oxidation. Adapted cells show marked increase in oxidation of phenylalanine, isoleucine and threonine, as well as acetate.

1. McCashland, B. W., *J. Protozool.*, 1955, v2, 97.
2. ———, *ibid.*, 1956, v3, 131.
3. McCashland, B. W., Marsh, W. R., *Growth*, 1958, v22, 99.
4. Kidder, G. W., Dewey, V. C., Heinrich, M. R., *Exp. Cell. Res.*, 1954, v7, 256.
5. Umbreit, W. W., Burris, R. H., Stauffer, J. F., *Manometric Techniques*, Burgess, Minneapolis, 1957, p31.
6. Krebs, H. A., *Biochem. J.*, 1935, v29, 1620.
7. Baker, S. B., Summerson, W. H., *J. Biol. Chem.*, 1941, v138, 535.
8. Block, R. J., Durrum, E. L., Zweig, G., *Paper Chromatography and Paper Electrophoresis*, Academic Press, Inc., New York, 1955, p81.
9. Block, R. J., *Anal. Chem.*, 1950, v22, 1327.
10. Kidder, G. W., Dewey, V. C., *Arch. Biochem.*, 1949, v20, 433.
11. Seaman, G. R., in S. H. Hutner, A. Lwoff, *Biochemistry and Physiology of Protozoa*, Academic Press, Inc., New York, 1955, v2, p100.
12. Elrod, L. M., *Growth*, 1956, v20, 187.
13. McKee, C. M., Dutcher, J. D., Groupe, V., Moore, M., PROC. SOC. EXP. BIOL. AND MED., 1947, v65, 326.
14. Wilber, C. G., Seaman, G. R., *Biol. Bull.*, 1948, v94, 29.
15. Aaronson, S., Baker, H., *J. Protozool.*, 1961, v8, 297.
16. Wu, C., Hogg, J. F., *Arch. Biochem. and Biophys.*, 1956, v62, 70.
17. Wells, G., *J. Protozool.*, 1960, v7, 7.
18. Loefer, J. B., Scherbaum, O. H., *ibid.*, 1961, v8, 184.
19. Roth, J. S., Eichel, H. J., Ginter, E., *Arch. Biochem. and Biophys.*, 1954, v48, 112.
20. Commoner, B., *Biol. Rev.*, 1940, v15, 168.

Received August 20, 1962. P.S.E.B.M., 1962, v111.

Studies on Dopa Decarboxylase Inhibitors *in vivo* by Use of C^{14}-Carboxyl-Labeled Dopa.* (27925)

ESKIL HANSSON† AND WILLIAM G. CLARK
Veterans Administration Hospital, Sepulveda, Calif., and the Medical Center, University of California, Los Angeles

Decarboxylation of amino acids is a minor but nevertheless important pathway in amino acid catabolism, since it is responsible for the formation in microorganisms, plants and animals, of biologically active amines such as histamine, 3,4-dihydroxyphenethylamine (dopamine, the precursor of norepinephrine and epinephrine), tyramine, tryptamine, and 5-hydroxytryptamine (serotonin, 5HT).

There have been many reports concerning compounds which inhibit the enzymatic decarboxylation, particularly of 3,4-dihydroxyphenylalanine (dopa). Most studies have been made *in vitro* and the variety of compounds tested has recently been reviewed (1,2). Inhibition *in vivo* has been studied by different methods, for example: pressor response of dopa on blood pressure(1-4), measurement of the dopamine formed after injection of dopa and decarboxylase inhibitors(3,5), broncho-dilation by 5HT(6), heart contractile force(7), and formation of 5HT in kidney after injection of 5-hydroxytryptophan(8).

We found that a rapid method of studying directly the effect of various compounds on decarboxylation of dopa *in vivo*, was to measure radioactivity in the respiratory carbon dioxide after injection of dopa labeled in the carboxyl group with C^{14}.

α-Methyldopa has been found to possess activity as an inhibitor of dopa decarboxylation(4,5,8,9). This report concerns mainly our investigation of different α-amino acids for dopa decarboxylase inhibition activity but also includes some other compounds which are known to possess inhibition *in vi-*

* Supported by grants from Am. Cancer Soc., U. S. Public Health Service, Nat. Assn. for Mental Health, and Am. Heart Assn.

† Present address: Dept. of Pharmacology, Royal Veterinary College, Stockholm, Sweden.

