the CO_2 yield from ^{14}C-1 glucose was considerably greater than from ^{14}C-6 glucose, and the fatty acid yield from ^{14}C-6 glucose was greater than from ^{14}C-1 glucose, indicating an operative HMP shunt in these organs. In brain the CO_2 yields from ^{14}C-1 glucose and ^{14}C-6 glucose were nearly equal, and the fatty acid yields from ^{14}C-1 glucose were greater than from ^{14}C-6 glucose, suggesting that in this tissue there was negligible HMP shunt activity. In all four tissues glucose catabolism appears to be via the EM pathway primarily, since lactate was the major metabolite. The presence of an active glycolytic pathway in B has been well documented (McIlwain, 1966).

The rate of exogenous glucose utilization was greater in the PP than in the PB (Table 9), based on the more accurate method of measuring glucose disappearance from the medium, rather than less as indicated by the data based on ^{14}C utilization (Table 8). Comparison of the total glucose utilized (Fig. 1) indicates that over a 4 hour incubation period the rate of glucose uptake in all 4 tissues was fairly constant. Since glucose disappearance from the medium was a more reliable measure of glucose utilization, we used this method to calculate the specific yield (relative specific activity) of CO_2 from ^{14}C-1- and ^{14}C-6-glucose for estimations of the relative activity of the HMP shunt as described by Katz and coworkers (1960; 1963). The results (Table 9) indicate that in the AP, PP, and PB 3-5% of total glucose is catabolized via the HMP shunt in vitro. In brain cortex the activity of this pathway is negligible. The contribution of non-triose-phosphate (NTP) pathways has been disregarded in these calculations. This simplification can be made if NTP contribution is not large, i.e., $< 20\%$ (Katz and Wood, 1963). Assuming that ^{14}C in glycogen, proteins, and nucleoproteins is a reflection of

NTP contribution (Table 10), this simplification would appear to be valid.

Although the glycerol yields were somewhat variable, in all four tissues considerably more glycerol was synthesized than was required for esterification of fatty acids. This apparent anomaly may be due to a recycling of endogenous fatty acids to form triglycerides. In AP, fatty acid and glycerol yields from both ^{14}C-1-and ^{14}C-6-glucose were much more greater than in any other organ suggesting high rate of lipid synthesis.

TABLE 9

CALCULATION OF HMP SHUNT CONTRIBUTION TO GLUCOSE CATABOLISM

Tissue	Glucose Label	Glucose Utilized (%)	^{14}C Recovered in $^{14}CO_2$ (%)	Specific Yield	% Contribution of the HMP shunt*
AP	1	7	1.0	.14	5
	6	9	.16	.02	
PB	1	17	1.9	.11	3
	6	19	.33	.02	
PP	1	34	4.9	.14	4
	6	32	.93	.03	
Brain	1	53	6.0	.11	< 1
	6	51	4.5	.09	

300 mg tissue in 3 ml. was incubated for 2 hours in the presence of either 2 μC of ^{14}C-1- or ^{14}C-6-glucose. The total glucose utilized was obtained by measuring the disappearance of non-labelled glucose from the medium.

Each value is mean of duplicates.

* According to the following equation of Katz and Wood (1960):

$$\frac{G1CO_2 - G6CO_2}{1 - G6CO_2} = \frac{3\ HMP}{1 + 2\ HMP}$$

Using their notation, the specific yields of CO_2 from ^{14}C-1 and ^{14}C-6 glucose are designated $G1CO_2$ and $G6CO_2$, respectively.

TABLE 10

^{14}C-6 GLUCOSE INCORPORATION INTO GLYCOGEN AND SUPPORTIVE TISSUE

Tissue	Total Glucose Utilization (CPM)	^{14}C in Glycogen and Supportive Tissue (CPM)	Specific Yield of ^{14}C in Glycogen and Supportive Tissue
AP	70,800	2,800	.04
PP	330,000	600	.002
PB	444,000	400	.001
Brain	1,050,000	1,000	.001

100 mg tissue in 3 ml was incubated for 2 hours in presence of 1 μC ^{14}C-6-glucose. After the lipid was extracted, the tissue residue was dissolved in 1 N KOH. Total glucose utilization was determined by totaling the ^{14}C in CO_2, lactate, lipid, and the tissue residue.

Each value is the mean of triplicates.

DISCUSSION

The PP gland, although comprised of cells embryologically related to other neurons within the central nervous system, possesses, in common with other endocrine glands, an active HMP pathway as indicated by our studies with ^{14}C-glucose. In fact, the C-1:C-6 oxidation ratios which range from 12 to 21 in different experiments are among the highest reported. Confirmation that the measured metabolic activity of the PP is due to the neuronal terminals in the gland and not to supporting elements was provided by similar findings on isolated nerve endings. Furthermore, the degree of stimulation of oxidation induced by epinephrine was similar for slices and isolated endings from the posterior lobe.

On the other hand, the cell bodies of the neurohypophysial neurons which reside in the hypothalamus, primarily in the supraoptic and paraventricular nuclei, apparently metabolize glucose almost exclusively through the EM and Krebs cycles. This conclusion was reached through the observation that the C-1:C-6 oxidation ratio determined on isolated PVN was close to unity, conforming to results obtained with all other areas of the brain which we have studied or which have been reported. Even if significant contamination by non-neurosecretory cells existed in the isolated PVN, the C-1:C-6 oxidation ratio would presumably have been altered, at least to some extent, by the neurosecretory cell bodies if they possess as active an HMP shunt as the nerve endings. It has been reported (Moss, 1964) that in the isolated, arterially perfused bovine brain high C-1:C-6 oxidation ratios were obtained with labelled glucose. This observation must await corroboration, since it contradicts previous findings of others (Sacks, 1957) which agree with all re-

ported *in vitro* data.

