TABLE 4. CONCENTRATION OF CYTOCHROME P-450 IN ISOLATED HEPATOCYTES AND 9000 *g* SUPERNATANT PREPARED FROM ISOLATED HEPATOCYTES OBTAINED FROM PHENOBARBITAL-TREATED MALE RATS*

Preparation	Cytochrome P-450	
	(nmoles/ml incubation mixture)	(nmoles/mg protein)
Hepatocytes	8·5	
9000 *g* supernatant	11·6	0·424
9000 *g* supernatant from cells incubated 60 min	14·9	0·595

* Preparations and assays are given in the text.

A second advantage of the technique described in this paper is the ease of obtaining the adult tissues in almost unlimited quantities. Use of adult tissues eliminates problems associated with procuring or breeding animals so that embryos of the desired age are available.

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Hydroxylation of phenylalanine by various areas of brain *in vitro**

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WE HAVE observed the formation of labeled tyrosine in several areas of brain *in vivo* following the injection of [^{14}C]phenylalanine into the lateral ventricle of rat brain.[1,2] The demonstration of the formation of tyrosine from phenylalanine in brain tissue *in vitro* is essential for a number of obvious

* Part of this work was presented at the annual meeting of the Federation of the American Societies for Experimental Biology, April 12–17, 1971, at Chicago.

reasons. In the present communication, we wish to report our observation of the hydroxylation in several areas of CNS *in vitro*.

Wistar rats (160–200 g) were killed by decapitation following which the pineal gland and the whole brain were quickly removed. The pineal gland, separated from the adherent connective tissue and blood, was immediately put in a tube containing ice-cold 0·1 M sodium phosphate buffer and 0·05 M 2-mercaptoethanol and then homogenized. The whole brain was chilled in ice-cold 0·25 M sucrose and dissected into various areas. The brain areas were homogenized in 9 vol. of 0·1 M sodium phosphate buffer. The tissue homogenates in phosphate buffer were then incubated at 37° for 30 min in air after the following additions: 110,000 dis./min of either [^{14}C]phenylalanine or [^{14}C]tyrosine (both uniformly labeled and of specific radioactivity 300–350 mc/mM; New England Nuclear, Boston) 0·05 M mercaptoethanol, 0·4 mM pargyline hydrochloride, and 1 mM $DMPH_4$* (2-amino-4-hydroxy-6,7-dimethyl-tetrahydropteridine HCl; CalBiochem, Los Angeles) as indicated in the results. The tissue weights used and the final incubation volumes were: three pooled pineals per tube (approximately 3·75 mg) in 0·23 ml, brainstem, 190 mg, in 2·2 ml and all other tissues 50 mg in 0·575 ml. The final concentration of the buffer was 0·087 M and the pH values for the various incubations were as mentioned in the results.

The incubation was terminated by adding 0·4 N perchloric acid and the extract was prepared by homogenizing and centrifuging twice. The fractionation of the tissue extract on the Dowex-50 ion-exchange column was carried out as described before.[2] The only modification employed was that 0·1 M phosphate buffer (pH 6·4) was substituted for acetate buffer for the elution of the amino acid fraction. The concentrate containing the amino acids was chromatographed bi-dimensionally on Whatman No. 3 MM paper employing *n*-butanol–2 N HCl (2:1, organic phase) for the first direction and isopropanol–formic acid–water (40:2:10) for the second. The areas containing phenylalanine and tyrosine were located by successively spraying with the isatin and the diazotized sulphanilic acid reagents. The regions of the chromatogram containing phenylalanine and tyrosine were cut out, eluted with 2·0 ml of water and counted in a liquid scintillation counter after mixing with 15 ml of Aquasol (New England Nuclear, Boston) scintillation fluid. The identity of the isolated tyrosine with authentic *p*-tyrosine in various chromatographic systems has been reported earlier.[1,2]

The catecholamines present in the tissue extract were eluted from the Dowex-50 ion-exchange column with 10 ml of 1 N HCl in 50% ethanol[3] following the removal of the amphoterics with the phosphate buffer. Further purification of the catecholamines was achieved by adsorption on acid alumina at pH 8·5 and subsequent elution with 3·0 ml of 0·5 N acetic acid. For determining the total ^{14}C in the catecholamines, the eluate was assayed at this stage by scintillation counting. For a number of samples, the separation of dopamine and norepinephrine present in the eluate was achieved by chromatography on Whatman No. 3 MM paper with *n*-butanol–2 N HCl solvent mixture. The catecholic compounds were visualized with the ethylenediamine-ferricyanide[1] spray reagent following which elution with water and scintillation counting was employed for radioactivity assay.