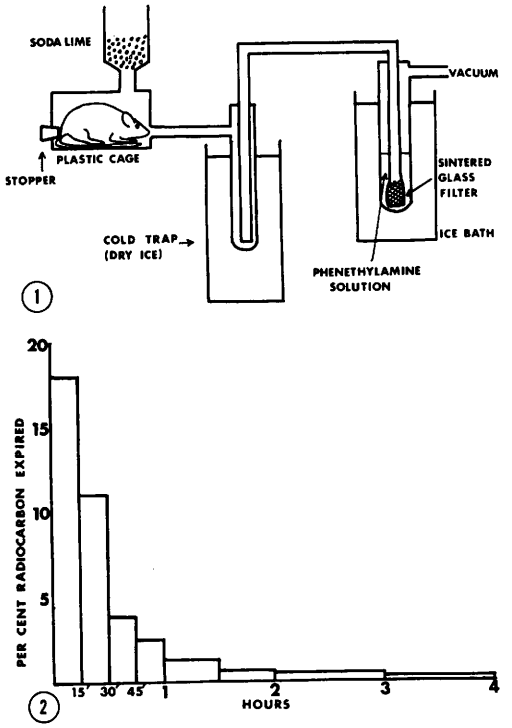

FIG. 1. Schematic diagram of apparatus for collecting respiratory CO_2.

FIG. 2. Radioactive CO_2 in respiratory air after intravenous injection of C^{14}-carboxyl-labeled dopa in a mouse. Radioactivity expressed as % of that administered which is expired in 15 min.

tro and/or *in vivo*.

Methods. Male albino mice weighing 18-22 g each were used in most experiments. In a few cases male DBA mice of the same weight were used. The mice were placed in a restrainer in a plastic metabolic cage (Fig. 1). The tail was kept outside the cage through a hole for injection. Carboxyl-labeled dopa (New England Nuclear Corp., Boston, Mass.) specific activity 1.89 mc/mM, was dissolved in saline and 0.2 ml of the solution containing 0.04 μc (4 μg) was injected intravenously into a tail vein. After injection the tail was put into the cage and the hole stoppered. Air which had passed through a soda lime filter was drawn through the cage by a vacuum pump, and the expiratory carbon dioxide collected in a phenethylamine solution (phenethylamine, Matheson, Coleman & Bell, Norwood, Ohio, Cat. No. 6066). Phenethylamine has been shown to absorb CO_2 quantitatively and the carbamate formed by the reaction of CO_2 with the amine quenches very little(10).

The CO_2 absorbing solution was the same as that used by Woeller(10) and contained methanol 27 ml, the phosphors, 2,5-diphenyloxazole (PPO) 500 mg, and 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) (Packard Instr. Co., La Grange, Ill.) 10 mg, phenethylamine 27 ml and toluene to 100 ml. The air from the metabolism cage was drawn through a sintered glass filter, porosity C, into 10 ml of the absorption-phosphor solution described above (Fig. 1). The absorbing solution was kept in an icebath to diminish evaporation, and a dry ice trap was put between the absorption fluid and the metabolism chamber to absorb the water in the respiratory air to avoid quenching (Fig. 1). At the end of the experiment the CO_2 absorbing solution was transferred to a counting vial and counted in a Packard Tri-Carb spectrometer. Quantitative data were obtained by use of an internal standard.

That $C^{14}O_2$ was quantitatively absorbed into the phenethylamine solution was tested by generating a known amount of $C^{14}O_2$ from a $NaHC^{14}O_3$ (Calbiochem., Los Angeles, Calif.) solution in the plastic cage under the same experimental conditions as in the animal experiment. A linear relationship was obtained after plotting the counting rate against the $C^{14}O_2$ used.

The test compounds were injected mostly intraperitoneally (i.p.) 15 minutes prior to dopa intravenously (i.v.), or i.v. immediately before dopa i.v., and the expiratory $C^{14}O_2$ was collected for 45 minutes, since previous experiments showed that most of the injected C^{14} was eliminated in the expired air in 30 minutes (see *Results*).

The inhibition was expressed as per cent depression in expired radioactivity in inhibitor-treated animals compared with control animals.

In a few cases the urine was collected and urine radioactivity was directly counted in the scintillation counter. Corrections for quenching were made by use of an internal standard.

Compounds used were generously provided by the following sources: L-α-methyl-

dopa, DL-α-methyldopa, DL-α-methyl-*m*-tyrosine, DL-α-methylhistidine, DL-α-methyl-3-methoxyphenylalanine and DL-α-hydrazino-α-methyl-3,4-dihydroxyphenylbutyric acid (MK-485), Merck, Sharp & Dohme, Inc., West Point, Pa.; DL-α-methyltryptophan, Sandoz, A.G., Basel, DL-α-methyl-5-hydroxytryptophan, Upjohn Co., Kalamazoo, Mich.; D-, and L-cycloserine, Eli Lilly and Co., Indianapolis; 3-hydroxybenzyloxyamine (NSD-1024), N(3-hydroxybenzyl)-N-methylhydrazine (NSD-1034) and 4-bromo-3-hydroxybenzyloxyamine (NSD-1055), Smith and Nephew Research Ltd., Ware, Herts, England. The other chemicals were purchased commercially.