A variety of experimental approaches have established that the neurohypophysial peptide hormones are synthesized in the cell bodies of the neurosecretory cells and are transported bound to a carrier protein to the axonal terminations located in the PP gland. Histological studies have shown the axonal flow of neurosecretion (Scharrer and Scharrer, 1954), and Sachs (1960) has provided evidence by the use of isotopic sulfur that vasopressin is synthesized in the cell bodies, although some additional synthesis takes place in the axoplasm and to a slight extent in the terminals. The PP serves as a storage depot for vasopressin and oxytocin and secretes the hormones upon appropriate stimulation. Our findings with the neurohypophysis would tend to support the hypothesis that an active HMP pathway in endocrine tissues is concerned with secretory activity, rather than with supplying NADPH in the synthesis of hormone as proposed by some workers (Field *et al.*, 1960a). Support for our hypothesis is provided by the work of Harding and Nelson (1964a; 1964b) who pointed out that no evidence is available to indicate that the concentration of NADPH is directly related to the rate of synthesis of adrenal steroids.

The C-1:C-6 oxidation ratio of the PB was found to be much higher than of any region of the brain which has been examined, including areas immediately adjacent to the pineal body. Ratios of 6.9 to 23.9 were found the PB, whereas the highest ratio found for brain in this study was 1.4. An active HMP pathway is thereby indicated in the PB, which thus is distinct from the brain as a whole and resembles the anterior and posterior pituitary with respect to this basic biochemical feature. Also, Thieblot *et al.*, (1966) have reported that the enzyme profile of PB and brain are quite different. These findings are consistent with the hypothesis that the PB

is an endocrine organ, since all known endocrine glands give high C-1:C-6 ratios. In total glucose oxidation activity it is intermediate to the AP, whose activity is much lower than that of brain tissue, and the posterior lobe in which the activity is somewhat higher than that of brain.

Oxidation of either glucose label per unit weight of tissue was lower in the PB of the adult than in the young animal. More significant, however, was the consistent finding that the C-1:C-6 ratio was lower in the adult. The PB is believed to be more active in the very young animal and to diminish in importance with age; in fact, some evidence suggests that the major role for this organ is exerted in prenatal life (Owman, 1960). There is some morphological evidence that the parenchymal mass of the PB decreases with age, in that progressive calcification of the pineal occurs with age, in some species at least (Bondareff, 1965; Kitay and Altschule, 1954). If the contribution of the HMP pathway to total glucose oxidation is, indeed, a direct reflection of the secretory potential of a tissue, then the lower C-1:C-6 ratio in the adult is consistent with the hypothesis of diminished activity of the PB with age. However, although the specific activity decreases with age, the total mass of the PB, as well as that of both hypophysial lobes, is greater in the adult. A shift with age in the proportion of parenchymal and stromal elements could account for the lower C-1:C-6 ratio. Clearly, quantitative relationships of glucose metabolism of these organs to functional capacity are only speculative.

There are only a few reports which deal with quantitative changes in the PB as a function of age. Hellman and Carlsson (1961) have reported that there is a decrease with age in oxygen consumption, glucose oxidation and conversion of labelled glucose to labelled amino acids in the PB of the goat. Our findings confirm their observations for glucose oxidation.

Zweens (1963) has reported a decrease in the phospholipid content with age in the PB of the rat, a finding which is consistent with our hypothesis that the level of activity of the HMP pathway represents the secretory and/or storage capacity of a particular tissue. The diminished lipid content with age may reflect a loss of membranous structures, whose synthesis is dependent upon the HMP pathway and may be related, as Zweens (1963) suggested, to the hypothesis of Hokin's group that phospholipid turnover is related to the secretory process. Wurtman et al., (1964c) reported, on the other hand, that the concentration of hydroxyindole-O-methyltransferase, monoamine oxidase, and histamine-N-methyltransferase remained constant in the PB's of humans aged 3 to 70 years. These enzymes may not be concerned with HMP pathway-dependent processes of secretion and/or storage. The finding that epinephrine stimulated the oxidation of both ^{14}C-1 and ^{14}C-6 of glucose by the PB, although the latter label was affected to a considerably lesser extent, is of interest. This is in contrast to ^{14}C-6 oxidation to $^{14}CO_2$ by brain, AP and PP, which was unaffected by epinephrine. Barondes et al., (1961) have reported that epinephrine and other neuroamines added to AP tissue in vitro stimulate oxidation of C-1 but not of C-6 of glucose to CO_2, and we have confirmed these findings. He suggested that a functional relationship exists between this metabolic pathway and hormone secretion, since the active amines are reported to stimulate adenohypophysial hormone secretion, also. However he failed to achieve any enhanced oxidation of glucose with PP hormones which are known to enhance release of AP hormones (LaBella, 1964; Martini et al., 1966). Furthermore, the concentrations of amines which were required in Barondes' studies to stimulate glucose metabolism render extrapolation of the in vitro findings to physiological situtations doubtful. Pastan and coworkers

(1962) have reported that epinephrine stimulates the oxidation of C-1, and to a lesser extent, C-6, by thyroid gland slices. They commented that the relatively high concentrations necessary to achieve this effect made it unlikely that epinephrine affects thyroid function by enhancing glucose oxidation. However, the rapid and specific effect of fairly low concentrations of TSH has led Field et al., (1960b) to speculate that the effect of TSH on the thyroid may be mediated by stimulation of HMP shunt activity.

The high ^{14}C-1:^{14}C-6 oxidation ratios, providing qualitative evidence for the presence of the HMP shunt, which were noted in the AP, PP, and PB are consistent with similar observations in other endocrine tissues, such as the parathyroid gland, adrenal cortex, ovary, and testes (Field et al., 1960a). We have also provided quantitative evidence that an active HMP shunt exists in the bovine AP, PP and PB. Dumont (1965), in an extensive study of thyroid metabolism, demonstrated an almost identical activity of the HMP shunt in thyroid slices as we found for AP, PP, and PB, and Weaver and Landau (1963) estimated that 10% of glucose is metabolized by this route in the adrenal cortex. Whereas the resting activity of this pathway in brain is very low, the finding that Synkavite, an electron acceptor (Hoskin, 1960), or catecholamines (Barondes, 1961) stimulate ^{14}C-1 glucose oxidation preferentially over ^{14}C-6 glucose suggests that the HMP shunt may be activated in this organ under certain conditions. The HMP shunt is undoubtedly present, albeit at low levels, since glucose-6-phosphate dehydrogenase has been demonstrated in many parts of the brain (Shimuzu and Abe, 1966).

