The Metabolism of Glycerol by Hypothalamic and Pituitary Tissues *in Vitro* in the Rat[1] (37378)

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(Introduced by John W. Ensinck)

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Specific chemical signals for hypothalamic regulation of appetite, and other metabolic events have been difficult to identify *in vivo*. Evidence for glucoreceptors is most convincing, but lipid, amino acid, hormonal and temperature receptors have also been postulated (1). On the premise that chemoreceptor function might be reflected in the metabolic pathways of the neurons in the receptor area, we have undertaken a search for biochemical differences between areas within the hypothalamus and other parts of the central nervous system.

Because some evidence suggests the participation of a signal from adipose tissue in long-term regulation of appetite (2) (the lipostatic theory), we have examined the possibility that free glycerol might be such a signal. Of the two immediate products of lipolysis (FFA and glycerol), glycerol seemed the more attractive because although its rate of transport across the blood–brain barrier appears to be quite limited (3), evidence has been presented for its metabolism by the brain (4–6), and because its entry into metabolic pathways depends upon glycerol kinase, an enzyme not widely distributed (7). Therefore, the finding of increased activity of this enzyme in a potential control site would suggest that glycerol could act as a metabolic signal.

Methods. Tissues for incubation with glycerol *in vitro* were obtained from female rats weighing 180–200 g, sacrificed by decapitation after an 18-hr fast. The brain, anterior pituitary (AP), and other tissues were quickly removed and placed in iced buffer solution. The chilled brain was placed on its cortical surface and sliced into four 0.5 mm frontal sections between the landmarks of the optic chiasm (anteriorly) and the anterior margin of the pons (posteriorly). These sections were laid flat on a chilled plastic plate in cold buffer, Krebs Ringer phosphate (KR P), and further dissected into specific segments with fine scissors under a 20× dissecting microscope. The margins used to define hypothalamus were the lateral sulcus between the hypothalamus and the cortex and the superior limit of the third ventricle. The margin between cortical grey and white tissue was easily identified at 20× magnification. The tissue segments were weighed to the nearest 0.1 mg on a torsion balance and minced into uniformly sized fragments using fine scissors. The tissue fragments were quantitatively transferred to micro-incubation vessels (8). Fragments were prepared to be about 0.5 mm in greatest thickness. Control tissues such as liver, kidney, and muscle were similarly minced.

Incubations and analyses were done as previously described (9). The medium volume was 0.50 ml. The buffer was KRP (pH 7.4) containing 1.0 mg/ml glucose, glycerol-1,3-^{14}C at a final concentration of 3.0 μCi/ml and 0.3 μmole/ml. The sealed flasks were incubated for 1 to 2 hr in room air at 37°. $^{14}CO_2$ was trapped in Hyamine (0.3 ml) contained in a suspended plastic cup. At the end of the incubation period, media were removed, and the tissues were homogenized in 0.1 *N* HCl. The tissue homogenates were extracted with chloroform methanol 2:1, washed 3 times

[1] This investigation was supported by Research Grant 1 RO1 AM10866 and Training Grant 2A 5331 03 from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health.

(Folch technique), and the lipid extract was taken to dryness for counting in a liquid scintillation system. Scintillation counting was performed in a conventional toluene system (9). Quench corrections were applied. In control experiments it was determined that glycerol metabolism was directly proportional to the wet weight of incubated tissue and that both oxidation and lipogenesis proceeded linearly for at least 2 hr.

To study uptake of glycerol *in vivo,* four rats were anesthetized with pentobarbital and infused iv with 10 μCi glycerol-1,3-^{14}C delivered at a constant rate by syringe pump over 60 min. The rats were sacrificed and tissues were collected for analysis of lipid radioactivity. Tissues were homogenized, extracted with chloroform methanol and counted for total lipid radioactivity. Aliquots of the lipid extracts from liver, kidney, hypothalamus and cerebral cortex were combined and analyzed by thin layer chromatography (tlc). The tlc was performed on silica gel (H) in two solvent systems. Phospholipids were separated first using chloroform:methanol:water, 150:50:8. Neutral lipids were then separated with hexane:ethyl ether:acetic acid, 90:10:1. Individual standards were run in parallel and lipid compounds were identified by exposure to iodine vapor. Compounds were scraped into scintillation vials for determination of radioactivity.