The results presented in Table 1 indicate that the homogenates of various areas of the CNS, the pineal gland, caudate nucleus, brainstem (pons and medulla) and hypothalamus (hypothalamus and thalamus) are able to hydroxylate phenylalanine. Several tissues, outside the CNS, such as spleen and lung did not contain measurable hydroxylating activity, and therefore may act as suitable controls for any non-enzymatic hydroxylation.

Our preliminary experiments with caudate tissue and the pineal gland have pointed out that a sharp difference exists between the two tissues with respect to the pH and activity relationship. The peak activity of the caudate system is at 6·0 and the pineal enzyme shows the maximum at pH 7·5. Results in Table 1 clearly show the effect of pH change on the enzyme activities in these two tissues. The alteration of the pH of incubation did not have any noticeable effect on the activity of brainstem (Table 1). Such a lack of effect of pH variation and the relatively low conversion of phenylalanine to tyrosine may raise some doubts about the enzymatic nature of the hydroxylation by brainstem homogenate. Some kind of enzyme activity is, however, indicated by the fact that pretreatment of the rats with *p*-chlorophenylalanine (PCPA) almost completely inhibited the hydroxylating activity (Table 1). We have previously reported that pretreatment with PCPA failed to inhibit completely the appearance of labeled tyrosine in brainstem *in vivo*.[1] The reason for this discrepancy between the *in vivo* and *in vitro* data is not clear and further experiments are needed for clarification.

We have used three different independent approaches to exclude the possibility that the observed hydroxylation may be due to non-enzymatic reactions. (1) The most useful method, which may be termed as tissue control, was to replace the brain area homogenate with a similar preparation of lung tissue in an otherwise identical incubation mixture. Following the incubation of such samples, either

* Small aliquots of aqueous solution of $DMPH_4$ were kept frozen at −15°. About 5 min before the start of the incubation, an aliquot was thawed, added to the incubation mixture and the rest of the solution discarded.

at pH 6·0 or at 7·5, the labeled tyrosine formed contained a very low level of radioactivity (Table 1). Similar substitution of brain tissue with spleen or plasma also led to the same low value of the radioactivities of tyrosine. (2) The homogenates of caudate and brainstem were heated at 100° for 75 sec and then incubated with the complete incubation mixture. The incubation of heated homogenates led to the same low level of tyrosine radioactivity as obtained from the tissue control experiments. (3) Well known inhibitors were used in *in vivo* and *in vitro* to inhibit the formation of labeled tyrosine by the brain areas. PCPA (acting *in vivo*) strongly inhibited the hydroxylation by the pineal gland and the brainstem (Table 1). The addition of alpha-methyl *p*-tyrosine (MPT) or tyrosine (TY) to 4×10^{-5} M