Comparison of the total ^{14}C utilized indicates that the AP metabolizes much less exogenous glucose than does brain (Fig. 1). This was

observed previously by Roberts and Keller (1953). The PP and PB, two organs embryologically derived from brain, lie midway between the AP and the brain in utilization of exogenous glucose. However, HMP shunt activities in the AP, PP and PB are nearly identical (Table 10), indicating a similar pattern of glucose catabolism in these tissues. Since the AP, PP, and PB differ in their anatomical characteristics and embryological origins, this common metabolic feature may be related to their common secretory function.

In the neurosecretory cells the components which synthesize the neurohypyphysial peptide hormones are anatomically distinct from those portions which store these secretory products. ^{14}C-6 oxidation ratios observed in the posterior lobe slices were due to neuronal and not to supporting elements, since isolated nerve endings had a pattern of glucose metabolism identical to that of slices of the posterior lobe and responded almost identically to added epinephrine. Because of the distinct metabolic difference between the cell bodies located in specific hypothalamic nuclei and the nerve endings, possibly an active HMP shunt in the PP and other endocrine glands is associated with a secretory and/or storage role. The HMP shunt provides ribose sugar for the synthesis of nucleic acids (Horecker and Mehler, 1955) and is the major source of NADPH, a rate-limiting co-factor in fatty acid synthesis (White et al., 1964). It is generally considered that glucose catabolism via the HMP shunt is not a major source of energy (Pon, 1964). Other intermediates formed via the HMP pathway may be used for the synthesis of aromatic amino acids (Neish, 1960) or ascorbic acid (Burns, 1960). Actively secreting cells may require NADPH or some other substance generated by the HMP shunt for synthesis of components which are rapidly turning over. For example, the reduced

coenzyme is required for the synthesis of lipids, substances which may comprise sites that are constantly being degraded and renewed on the membranes of secretory cells. The failure to find any immediate correlation between HMP shunt activity and hormone production and/or secretion (Barondes et al., 1961; Harding and Nelson, 1964a) may be explained on the basis that this metabolic pathway is concerned with long-term renewal of secretion-linked constitutents of endocrine tissue cells. Alternatively, HMP shunt activity may be concerned with storage mechanisms of endocrine cells, perhaps to provide substrates for secretory granule or granule-membrane formation and/or maintenance. This last hypothesis has support in the conclusions of Hokin and Hokin (1966) who have recently reviewed their investigations. They believe that phospholipid synthesis which is accelerated during active zymogen secretion by pancreatic cells is concerned, not with the extrusion process per se, but with some step in the segregation of proteins in preparation for zymogen granule formation. It seems likely that substrates or cofactors generated by this pathway in actively secreting cells are necessary for the renewal of cellular components which are rapidly turning over.

Consistent with this hypothesis are the findings that in cells engaged either in phagocytosis or 'emiocytosis' (Lacy and Hartcroft, 1959) (i.e. the extrusion of secretory material by the fusion of the granule membrane with the plasma membrane) there is enhanced catabolism of glucose via the HMP shunt. Sbarra and Karnovsky (1959) demonstrated an increased ^{14}C-1:^{14}C-6 oxidation ratio in actively phagocytosing leucocytes. Recently, Rossi and Zatti (1966) confirmed this finding and concluded that stimulation of the HMP pathway is effected by an increase in the ratio of NADP/NADPH and occurs before any change is apparent in the rate of glucose metabolism

via the EM and Krebs Cycles. Dumont and Elroy (1966) have reported that HMP shunt activity is stimulated in thyroid slices by the addition of thyrotrophin, which increases the NADP/NADPH ratio. These changes were apparently coupled to resorption of the intrafollicular colloid and subsequent secretion (Dumont, 1965).

Hokin and Hokin (1965) have demonstrated a relationship between phospholipid turnover and secretion in a variety of tissues. They have implicated this phospholipid effect with some step in the secretory process, possibly membrane changes during intracellular transport prior to the extrusion of secretory material (Hokin and Hokin, 1965). Recently, they suggested that activation of glycerol kinase enzyme might be responsible for the increased turnover of phospholipid (Sastry and Hokin, 1966). Since NADPH generated via the HMP shunt is necessary for fatty acid synthesis, enhanced phospholipid turnover may represent a final common pathway for the metabolic changes accompanying secretion. The importance of the HMP shunt in lipid metabolism and the enhancement of this pathway and phospholipid metabolism during secretion lend support to the hypothesis that changes in the functional and metabolic status of secreting cells may be related to the turnover of lipid during active membrane phenomena.

IV. THE ROLE OF METABOLISM IN THE SECRETORY RESPONSE OF ANTERIOR PITUITARY SLICES TO VASOPRESSIN, OXYTOCIN, AND EPINEPHRINE

INTRODUCTION

The release of trophic hormones from the AP gland *in vitro* as influenced by neurohypophysial peptides has been under investigation in our laboratory (LaBella, 1964a; LaBella, 1964b; Pollock, 1965). These particular studies have had two major aims: first, to gain an understanding of the mechanisms whereby the trophic hormones are transformed from cytoplasmic storage granules into soluble extracellular hormones, and, second, to determine how the highly potent posterior lobe peptides initiate the secretory response.

The important role that metabolism plays in secretory glands has been reviewed in previous sections of this thesis, and smooth and skeletal muscle (Huxley, 1960) and nervous tissue (McIlwain, 1966) are greatly dependent on glucose substrate, and on energy metabolism in general, for normal function. In the present investigation attempts were made to assess the role, if any, of cell metabolism in the release of trophic hormones from the AP *in vitro*, by performing incubations at different temperatures and under different gaseous environments and measuring both TSH release and glucose oxidation. The effects of vasopressin and oxytocin, and epinephrine as well, on both parameters of adenohypophysial cellular activity were examined.

MATERIALS AND METHODS

Hormones.

Synthetic oxytocin, 410 U/mg, was supplied by Sandoz Pharmaceutical Co., purified lysine vasopressin, 260 U/mg, from NIH, and thyrotrophin pellets, 0.074 U/mg, from USP Reference Standards. The peptide hormones and TSH were kept frozen in acidified 0.9% NaCl in small aliquots, thawed once when required, and the unused portions of the solutions discarded. Solutions of epinephrine and phenoxybenzamine (POB) were freshly prepared for each experiment. POB was dissolved in ethylene glycol monomethyl ether and then diluted with Krebs-Henseleit buffer.