For assay of glycerol kinase, tissues were frozen on powdered solid CO_2. Assay was done by the method of Newsholme, Robinson and Taylor (7). Tissue extracts were prepared by homogenizing the freshly frozen tissue in 4 ml 1% KCl, 1 m*M* EDTA (pH 7.5)/g tissue. The assay is based upon incubating unknown enzyme (tissue extract) with ATP (6 m*M*) and labeled glycerol-1,3-^{14}C (3 m*M*) in the presence of mercaptoethanol (20 m*M*), NaF (25 m*M*), $MgSO_4$ (4 m*M*), and EDTA (1 m*M*) in Tris buffer (100 m*M*) (pH 7.5) at 25°. After varying intervals the reaction was stopped with ethanol (95%), and the radioactive product, L-glycerol-3-phosphate, was separated for counting by passing the reaction mixture through a filter disc of DEAE-cellulose. The disc removed the charged product from the uncharged precursor. It was counted directly in a liquid scintillation system after washing and drying. Volumes employed were as follows: (a) buffer containing ATP and radioglycerol, 100 μl; (b) tissue extract, 20 μl; (c) ethanol at end of time period, 100 μl; (d) aliquot for separation on DEAE disc, 20 μl; (e) wash volume for each disc, 150 ml distilled water.

With each assay, controls lacking ATP or lacking enzyme were included and appropriate correction was made. Specific activity of ^{14}C-glycerol was adjusted to provide about 100,000 cpm in each 20 μl aliquot of the final reaction mixture (unreacted substrate).

Results. Metabolism of glycerol-^{14}C in vitro *by minced rat tissues.* The relative activity of brain tissues and various control tissues was assessed in two experiments. The results are presented in Table I. Oxidation of

TABLE I. The Metabolism of Glycerol-1,3-^{14}C by Rat Tissues *in Vitro* Incubation was in KRP, pH 7.4 at 37° for 2 hr.[a]

Tissue	Tissue wt (mg)	cpm $\times 10^{-3}$		μmoles/mg/2 hr		% of liver activity	
		$^{14}CO_2$	Lipid ^{14}C	$^{14}CO_2$	Lipid ^{14}C	$^{14}CO_2$	Lipid ^{14}C
Liver	65.5	10.3	28.0	0.0161	0.0418	100	100
Kidney	65.4	41.3	11.5	0.0633	0.0178	393	43
Diaphragm	60.5	4.01	2.51	0.0065	0.0041	40	10
Adipose tissue	75.5	2.39	8.65	0.0031	0.0116	19	28
Anterior pituitary	10.8	1.08	0.85	0.0092	0.0074	57	18
Hypothalamus	70.4	1.38	0.88	0.0019	0.0012	12	3
Cortical white	65.5	1.54	0.63	0.0022	0.0009	14	2
Cortical grey	70.3	1.25	0.32	0.0016	0.0004	10	1
Blank	—	0.10	0.05	—	—	—	—

[a] Data are the mean of two experiments.

TABLE II. The Metabolism of Glycerol-1,3-^{14}C by Rat Hypothalamic Segments *in Vitro* Incubation was in KRP, pH 7.4 at 37° for 60 or 120 min.[a]

Tissue	(min):	$^{14}CO_2$						Lipid-^{14}C					
		60			120			60			120		
		Mean	SD	*N*	Mean	SD	*N*	Mean	SD	*N*	Mean	SD	*N*
Hypothalamic segments[b]	A	0.0056	±0.0012	(3)	0.0089	±0.0040	(9)	0.0031	±0.0006	(3)	0.0043	±0.0020	(9)
	B	0.0035	±0.0005	(3)	0.0079	±0.0027	(9)	0.0038	±0.0006	(3)	0.0059	±0.0022	(9)
	C	0.0057	±0.0007	(3)	0.0098	±0.0037	(9)	0.0048	±0.0004	(3)	0.0057	±0.0028	(9)
	D	0.0062	±0.0008	(3)	0.0109	±0.0053	(9)	0.0022	±0.0003	(3)	0.0042	±0.0028	(9)
Cortical grey		0.0035	±0.0007	(3)	0.0049	±0.0023	(9)	0.0010	±0.0001	(3)	0.0012	±0.0006	(9)
Anterior pituitary		0.0025	±0.0003	(3)	0.0120	±0.0037	(9)	0.0071	±0.0005	(3)	0.0161	±0.0057	(9)

[a] Results are expressed as μmoles ^{14}C-glycerol/g tissue.