The compounds were dissolved in water for injection, facilitated when necessary by addition of dilute hydrochloric acid. In a few cases the compound was suspended in saline and the suspension injected i.p. The doses were calculated on the basis of the free base except where otherwise indicated.

Results. Appearance of respiratory radiocarbon. The rate of appearance of $C^{14}O_2$ in the respiratory air was determined at various times after injection of C^{14}-dopa. Fig. 2 shows the appearance of radioactivity expressed as per cent of injected dose. Thirty per cent of injected radioactivity was expired during the first 30 minutes; during the next 3.5 hour period only about 9% more was expired. In experiments with 18 mice without pretreatment with inhibitor, the mean value of expired $C^{14}O_2$ was 35.9 ± 3.8% (mean ± standard deviation) in 45 minutes. The DBA mice consistently showed a much higher decarboxylating activity, the expired radiocarbon amounting to 40.8 ± 2.3 in experiments with 10 mice.

Dopa decarboxylase inhibitors. 100 mg/kg DL-α-methyldopa was injected i.v. into the tail vein of mice in a volume of 0.2 ml. Immediately afterwards the C^{14}-dopa was injected i.v. The results show that DL-α-methyldopa inhibits the decarboxylation 65.7% (Table I). Experiments were also performed to investigate whether a large dose of unlabeled DL-dopa injected immediately before injection of the C^{14}-dopa had any effect. 100 mg/kg DL-dopa dissolved in saline was injected followed by the C^{14}-dopa. An inhibition of 18.2% was obtained (Table I).

TABLE I. Effects of Intravenously Administered DL-α-Methyldopa and DL-Dopa on Decarboxylation of Dopa.

Compound	Dose, mg/kg i.v.	% inhibition Mean value	Range
DL-α-methyldopa	100	65.7*	81.3-50.1
DL-dopa	100	18.2†	24.5-13.9

* Mean of 5 animals. † Mean of 4 animals.

To ascertain whether a change in urinary excretion of radioactivity occurred after administration of DL-α-methyldopa or a large dose of unlabeled DL-dopa, urine samples were in a few cases analyzed for radioactivity (Table II). The control animals excreted approximately 27% of the injected radioactivity in the urine within 45 minutes. Animals receiving α-methyldopa, although exhibiting a decreased respiratory excretion, showed no increase in urinary radioactivity. Injection of the same large dose of labeled DL-dopa also had no effect.

Since intravenous injection was somewhat cumbersome, intraperitoneal injection of presumed inhibitors was used in most experiments. A number of α-amino acids were tested. Quantitatively, L-α-methyldopa was the most potent, followed in order of decreasing potency by DL-α-methyldopa, DL-α-methyl-*m*-tyrosine, DL-α-methyl-5-hydroxytryptophan and DL-α-methyltryptophan. DL-α-methylhistidine and DL-α-methyl-3-methoxyphenylalanine were devoid of inhibitory activity (Table III).

Some experiments were also performed with compounds other than α-methylamino acids, which were known to inhibit dopa decarboxylation *in vitro* and/or *in vivo*. The results are shown in Table IV.

Of the compounds tested, the hydrazine

TABLE II. Radioactivity in Urine and Respiratory Air Collected During 45 Minutes After Injection of C^{14}-Carboxyl-labeled Dopa in DL-α-Methyldopa and DL-Dopa Pretreated Animals. Mean values of 3 animals.

Pretreatment	Dose mg/kg i.p.	% expired	% urine	Total
Control		38.6	26.9	65.5
DL-α-methyldopa	100	18.6	27.4	46.0
DL-dopa	100	34.3	27.1	63.4

TABLE III. Comparison of Dopa Decarboxylase Inhibitory Effect of Various α-Methyl-amino Acids.

Compound	Dose mg/kg i.p.	% inhibition Mean value*	Range	ED_{50}†
DL-α-methyldopa	100	41.6	61.7–24.6	
L-α-methyldopa	100	61.2	68.3–51.4	82
DL-α-methyl-*m*-tyrosine	100	36.9	51.0–12.0	176
DL-α-methyl-5-hydroxy-tryptophan	100	22.4	24.2–19.7	
DL-α-methyltryptophan	100	7.2	21.3– 2.3	
DL-α-methyl-3-methoxy-phenylalanine	100	0		
DL-α-methylhistidine	100	0		

* Mean of at least 3 animals.