Incubation of bovine anterior pituitary tissue and determination of $^{14}CO_2$ from ^{14}C-glucose.

These procedures were carried out as described in the previous section. The gas phase was 95% O_2/5% CO_2 where "O_2" is indicated and 95% N_2/5% CO_2 where "N_2" is indicated. 50 mg aliquots of tissue were incubated for 30 minutes in 5 ml of Krebs-Henseleit buffer containing 2 mg glucose/ml. The concentration of each drug was adjusted so that the desired amount was contained in 0.2 ml of incubation medium.

Assay of TSH released in vitro

Aliquots of 4.0 ml of incubation medium was dialyzed in Visking dialysis tubing to remove vasopressin, oxytocin, or epinephrine and subsequently diluted to 10 ml with saline. Aliquots of 0.25 ml of dialyzed incubation medium were assayed for TSH by determining the degree of depletion of thyroid I^{131} in the day-old chick, similar to the method described by Bates and Cornfield (1957).

Two μC of carrier-free I^{131} was injected i.p. into day-old

cockerels on the first day of hatching. On the second day, the thyroid region of each chick was counted by positioning the chick over a scintillation well-counter. Thyroid uptake of I^{131} generally ranged from 10-25%. Chicks with counts less than 1000 counts/sec. or greater than 3500 counts/sec. did not give satisfactory results and were discarded. Arbitrarily, 8-9 chicks were assigned to each group. Each chick was then tagged and injected i.m. in the leg with 0.2 ml of solution containing 250 μg DNP. In the original assay described by Bates and Cornfield (1957) thyroxine was used, but the substitution of DNP yielded results which were equally as good, and DNP is cheaper and easier to use. DNP has been reported to inhibit TSH release from the pituitary in vivo and is thought to have a PTU-like action on the thyroid gland (D'Angelo, 1963). After counting, 0.2 ml of standard TSH solution or 0.25 ml unknown TSH solution were injected i.m. into the leg. On the third day the thyroid was again monitored for radioactivity, to determine the depletion of I^{131}.

The statistical analysis for individual assays was done as follows: first the day 1 and day 2 thyroid counts were corrected for background and decay of I^{131}. The day 2 count was then expressed as a percentage of the day 1 count (relative per cent remaining). As these percentages covered a wide range, they were transformed using the inverse sine transformation (Steel and Torrie, 1960a). The estimates of relative TSH activity and the standard error of this activity were calculated as outlined by Bliss (1960a, 1960b). The log of the estimate of relative TSH activity was used in the statistical analysis of the pooled experiments, since the error of the log-transformed estimates was homogenous.

The statistical analysis for the pooled experiments was done by randomized block analysis of variance with the treatment effects partitioned into individual degree of freedom comparisons (Steel and Torrie, 1960b). These were designed such that the nature of the dose-response curve could be evaluated as to linearity and curvature (Steel and Torrie, 1960c).

RESULTS

TSH release and glucose oxidation at 37^{o} and in 0_2 atmosphere.

The dose-response curve for vasopressin on TSH release at $37^{o}/0_2$ (Fig. 2a) is both significantly quadratic and quartic, and for oxytocin and epinephrine (Figs. 3a, 4a) significantly quadratic, i.e. the dose-response curves were symmetrical or "bell-shaped" (Table 11).

The effects of these three hormones on glucose oxidation at $37^{o}/0_2$ are shown in Tables 12, 13, and 14. Oxidation of ^{14}C-1-glucose was unaffected by concentrations of vasopressin and oxytocin which ranged over the whole dose-response curve for TSH release (Tables 12 and 13). Epinephrine was effective in stimulating glucose oxidation only when the concentration reached 10^{-6}M (Table 12). This threshold concentration of the catecholamine for enhancing glucose oxidation was equal to or above that concentration which was usually most effective in enhancing TSH release and 1000 times the molar concentrations of vasopressin and oxytocin which were maximally effective in stimulating trophin release. Oxidation of ^{14}C-6-glucose was also unaffected by vasopressin or oxytocin (Table 13). (Epinephrine has been previously shown (Table 3) to be without effect on ^{14}C-6 oxidation by the AP, even at concentrations well above those which markedly enhanced ^{14}C-1 oxidation).

The effect of epinephrine in enhancing the release of TSH was significantly blocked by 10^{-8}M POB (Fig. 4a, 4b). The stimulation of TSH release by vasopressin, however, was unaffected by this concentration of POB (Fig. 4c, 4d), suggesting that POB is relatively specific for the epinephrine "receptors" concerned with stimulation of TSH release.

TABLE 11

ANOVA

Source	DF	SS	MS	F
Blocks	29	6.6485	.2289	6.29 **
Treatments	4	.8181	.2045	5.62 **
Linear	1	.0012	.0012	< 1
Quadratic	1	.4705	.4705	12.93 **
Cubic	1	.0890	.0890	2.45 n.s.
Quartic	1	.2574	.2574	7.07 **
ERROR	116	4.2235	.0365	
TOTAL	149	11.6801		

The highly significant quadratic term for the polynomial in the absence of any linearity can be interpreted as indicating that a symmetric single maximum curve best fits the data. The presence of the somewhat lesser, yet still highly significant, quartic term can be interpreted as meaning that the tails of the dose-response curve can best be considered as inflecting upwards, yielding a 'bell-shaped' or gaussian curve.