[b] Segments A–D were obtained from serial 0.5 mm sections through the region of the hypothalamus. Segment A was most anterior at the level of the optic chiasm while segment D passed through the mammilary bodies.

glycerol to $^{14}CO_2$ was most active in kidney while lipogenesis was greatest in liver. Skeletal muscle, adipose tissue, and anterior pituitary (AP) displayed appreciable activity with individual differences in distribution of glycerol carbon to the two products. Hypothalamus and cortical grey and white matter were less active. Each displayed a preponderance of oxidation over lipogenesis.

The activity of 4 subsections of the hypothalamus (segments A–D subdividing the hypothalamus from anterior to posterior) was compared to cortical grey tissue and AP in 9 experiments. The results are presented in Table II. Conversion of glycerol-^{14}C to $^{14}CO_2$ and lipid-^{14}C was significantly greater by AP compared to either brain tissue. Similarly, the activity of the 4 hypothalamic segments significantly exceeded the activity of cortical grey for both products. The hypothalamic segments (A–D) did not differ significantly in activity among themselves. The most striking difference between cortical grey matter and hypothalamic tissue was in the relatively greater rate of lipogenesis from glycerol in the hypothalamic segments.

Uptake of glycerol-^{14}C into tissue lipids after constant infusion for 60 min. Table III presents the mean uptake of glycerol-^{14}C into tissue lipid in four rats at the end of the infusion. Uptake by hypothalamus and cerebral cortex was small compared to liver, kidney and muscle. AP and adipose tissue took up slightly greater amounts of glycerol (as judged from lipid labeling). The major product of lipogenesis from glycerol was phosphatidyl choline in hypothalamus, cerebral cortex and kidney, Table IV. The pattern of incorporation of glycerol into individual lipids was somewhat different in the two brain areas studied (Table IV). The demonstration by tlc of incorporation of glycerol-^{14}C into specific lipid compounds establishes the activity of glycerol kinase *in vivo* in brain tissues. The relatively small uptake by brain compared to liver, kidney and muscle could reflect limited transport from blood to brain and/or the lower activity of glycerol kinase in brain tissue (*vide infra*).

Glycerol kinase activity. The preceding experiments showing glycerol metabolism in

TABLE III. Total Lipid Radioactivity in Tissues After Constant Infusion[a] of ^{14}C-Glycerol for 60 min.[a]

Tissue	Mean (cpm/mg)	±SE	Uptake relative to liver (100)
Liver	75.0	9	100
Kidney	26.0	4	35
Diaphragm	11.0	0.6	15
Adipose tissue	2.8	0.9	3.7
Anterior pituitary	3.3	0.6	4.4
Hypothalamus (anterior)	2.0	0.4	2.7
(posterior)	2.3	0.5	3.1
Cortical white	1.5	0.3	2.0
Cortical grey	1.5	0.3	2.0

[a] Four rats infused iv with a total dose of 10 μCi glycerol-1,3-^{14}C/rat.

brain tissues indicated the presence of glycerol kinase, since this enzyme is the only known route for entry of glycerol into the metabolic pathways in mammalian cells. The relative activity of glycerol kinase was compared in crude tissue extracts and expressed as cpm appearing as product per milligram of tissue in the assay system. Figure 1 presents a comparison of activity between several tissues. Although the order of activity among the tissues is similar to that found for glycerol-^{14}C metabolism *in vitro* (Table I), the quantitative disparity in activity between liver and kidney and the other tissues is much more marked. Liver and kidney are at least 10 times as active as any other tissue tested, results which are similar to the findings of previous workers (10).

The activity of glycerol kinase in hypothalamus, cerebral cortex and AP was compared in a series of rats. The results, Fig. 2, were similar to those for glycerol-^{14}C metabolism (Table II) with AP activity being greater than hypothalamus, which in turn was greater than cerebral cortex. These differences were significant when tested by the paired t test ($p < 0.01$).

With all tissues tested, crude tissue extracts produced a nonlinear reaction, the reaction rate falling with time. In contrast, purified glycerol kinase (Boeringer) always produced a linear reaction for all intervals when substrate was present in excess (data not presented). The apparent fall in reaction rate with time could reflect the activity of other enzymes in the extract, which caused further transformation of the product (L-glycerol-phosphate) to intermediates with less affinity for the DEAE-cellulose discs. Bublitz and Kennedy (11) demonstrated that glycerol kinase was relatively heat stable in the presence of free glycerol. Accordingly, the tissue extracts were heated at 60° for 15 min in the presence of 3 m*M* glycerol, before assay. After heat treatment the reaction became linear for all tissues (Fig. 3). The rates of reaction were, however, greatly reduced compared to the initial rates for the

TABLE IV. Percentage of Total Lipid Radioactivity in Individual Lipids After Infusion of Glycerol-1,3-^{14}C *in Vivo*.