† Intraperitoneal dose required to depress the expired CO_2 to 50% compared with control animals. ED_{50}'s were determined graphically from a dose response curve.

compounds DL-α-hydrazino-α-methyl,3,4-dihydroxyphenylbutyric acid (MK-485) and N(3 - hydroxybenzyl) - N - methylhydrazine (NSD-1034) had very strong inhibitory activity. The ED_{50}'s were calculated to be 1.8 and 2.2 mg/kg respectively, and the potency of these drugs was thus approximately 40 times stronger than that of L-α-methyldopa.

The 2 oxyamines tested, 3-hydroxybenzyloxyamine (NSD-1024) and 4-bromo-3-hydroxybenzyloxyamine (NSD-1055), show less inhibition. NSD-1024 had approximately the same activity as L-α-methyldopa. 5-(3-hydroxycinnamoyl)salicylic acid(1), hydrazine sulfate and semicarbazide hydrochloride show inhibitory activity while the other compounds tested do not.

TABLE IV. Comparison of Dopa Decarboxylase Inhibitory Effects of Various Compounds.

Compound	Dose mg/kg i.p.	% inhibition*	ED_{50}†
NSD-1034 (See text)	100	95.6	2.2
NSD-1024 " "	100	34.3	
NSD-1055 " "	100	20.1	
MK-485 " "	100	93.7	1.8
5-(3-hydroxycinnamoyl) salicylic acid	100	17.4	
L-cycloserine	100	0	
D-cycloserine	100	0	
Semicarbazide • HCl	100	8.3	
Iproniazid	100	0‡	
Hydrazine • H_2SO_4	100	26.1	

* Mean of at least 3 animals.

† Intraperitoneal dose required to depress the expired CO_2 to 50% compared with control animals. ED_{50}'s were determined graphically from a dose response curve.

‡ A slight increase of respiratory CO_2 was observed after injection of iproniazid.

Duration of inhibition by the 3 most potent inhibitors is shown in Table V. The dose used approximated the ED_{50}. The mice were injected i.p. and inhibitory activity determined at 15 minutes, 1, 2, 4, and 8 hours after injection. Although the number of mice is limited it is evident that the inhibitory effect of NSD-1034 is of rather short duration. The effect decreased more than 50% one hour after injection, compared with the 15 minute test. L-α-methyldopa and MK-485, on the other hand, had almost the same activity 2 hours after injection, and significant activity remained after 8 hours.

Discussion. Published methods for demonstrating inhibition of amino acid decarboxylase *in vivo* are limited and mostly indirect. No reports have been found similar to the present method, although Baxter and Roberts (personal communication) have measured glutamic acid-$C^{14}OOH$ decarboxylation and its inhibition *in vitro*, and in intact rats, using hyamine as CO_2 absorbent.

There are of course other pathways which can yield $C^{14}O_2$ from carboxyl-labeled amino acids besides direct decarboxylation. Dopa, for example, could be transaminated to 3,4-dihydroxyphenylpyruvic acid followed by oxidative decarboxylation to 3,4-dihydroxyphenylacetic acid and CO_2(11-13).

Certain precautions must be taken in using the present method. The radioactive CO_2 released by decarboxylation of dopa *in vivo* can be reutilized and, depending on the condition of the animals, varying amounts of the radiocarbon may be expired. The con-

TABLE V. Duration of Dopa Decarboxylase Inhibitory Effect of L-α-Methyldopa, NSD-1034 and MK-485 (See Text). Each value represents a single experiment.

Compound	Dose, mg/kg i.p.	% inhibition at various times after inj. of inhibitor				
		15 min	1 hr	2 hr	4 hr	8 hr
L-α-methyldopa	100	59.5	56.6	56.9	26.8	10.3
NSD-1034	5	76.0	20.4	9.3	5.6	0
MK-485	5	73.8	61.5	43.4	22.8	11.2

trol animals show, however, surprisingly small individual variation and it can therefore be expected that this possible error is minor and it can be decreased by using mice of the same strain, sex, weight and nutritional state. It is important to use the same strain since a consistent difference of approximately 15% has been observed between albino mice and DBA mice. It is possible that the increased activity in DBA mice is related to melanin formation.

Another possible error may arise from low respiratory rates caused, for example, by such drugs as hypnotics and sedatives which could result in a decreased respiratory CO_2, thus reflecting a false "inhibition" although decarboxylation itself actually may remain unaltered. Also, if injected drugs cause excessive hyperventilation, more $C^{14}O_2$ might be expired, masking possible inhibition of decarboxylation. Although this probably would not be a large effect, routine spirometer measurements should be done with suspect drugs.