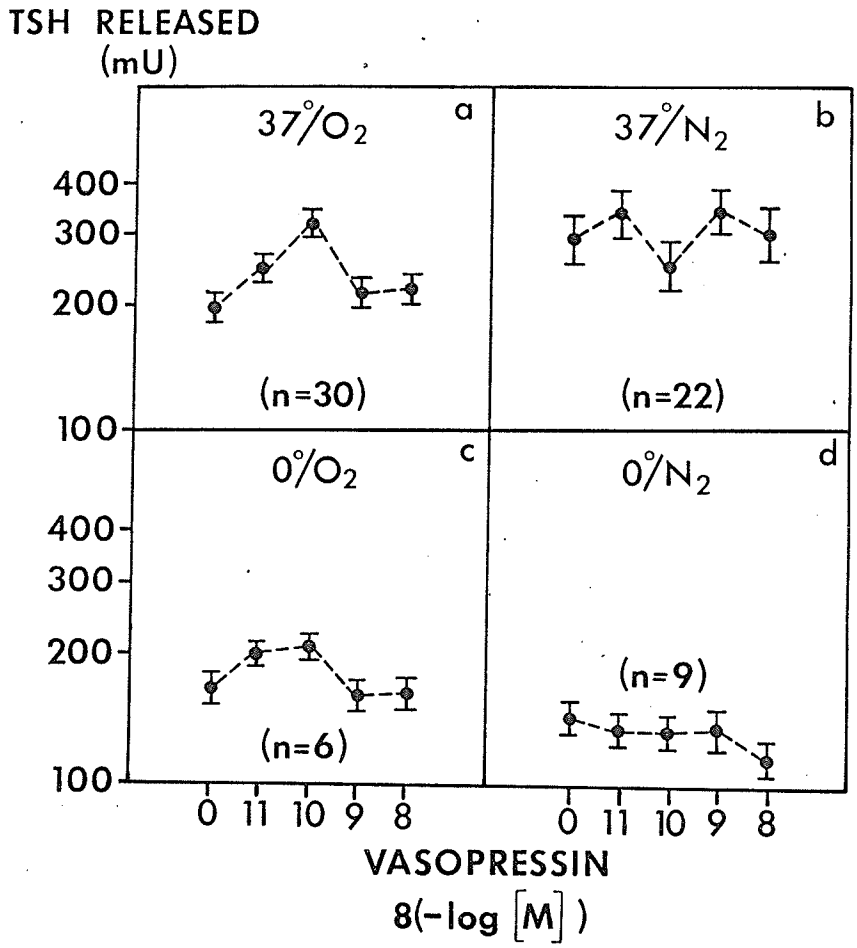


Figure 2. The effect of temperature and anoxia on the stimulation of TSH release by vasopressin from anterior pituitary slices in vitro. Each point represents the mean (± S.E.) of a number of observations (n) indicated in parenthesis under each condition. At $37^{\circ}/0_2$ the dose-response curve has highly significant quadratic and quartic components, F(1,116) 7.07 **, F(1,116), 12.92 ** ($p < .01$), respectively (Table 11). At $0^{\circ}/0_2$ the dose-response curve has a significant quartic component, F(1,184) 5.44 * ($p < .05$). At $37^{\circ}/N_2$ and $0^{\circ}/N_2$ no significant effect was seen ($p > .05$).

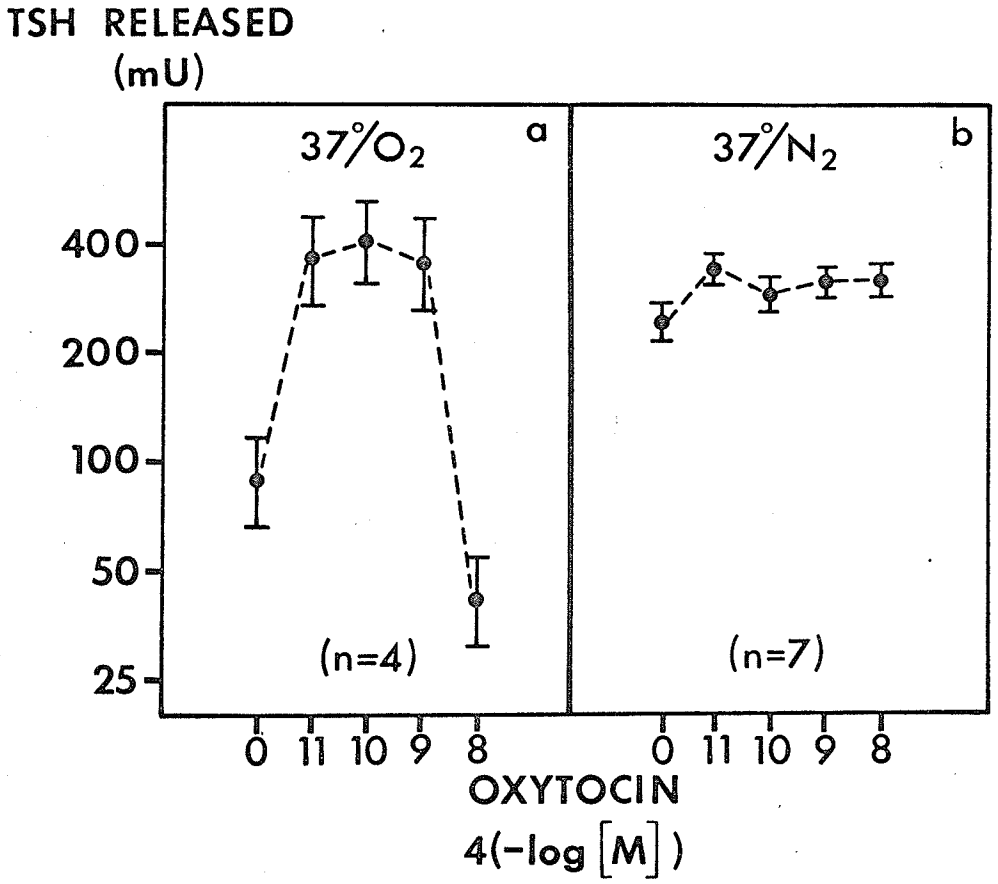
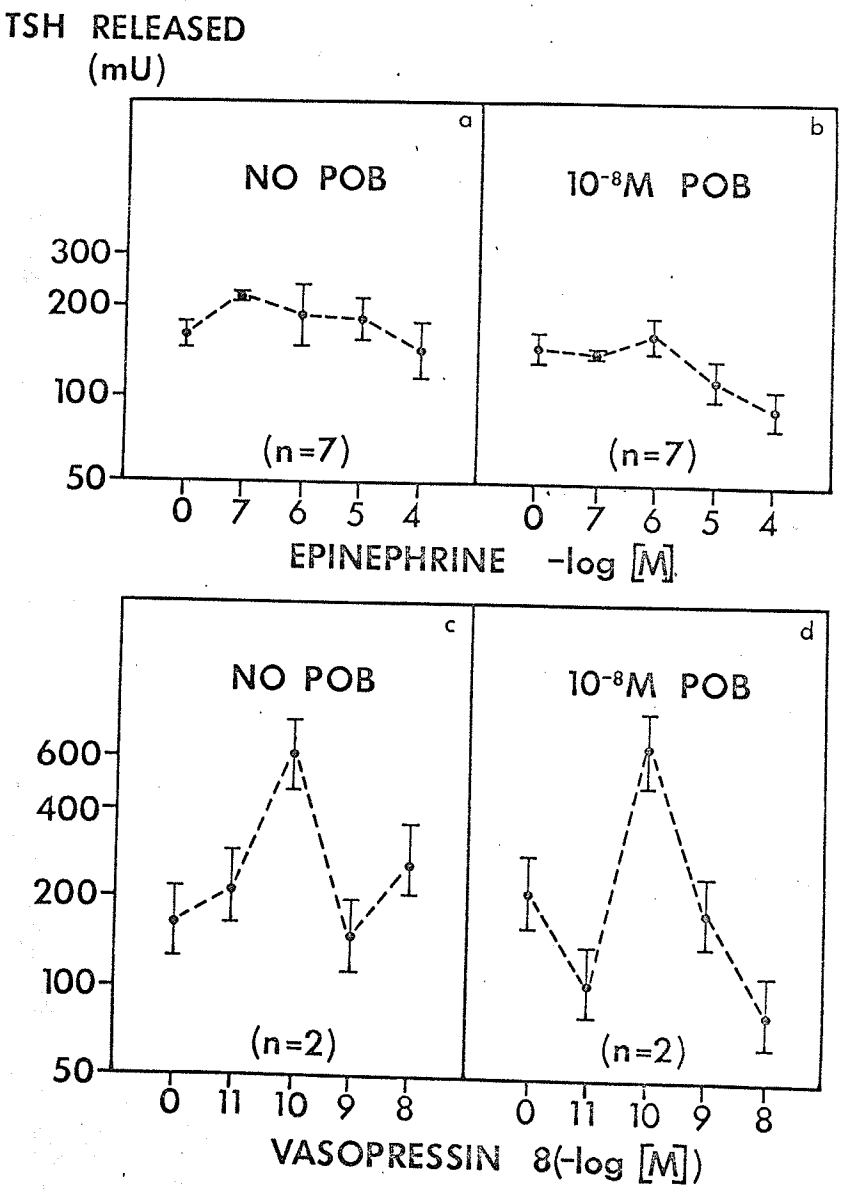