Lipid fraction	Liver	Kidney	Hypothalamus	Cerebral cortex
Sphingomyelin	2	6	5	9
Phosphatidyl choline	20	48	49	35
Phosphatidyl ethanolamine	15	13	16	8
Mono- and diglycerides	15	10	19	26
Triglycerides	48	23	11	13
Cholesterol esters	0	0	0	9

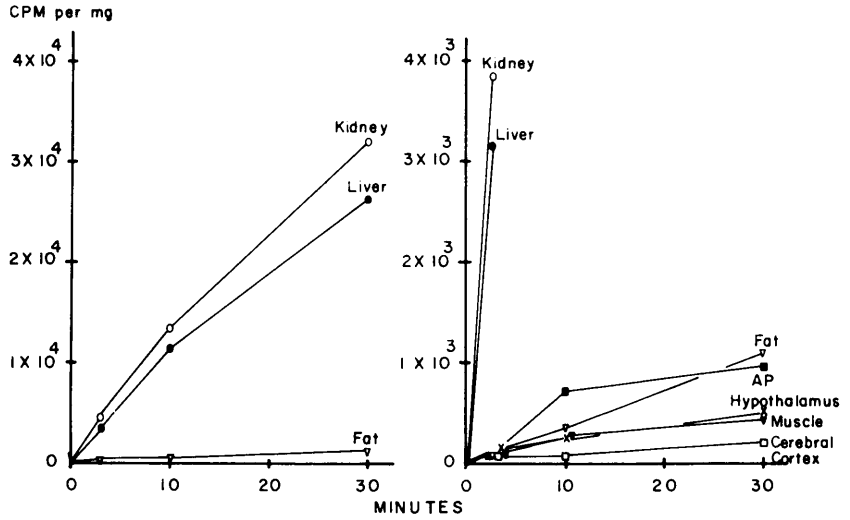

FIG. 1. Glycerol kinase assay: comparison of brain tissue and AP with other representative tissues. Enzyme activity was assayed in tissue extracts identically prepared from fresh material. Activities are expressed as cpm glycerol-1,3-^{14}C converted to product per milligram of tissue in the assay system. Note that the activity scale of the left graph is 10 times that of the right. Data for liver, kidney and fat tissue are presented on both scales for ease of comparison.

unheated extract. These findings suggest that the nonlinear reaction of crude extracts was due to competing reactions for the product. Accordingly, the initial reaction rates for crude extracts would more closely approximate glycerol kinase activity within the tissue. The predicted behavior of the activity in heated tissue extracts helps to confirm the authenticity of the glycerol kinase activity observed.

Discussion. We have demonstrated that glycerol kinase is present in hypothalamic tissue, and that glycerol can be metabolized by this tissue *in vitro*. On this basis alone, it can be concluded that glycerol could potentially serve as a signal for hypothalamic control systems. If in some portion of the hypothalamus, glycerol kinase had been very active in the range of liver or kidney, while other areas of brain were less active, a strong circumstantial case would have been made for a specific glycerol receptor area. However, the results are less dramatic. Although both the ability to metabolize glycerol and the activity of glycerol kinase were significantly higher in the hypothalamus compared to the cerebral cortex, both areas displayed low levels of activity comparable to a number of other tissues thought not to significantly contribute to glycerol economy *in vivo*. We are, therefore, unable to draw any more definite conclusions than the existence of the potential for glycerol chemoreceptor as stated at the outset. We can, however, make note of further consideration, that is, the presence of *any* measurable activity in hypothalamic tissue raises the possibility that a specific group of chemoreceptor neurons with a small mass compared to the total tissue studied, might have contained a high degree of activity, but escaped detection in our studies.

A further consideration in assessing the potential of a substance to act as a central nervous system signal, concerns its ability to cross the blood–brain barrier. Sloviter, Shimkin, and Suhara (6) previously presented evidence that glycerol can support cerebral cortical function during insulin hypoglycemia when infused directly into the carotid artery. Others found slow rates of penetration of glycerol into brain in the rabbit (3) and the cat (5). We found that infused ^{14}C-glycerol crosses from blood to hypothalamus during 1 hr constant infusion, but the relative incorporation of label into

lipid was small compared to liver, kidney, and muscle and did not differ significantly from cerebral cortex. Thus, these data do not support the possibility that the hypothalamus displays selective permeability for glycerol as a mechanism for receptor specificity. However, just as glycerol kinase might be more active in a small specialized group of neurons, it is also possible that the blood–brain barrier in the vicinity of a small group of neurons might be selectively more permeable to glycerol and thereby confer chemoreceptor specificity to that region.