TABLE 1. HYDROXYLATION OF [^{14}C]PHENYLALANINE IN VARIOUS RAT TISSUES *in vitro**

Tissue	Incubation pH (No. of expts)	Addition†/ condition	Radioactivity in the isolated	
			Tyrosine (dis./min)	Catecholamines (dis./min)
Pineal	7·5 (5)	None	1978	0
	7·5 (2)	PCPA	695	0
	7·5 (2)	$DMPH_4$	3536	0
	6·0 (1)	$DMPH_4$	232	0
Caudate	6·0 (7)	None	3689	2400
	6·0 (1)	TY	1104	100
	6·0 (3)	PCPA	2579	787
	6·0 (1)	MPT	604	0
	6·0 (2)	Heated	154	0
	7·5 (2)	None	380	0
Brainstem	6·0 (2)	None	754	
	6·5 (2)	None	806	
	7·0 (3)	None	709	
	7·5 (9)	None	745	0
	7·5 (1)	Slice	288	
	7·5 (4)	$DMPH_4$	1217	0
	7·5 (6)	PCPA	177	0
	7·5 (1)	Heated	122	0
Hypothalamus (plus thalamus)	6·0 (3)	None	778	0
	6·0 (1)	$DMPH_4$	772	0
	7·5 (5)	None	796	0
	7·5 (3)	$DMPH_4$	1220	0
Plasma	6·2 (2)	None	97	0
	7·5 (2)	None	81	0
Spleen	6·0 (2)	None	165	0
	6·0 (1)	$DMPH_4$	270	0
	7·5 (1)	None	143	0
Lung	6·0 (1)	None	144	0
	7·5 (2)	None	116	0
	7·5 (1)	Heated	145	0

* Tissue was homogenized in phosphate buffer (0·1 M; pH same as given for incubation) and then incubated with the standard additions of labeled phenylalanine (110,000 dis./min), pargyline (0·4 mM) and mercaptoethanol (0·05 M). Final phosphate concentration was 0·087 M. The number of experiments for each tissue is given in the parentheses and the average result is reported. Usually the experiments with a given tissue were done on 2–3 different days and yielded very similar values. The radioactivity of the isolated tyrosine and catecholamines is as formed from the labeled phenylalanine substrate.

† Any variation of the described procedure. Heated: the homogenate heated for 75 sec at 100° and then incubated with the additions. PCPA: rats pretreated with 316 mg/kg PCPA.[2] Slice: approximately 0·5–1·0 mm thick slices incubated. $DMPH_4$: addition of $DMPH_4$ to the incubation medium to 1 mM concentration. MPT and TY: addition of MPT and TY to 4×10^{-5} M incubation concentration.

final concentration in the incubation medium largely inhibited the formation of labeled tyrosine and labeled catecholamines by caudate homogenate (Table 1). The enzymatic nature of the hydroxylation is therefore confirmed by each of the three different methods. It should be mentioned, however, that the addition of $DMPH_4$ to the heated homogenate caused non-enzymatic formation of labeled tyrosine (Table 2). Such non-enzymatic oxidation in the presence of $DMPH_4$ has been previously reported.[4]

The only additions routinely employed were 0·05 M mercaptoethanol and 0·4 mM pargyline. The sulfhydryl reagent was added since it may have a stabilizing effect on hydroxylating enzymes[5,6] and help to regenerate the cofactor.[7] The addition of pargyline was for preventing the oxidation by monoamine oxidase (MAO) of any catecholamines formed.

TABLE 2. CONVERSION OF [^{14}C]TYROSINE TO CATECHOLAMINES AND THAT OF [^{14}C]PHENYLALANINE TO TYROSINE AND CATECHOLAMINES BY THE HOMOGENATE OF CAUDATE NUCLEUS IN THE PRESENCE OF ADDED $DMPH_4$*

$DMPH_4$ addition (No. of expts)	Radioactivity in the isolated: Tyrosine (dis./min)	(%)	Catecholamines (dis./min)	(%)	Total product (dis./min)	(%)
[^{14}C]Phenylalanine substrate						
None (7)†	3689	100†	2400	100†	6089	100†
None (heated) (2)	154	4	0	0	154	2
0·05 mM (4)	3207	87	2128	89	5335	88
1·0 mM (2)	1839	50	1370	57	3209	53
1·0 mM (heated) (1)	443	12	0	0	443	7
4·0 mM (3)	2340	63	446	18	2786	46
4·0 mM (heated) (2)	1345	36	0	0	1345	22
[^{14}C]Tyrosine substrate						
None (4)			9038	100	9038	100
1·0 mM (4)			8832	98	8832	98
4·0 mM (3)			8130	90	8130	90
4·0 mM (heated) (2)			0	0	0	0

* The homogenate of caudate tissue in phosphate buffer (pH 6·0) was incubated with either [^{14}C]phenylalanine or [^{14}C]tyrosine as the substrate in presence of pargyline and mercaptoethanol, and $DMPH_4$ was added to the indicated concentration. The total products formed from [^{14}C]phenylalanine are the sum of the radioactivities in the isolated tyrosine and catecholamine. The other details are as mentioned under Table 1.