Figure 3. The effect of anoxia on the stimulation of TSH release by oxytocin from bovine anterior pituitary slices in vitro. Each point represents the mean (± S.E.) of a number of observations (n) indicated in parentheses under each condition. At $37^{o}/0_{2}$ the dose-response curve was significantly quadratic, F(1,10) 47.0 **; at $37^{o}/N_{2}$ no significant effect was seen.


TSH RELEASED
(mU)
a
NO POB
b
10-8M POB
300
200
100
50
(n=7)
(n=7)
0 7 6 5 4
0 7 6 5 4
EPINEPHRINE -log [M]
c
NO POB
d
10-8M POB
600
400
200
100
50
(n=2)
(n=2)
0 11 10 9 8
0 11 10 9 8
VASOPRESSIN 8(-log [M])

TSH release and glucose oxidation in the cold and in O_2 atmosphere.

Glucose oxidation was decreased by about 85% when anterior pituitary tissues was incubated at $15^{\circ}/O_2$, when compared to activity at $37^{\circ}/O_2$, and to an even greater extent at $0^{\circ}/O_2$ (Table 15).

In contrast to the findings on glucose oxidation, the effects of vasopressin on TSH release at $0^{\circ}/O_2$ (Fig. 2c) and $37^{\circ}/O_2$ were quite similar. At $0^{\circ}/O_2$ the dose-response curve was significantly quadratic, with the peak effect at 8×10^{-10}M.

TSH release and glucose oxidation at 37° and 0° in N_2 atmosphere.

Glucose oxidation, either spontaneously or in response to epinephrine was markedly depressed at $37^{\circ}/N_2$. Representative data showing the degree of glucose oxidation under 0° or N_2 atmosphere by control tissue, and tissue exposed to different concentrations of epinephrine are presented in Table 14.

When tissue incubations were carried out at $37^{\circ}/N_2$, the spontaneous release of TSH tended to be higher than at $37^{\circ}/O_2$ (Fig. 2a and 2b; Fig. 3a and 3b), although this effect was not statistically significant ($p > .05$). Neither vasopressin or oxytocin significantly enhanced the release of TSH at $37^{\circ}/N_2$. At $0^{\circ}/N_2$ vasopressin had no significant effect on the release of TSH (Fig. 2d).

TABLE 12

EFFECTS OF VASOPRESSIN, OXYTOCIN, AND EPINEPHRINE ON ^{14}C-1-GLUCOSE OXIDATION BY ANTERIOR PITUITARY TISSUE IN VITRO AT 37°/O_2

Reagent	[M]	$^{14}CO_2$ CPM/GM	% of Control	$^{14}CO_2$ CPM/GM	% of Control
		Expt. 1		Expt. 2	
Vaso-pressin	0	23,800		8,900	
	8×10^{-11}	23,500	98	9,400	105
	8×10^{-10}	25,800	108	8,700	98
	8×10^{-9}	23,900	100	10,300	116
	8×10^{-8}	22,200	93	9,300	105
		Expt. 3		Expt. 4	
Oxytocin	0	31,600		5,000	
	4×10^{-11}	33,400	106	5,100	101
	4×10^{-10}	30,200	95	5,100	104
	4×10^{-9}	33,400	106	4,600	91
	4×10^{-8}	31,800	101	5,200	104
		Expt. 5		Expt. 6	
Epine-phrine	0	13,000		30,900	
	10^{-7}	11,400	81	31,000	89
	10^{-6}	15,200	117	35,100	113
	10^{-5}	15,800	122	40,000	129
	10^{-4}	35,900	277	66,000	214

Each value is the average of duplicates.