The anterior pituitary metabolized glycerol at significant rates relative to the most active tissues, liver and kidney. This capacity for AP to oxidize glycerol and labeled palmitate (unpublished observations) is in keeping with its previously demonstrated ability to normally synthesize protein in fasting (12). As in hypothalamus the demonstration of active glycerol metabolism raises the possibility that glycerol could play a regulatory role in anterior pituitary function.

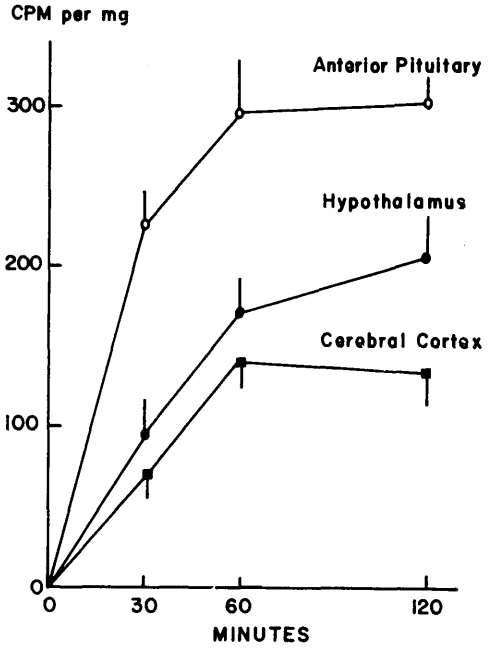

FIG. 2. Glycerol kinase activity: comparison of AP, hypothalamus, and cerebral cortex. Fresh tissue extracts were prepared and assayed in duplicate in three experiments at 30, 60 and 90 min. The initial reaction rates were greater in AP than hypothalamus and greater in hypothalamus than cerebral cortex. The plotted data are means and SE.

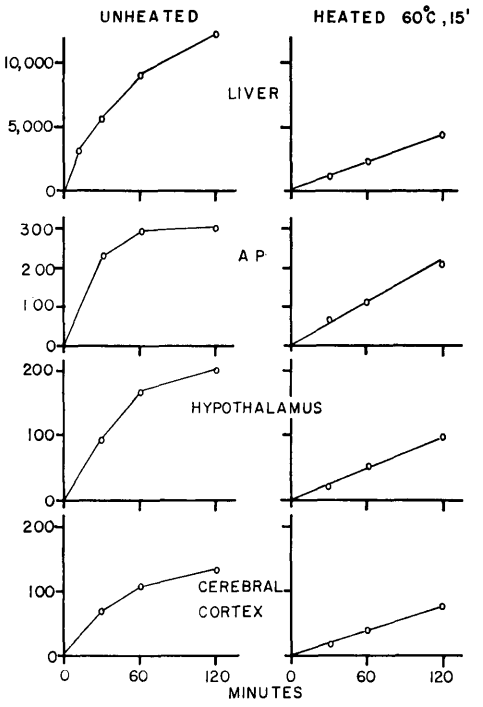

FIG. 3. The effect of heating upon the glycerol kinase activity of tissue extracts. Tissue extracts were assayed before and after heating for 15 min at 60° in the presence of 3 m*M* glycerol. Heating converted the nonlinear reaction characteristic of all crude fresh extracts to a linear reaction. Purified glycerol kinase has been shown to be heat stable under similar conditions. The relative rank order of reaction rates among the four tissues was not altered by heating. Ordinate: cpm/mg.

Summary. The metabolism of labeled glycerol *in vitro* and the activity of glycerol kinase (EC 2.7.1.30) were measured in rat tissues. Although glycerol was actively oxidized and converted to lipids by hypothalamus the rates were low compared to liver and kidney. The activity of anterior pituitary was significantly greater than hypothalamus which was in turn greater than cerebral cortex. After infusion of glycerol-^{14}C iv label was recovered in phospholipids of brain tissues, direct assay of glycerol kinase provided similar results with the same order of activity among the tissues tested. Because glycerol kinase was present

in hypothalamus, it is concluded that glycerol could potentially function as a signal for hypothalamic control systems. Anterior pituitary metabolism of glycerol was sufficiently active to classify glycerol as an energy substrate in this tissue.

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Received Feb. 15, 1973. P.S.E.B.M., 1973, Vol. 143.