† Data from Table 1.

The homogenate of caudate nucleus was found to be highly active and, unlike the other tissues, formed catecholamines (Table 1). That catecholamines may be formed from phenylalanine *in vivo* as well has been observed before.[1] The implication of caudate catecholamines with Parkinson's disease prompted us to investigate the hydroxylation of phenylalanine in comparison with that of tyrosine by caudate homogenate. The results (Table 2) show that when no external $DMPH_4$ was added the total products (sum of radioactivities in the isolated tyrosine and catecholamines) formed from [^{14}C]phenylalanine was as high as 67 per cent of the products ([^{14}C]catecholamine) appearing from [^{14}C]tyrosine substrate under identical conditions. One-fourth as much labeled catecholamines was formed from [^{14}C]phenylalanine as from labeled tyrosine. Whether phenylalanine or tyrosine was used as the labeled precursor, [^{14}C]dopamine represented about 90 per cent of the radioactivity in the catecholamines and only a trace of [^{14}C]norepinephrine was present. Decarboxylase activity is present in the caudate nucleus homogenate and therefore any accumulation of labeled dopa to a significant extent is unlikely as has been observed before.[8] In the present experiments with [^{14}C]phenylalanine and [^{14}C]tyrosine, a number of samples were analyzed for the radioactivity appearing in

dopa. It was eluted from the Dowex-50 ion-exchange column with the phosphate buffer and further chromatographed on an acid alumina column as described for the analysis of the catecholamines. The label in dopa amounted to only 10–20 per cent of the radioactivity present in the catecholamines.

We have studied the hydroxylation of both the substrates also in the presence of $DMPH_4$ of varied concentrations, since various aromatic hydroxylations have been found to be dependent on this cofactor.[9] From the results in Table 2, it is apparent that the addition of $DMPH_4$ did not have any noticeable effect on the catecholamine formation from [^{14}C]tyrosine. This is possibly due to the adequate presence of the endogenous cofactor. In contrast, $DMPH_4$ was observed to have an inhibitory effect on the phenylalanine hydroxylation. While the inhibition of [^{14}C]tyrosine formation was significant, a much stronger effect on the catecholamine formation was observed (82 per cent inhibition at 4 mM).

The present results clearly demonstrate that the hydroxylation of phenylalanine may occur *in vitro* in various brain regions. Our previous findings from *in vivo* experiments[1,2] are in agreement with the present results.

The identity of the enzyme involved is not clear at this time. The possible actions of three different aromatic ring hydroxylases should be considered. The phenylalanine hydroxylase that is characteristic of liver tissue does not occur in brain.[10] The optimum pH of this enzyme is about 7·4, a property not clearly shared by most of the brain areas we have investigated. Tyrosine hydroxylase has been reported to act on phenylalanine very slowly. Only about one-tenth as much product appears from phenylalanine substrate as from tyrosine.[11] We have not at this time investigated tyrosine hydroxylation by all the areas, but it is evident from our studies with caudate (Table 2) that phenylalanine hydroxylation is relatively much greater in this tissue. Furthermore, noticeable difference exists between caudate and brainstem regarding pH activity relationship, effect of $DMPH_4$ and inhibition by PCPA (Table 1), though both of these areas contain tyrosine hydroxylase.[6,8] While the preparation of this manuscript was under progress, Shiman *et al.*[12] published results which indicate that highly purified adrenal tyrosine hydroxylase is able to hydroxylate phenylalanine actively in the presence of the tetrahydrobiopterin cofactor. The possible significance of this finding with respect to the hydroxylation of phenylalanine by brain areas is being investigated in our laboratory.