TABLE 13

EFFECT OF VASOPRESSIN ON ^{14}C-1- AND ^{14}C-6-GLUCOSE OXIDATION BY ANTERIOR PITUITARY TISSUE IN VITRO AT 37°/O_2

Vasopressin	$^{14}CO_2$	CPM/GM
[M] x 8	^{14}C-1	^{14}C-6
0	12,600	620
10^{-11}	11,400	780
10^{-10}	13,100	720
10^{-9}	11,200	760
10^{-8}	11,900	600

Each value is the average of duplicates.

TABLE 14

^{14}C-1-GLUCOSE OXIDATION BY ANTERIOR PITUITARY TISSUE IN VITRO AT $37^{o}/O_2$ OR $37^{o}/N_2$ AND THE EFFECT OF EPINEPHRINE

Epinephrine	$^{14}CO_2$ cpm/gm	
[M]	O_2	N_2
0	30,900	7,000
10^{-6}	40,000	7,700
10^{-5}	36,000	10,300
10^{-4}	66,000	10,900

Each value is the average of duplicates.

TABLE 15

EFFECT OF TEMPERATURE ON ^{14}C-1-GLUCOSE OXIDATION BY ANTERIOR PITUITARY TISSUE IN VITRO

	TEMP. (^{o}C)	$^{14}CO_2$ cpm/gm
EXPT. 1	37	9,500
	15	1,500
	0	1,200
EXPT. 2	37	7,700
	27	4,600
	15	1,100

Each value is the average of duplicates.

DISCUSSION

Vasopressin and oxytocin are the most potent agents which we have found to stimulate the release of TSH from AP tissue *in vitro*. Maximal effects with these agents occur generally at concentrations of 10^{-9} and 10^{-10}M, and LaBella (1964a) has reported stimulation of TSH release at even lower concentrations. Epinephrine, on the other hand, stimulates TSH release maximally at about 10^{-7}M. The dose-response curve for all three agents is bell-shaped, progressively higher concentrations being less effective and finally ineffective with respect to control levels. In some experiments (Fig. 2a, 2b, 4c, 4d) a quartic component was obtained, a finding which we interpret as indicating inflection of the tails of the dose-response curve. This is the first report, to our knowledge, that epinephrine acts directly on the AP to stimulate the release of TSH. The fact that POB blocked TSH release due to epinephrine, but not vasopressin, suggests that the AP cell adrenergic "receptors" conform to the classification proposed for smooth muscle (Nickerson, 1949; Dekanski, 1952). Hokin's group (1953) has reported that atropine blocks the stimulation of pancreatic enzyme secretion and the stimulation of adrenomedullary hormone secretion Hokin *et al.*, 1958) induced by cholinergic drugs *in vitro*. There is *in vitro* evidence that in some species epinephrine stimulates AP trophin secretion (Ganong, 1963; D'Angelo, 1963), and Guillemin (1955) has reported that ACTH release in the rat by epinephrine or acetylcholine is blocked by POB and atropine, respectively. The site of action of these agents was not established, however. Our findings suggest that studies of classical drug-receptor interactions directly at the pituitary level in an *in vitro* system are a feasible and possibly useful tool in elucidating secretory mechanisms.

Vasopressin, oxytocin and epinephrine stimulate TSH release at

$37^{o}/0_2$ and at concentrations that do not affect glucose oxidation as determined by the production of $^{14}CO_2$ from ^{14}C-labelled glucose. At $0^{o}/0_2$, whereas $^{14}CO_2$ production from labelled glucose was greatly diminished, the stimulation of TSH release by vasopressin was manifest. When AP tissue was incubated at $37^{o}/N_2$, vasopressin and oxytocin had no significant effect on TSH release. These results indicate that there is no direct coupling between TSH release and the oxidation of glucose and that peptide-induced TSH release has a low apparent Q_{10}. Anaerobiosis may interfere with some aspect of general cellular activity which indirectly affects trophin release. The greater release of TSH from control tissue at $37^{o}/N_2$ may be a result of trophin leakage due to a breakdown of cell integrity. We did not observe an increased trophin release in the cold, as has been reported by Schramm et al., (1967) for amylase release from parotid gland slice and by Douglas and Ishida (1965) for vasopressin release from the PP in vitro.

Saffran's findings (1959) on the effects of neurohypophysial peptides on ACTH release in vitro are particularly relevant to our work. Saffran reported that anaerobiosis blocked ACTH release by pitressin and arginine vasopressin. However, a purified corticotrophin-releasing factor (CRF) preparation had a slight, although erratic, effect on ACTH release in N_2 atmosphere. Saffran also reported an increase in basal release at $37^{o}/N_2$, similar to our findings with TSH release. In this context, the increased permeability of the toad bladder to water and urea induced by vasopressin may also be relevant to our findings with AP tissue. Rasmussen et al., (1960) reported that cold, lack of glucose, cyanide, fluoroacetate, 2,4-dinitrophenol, and iodoacetamide had no effect on water movement across to toad bladder. In contrast to our findings, anoxia also was without effect on toad bladder permeability. They suggested that a chemical, non-

enzymatic process may be influenced by vasopressin. The similarities between Rasmussen's findings on the toad bladder, and our own suggest that a common mechanism of action for vasopressin may be operative. However, the findings of Rasmussen et al., have been disputed by Bentley (1958) and recently by Handler et al., (1966).

The overall energy requirements for secretion have not been thoroughly studied and are little understood. However, it is generally thought that oxidative metabolism is essential for secretion to occur. For example, in vitro, cholinergic stimulation of pancreatic enzyme secretion (Hokin, 1951) adrenergic stimulation of amylase secretion from the parotid gland (Bdolah and Schramm, 1962) and stimulation of vasopressin secretion by excess K^+ (Douglas et al., 1965), are blocked by cyanide and 2,4-dinitrophenol, and stimulation of pancreatic enzymes by cholinergic agents (Hokin, 1951) and amylase secretion by adrenergic agents (Bdolah and Schramm, 1962) could not be elicited in N_2 atmosphere. Coore and Randle (1964) reported that insulin secretion stimulated in vitro in the presence of excess glucose, was blocked by anoxia and 2,4-dinitrophenol. Thus, in some cells energy may be required for the actual extrusion of secretory material, whereas in others, such as the AP, release of hormone is neither immediately nor markedly dependent on metabolic energy. Douglas and Poisner (1964) have suggested that for glands under direct neural control a calcium-coupled secretion mechanism may be triggered by nerve impulse. It would be of interest to determine whether calcium is involved with secretory processes of the adenohypophysis in which secretion of protein products is regulated, not by direct nervous control, but by blood-borne chemical mediators originating from other endocrine glands and the central nervous system.

Studies concerned with the release of TSH from AP glands by vasopressin and oxytocin are of interest from another aspect. There is a large body of evidence indicating that the neurohypophysial hormones are effective *in vitro* and *in vivo* in stimulating TSH, ACTH, and other AP hormones (Martini, 1966). Recurrent suggestions have been made that the neurohypophysial hormones are concerned with the physiological regulation of adenohypophysial secretion (Guillemin, 1963), although these proposals have not gained much favor. However, some evidence (McCann *et al.*, 1966) suggests that the PP peptides may be involved in the promotion of enhanced secretion of AP hormones under special conditions. Several substances, distinct from vasopressin and oxytocin, have been isolated and proposed as specific trophic hormone releasing factors (Ganong, 1963; D'Angelo, 1963; Saffran, 1959, Guillemin, 1963; Guillemin, 1967). Some of these presumed releasing factors have been thought to be similar structurally to the neurohypophysial hormones, although recent findings by Guillemin *et al.* (1966) indicates that at least some of the releasing factors are not peptide in nature. The possibility that these compounds are artifactual products of chemical manipulation of physiologically inactive precursors or products of certain metabolic processes has not been entirely ruled out. In any case, vasopressin and oxytocin appear to be at least as potent, and often much more potent, than the purified releasing factors. Furthermore, the numerous claims that vasopressin and oxytocin are ineffective in causing trophic hormone release in a particular test system are often based upon the use of one or two doses of either of these peptides, usually at relatively high concentration. The present report and previous ones from this laboratory (LaBella, 1964a; LaBella, 1964b; Pollock, 1965) and others have pointed out that an apparently ineffective dose of

trophin-releasing agent may be one that is on the descending portion of the dose-response curve.

V. SUMMARY AND CONCLUSIONS

1. High C-1:C-6 glucose oxidation ratios, indicative of the presence of the HMP shunt, were found for bovine AP, PP, and PB, as has been reported for other endocrine glands. The low C-1:C-6 oxidations ratio seen in brain suggest that in this tissue glucose catabolism via the HMP shunt is negligible.

2. High C-1:C-6 oxidation ratios were observed in the PP, while ratios close to unity were found in the PVN, indicating that the HMP shunt in the hormone storing and secreting portion of the neurohypophysial neuron is relatively more active than in the hormone synthesizing portion.

3. Nerve endings of the PP were isolated by fractionating a PP homogenate. The nerve ending fraction gave C-1:C-6 oxidation ratios similar to those obtained with slices of the whole gland, and epinephrine stimulated the oxidation of ^{14}C-1-glucose to $^{14}CO_2$ to a similar extent in the nerve endings and the PP slices, indicating that the metabolic activity in the PP is due mainly to nerve endings and not to supporting elements. In the more slowly sedimenting fractions (heavy and light mitochondrial fractions) the progressively decreasing C-1:C-6 oxidation ratios reflects the greater number of mitochondria (which do not contain HMP shunt enzymes) to nerve endings.

4. C-1:C-6 oxidation ratios were higher in bovine PB obtained from young (5-10 months) animals than from PB's obtained from adults (3-8 years), and total glucose oxidation (both C-1 and C-6) was greatest in PB's obtained from young animals. While total glucose oxidation was lower in bovine AP and PP from young animals, C-1:C-6 oxidations ratios tended to be higher in these tissues from adult animals. In brain, however, C-1:C-6

ratios were similar in young and adult animals, while total glucose oxidation was greater in the adult. The PB is thus similar to the AP and PP, and distinct from the brain, with respect to the presence of a high C-1:C-6 ratio, and to the decrease in total glucose oxidation with age.

While it is speculative to relate changes in glucose catabolism to changes in functional capacity, assuming that the C-1:C-6 ratio reflects the secretory potential of gland cells, then the decreased ratio in the adult PB support the hypothesis that secretory activity of the PB decreases with age.

5. Since C-1:C-6 oxidation ratios provide only qualitative information regarding the activity of the HMP shunt, quantitative estimation of the activity the HMP shunt has been made in the AP, PP, PB, and brain. The results indicate that approximately 3-5% glucose is catabolized via the HMP shunt in the AP, PP, and PB, and that the activity of this pathway is negligible in brain.

6. Total glucose utilization for the AP, PP, PB, and brain was fairly constant over a four hour incubation period. Brain had the highest rate of glucose uptake, with PP, PB, and AP having respectively lower rates.

7. The dose-response curves for TSH release <u>in vitro</u> by vasopressin, oxytocin and epinephrine were roughly bell-shaped, with vasopressin and oxytocin stimulating TSH release maximally at 10^{-9} and 10^{-10}M, and epinephrine at 10^{-7}M. The stimulation of TSH release was not markedly temperature dependent, since significant stimulation of TSH release by vasopressin occurred even at $0^{o}/0_2$, but was dependent on 0_2, since in N_2 atmosphere neither oxytocin nor vasopressin had a significant effect on TSH release.

8. ^{14}C-1 or ^{14}C-6-glucose oxidation to $^{14}CO_2$ was unaffected by concentrations of vasopressin, oxytocin, or epinephrine which were effective in stimulating TSH release. Although $^{14}CO_2$ production was reduced to 10-15% of control level at $0^{\circ}/0_2$, enhancement of TSH release by vasopressin was still effected, and there was little difference in basal levels of TSH release between $0^{\circ}/0_2$ and $37^{\circ}/0_2$. At $37^{\circ}/N_2$, $^{14}CO_2$ production was greatly depressed and basal release of TSH tended to higher than at $37^{\circ}/0_2$. Thus, stimulated or basal release of TSH and glucose oxidation do not appear to be directly coupled.

9. Phenoxybenzamine, a classical adrenergic blocking agent, at $10^{-8}M$ blocked the stimulatory effect of epinephrine but not of vasopressin on TSH release. This finding suggests that a certain degree of "receptor" specificity exists at the cell membrane of pituitary cells as in other organs.

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