Phenylalanine has been found to be the substrate of tryptophan hydroxylase of the pineal gland. But this appears to be a specific property of the pineal enzyme only since the same enzyme purified from brainstem was devoid of any activity toward phenylalanine.[13] There is no published report of phenylalanine hydroxylation by tryptophan hydroxylase of any tissue other than the pineal gland. In view of these unresolved questions it is difficult at this time either to rule out or to confirm the role of any of these ring hydroxylases. Further work is necessary in order to identify the participating enzyme(s).

Whatever may be the identity of the enzyme, an important issue is the physiological relevance of this reaction in the nervous tissue. Any suggestion regarding a possible metabolic role of phenylalanine hydroxylation in nervous tissue has not been made to date, but the demonstration of this reaction, both *in vitro* (present data) and *in vivo*,[1,2] suggest such a role.

The present communication, to our knowledge demonstrates for the first time phenylalanine hydroxylation by the tissue homogenates of various brain areas. Some previous attempts have produced either negative results,[14,15] or ambiguous data.[16] The observation of the enzymatic step in simple homogenates should prove helpful for further studies.

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Adrenergic receptors in human adipocytes—Divergent effects on adenosine 3′,5′-monophosphate and lipolysis*

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THE ROLE of adenosine 3′,5′-monophosphate (cyclic AMP) in mediating many of the effects of a number of hormones, including the catecholamines, is now widely accepted. The catecholamines are capable of interacting with at least two types of adrenergic receptors, known as alpha and beta receptors, and it has been postulated that cyclic AMP may be involved in both types of interaction.[1] According to this hypothesis, beta receptors mediate an increase in the intracellular level of cyclic AMP, secondary to the stimulation of adenyl cyclase, whereas an interaction with alpha receptors leads to a fall in the level of cyclic AMP, and consequently an opposite effect on cell function. In a previous study,[2] the effects of phentolamine (an alpha adrenergic blocking agent) and propranolol (a beta adrenergic blocking agent) on the basal and epinephrine-stimulated rates of lipolysis of human and rat adipocytes were observed. The results of these observations indicated that human fat cells possessed both alpha and beta adrenergic receptors, mediating inhibition and stimulation of lipolysis, respectively, whereas rat cells possessed only beta receptors. Comparable results have since been reported by others.[3] To obtain more direct information about the possible role of cyclic AMP in these responses, we have now carried out experiments with human fat cells in which both cyclic AMP levels and glycerol release have been measured. As predicted on the basis of the above hypothesis, the results indicate that cyclic AMP levels and lipolysis are indeed influenced by alpha and beta receptors in a closely parallel fashion.

Isolated human fat cells were prepared from subcutaneous adipose tissue obtained from surgical patients using a method previously described.[4] Krebs albumin buffer, cells, and test substances (epinephrine, phentolamine, and propranolol, each at a final concentration of 10^{-5} M) were placed in plastic flasks and incubated with gentle shaking at 37° under an atmosphere of 95% O_2 and 5% CO_2. At the end of 30 min, the contents of one set of flasks were rapidly frozen in liquid nitrogen and stored at −70°. They were later homogenized in the presence of 0·1 M HCl, and cyclic AMP was measured in purified extracts by a method based on the activation of liver phosphorylase.[5] The remaining flasks were incubated for 4 hr, following which filtrates were prepared for glycerol analysis by the method of Garland and Randle.[6]

Prior to the basic study just described, a series of preliminary experiments were done in which both parameters were measured at various intervals up to 4 hr. The effect of epinephrine in the presence of phentolamine is illustrated in Fig. 1. The level of cyclic AMP under these conditions reaches a peak at around 30 min, whereas glycerol continues to be released at an increased rate for at least 4 hr. This type of relationship is compatible with a mechanism involving the phosphorylation and hence activation of a lipase under the catalytic influence of a cyclic AMP-sensitive protein kinase.[7,8] Cyclic AMP was detected in increasing concentrations in the buffer during the first hour of incubation, but thereafter its concentration remained relatively stable.

The results of an experiment involving both blocking agents are shown in Fig. 2. The addition of epinephrine by itself increased the level of cyclic AMP, and this effect was greatly enhanced in the presence of phentolamine. In the presence of propranolol, by contrast, epinephrine reduced the level below that seen in control flasks. Neither phentolamine nor propranolol at these concentrations had

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