The results show that α-methyldopa is the strongest inhibitor among the α-methylamino acids tested, and confirm earlier investigations using different methods, that several of the latter also are active(5,8) as well as the fact that only the L form is active(8). Thus the present method provides a quantitative assay for determining dopa decarboxylase inhibition *in vivo* which compares favorably with the other methods in terms of sensitivity but has the advantage of simplicity and speed. Unfortunately the carboxyl-labeled method has not revealed any inhibitors of histidine decarboxylase in intact mice, at least of the compounds mentioned here. Further work on this is in progress, as well as on carboxyl-labeled 5-hydroxytryptophan.

The results also confirm earlier reports that NSD-1034 and MK-485 are the most potent decarboxylase inhibitors yet described *in vivo*(14-16). The 2 oxyamines tested, NSD-1024 and NSD-1055, are less potent. At first it was thought that the high activity of the former 2 compounds possibly was related to the hydrazine moiety. However, under the conditions of these experiments hydrazine and semicarbazide, known inhibitors of dopa decarboxylase(1), do not inhibit sufficiently to support this view. Iproniazid pretreatment, in contrast, caused an *increased* output of respiratory $C^{14}O_2$ in comparison with controls. This problem is being studied, and other monoamine oxidase inhibitors and amine releasers are being examined.

It is of interest that other compounds such as cycloserine and 5-(3-hydroxycinnamoyl)salicylic acid which have been shown to inhibit *in vitro* and *in vivo*(17,1) by other methods, have little or no effect in the present method. The reason for this is not clear, but it may be that some of the compounds which are potent *in vitro* are more rapidly metabolized and/or unable to pass cell barriers or other barriers to reach the enzyme.

Summary. A study has been made on the effects of some compounds on inhibition of decarboxylation of 3,4-dihydroxyphenylalanine (dopa) *in vivo*. A new rapid and simple method for determination of the inhibition has been developed which yields satisfactory results in intact unanesthetized mice. C^{14}-carboxyl-labeled dopa is injected and the respiratory $C^{14}O_2$ trapped in a phenethylamine solution containing fluorophore. Radioactivity is then counted directly in a liquid scintillation counter. The most active decarboxylase inhibitors of the compounds tested are DL-α-hydrazino-α-methyl,3,4-dihy-

droxyphenylbutyric acid and N(3-hydroxybenzyl)-N-methylhydrazine. Their inhibitory action is approximately 40 times higher than, *e.g.*, that of L-α-methyldopa.

1. Clark, W. G., *Pharmacol. Rev.*, 1959, v11, 330.
2. Clark, W. G., Pogrund, R. S., *Circulation Res.*, 1961, v9, 721.
3. Pogrund, R. S., Drell, W., Clark, W. G., *J. Pharmacol. and Exp. Therap.*, 1961, v131, 294.
4. Dengler, H., Reichel, G., *Naunyn-Schmiedeberg's Arch. Exp. Path. Pharmak.*, 1958, v234, 275.
5. Murphy, G. F., Sourkes, T. L., *Arch. Biochem.*, 1961, v93, 338.
6. Westermann, E., Balzer, H., Knell, J., *Naunyn-Schmiedeberg's Arch. Exp. Path. Pharmak.*, 1958, v234, 194.
7. Goldberg, L. I., DaCosta, F. M., Osaki, M., *Nature*, 1960, v188, 502.
8. Porter, C. C., Totaro, J. A., Leiby, C. M., *J. Pharmacol. Exp. Ther.*, 1961, v134, 139.
9. Sourkes, T. L., *Arch. Biochem.*, 1954, v51, 444.
10. Woeller, F. H., *Annal. Biochem.*, 1961, v2, 508.
11. Pellerin, J., D'Iorio, A., *Canad. J. Biochem.*, 1955, v33, 1055.
12. ———, *Rev. Canad. Biol.*, 1957, v15, 371.
13. Shaw, K. N. F., McMillan, A., Armstrong, M. D., *J. Biol. Chem.*, 1957, v226, 255.
14. Drain, D. J., Horlington, M., Lazare, R., Poulter, G. A., *Life Sciences*, 1962, v1, 93-97.
15. Hirsch, C. W., Kuntzman, R., Costa, E., *Fed. Proc.*, 1962, v21, 364.
16. Udenfriend, S., Connamacher, R., Hess, S. M., *Biochem. Pharmacol.*, 1961, v8, 419.
17. Dengler, H. J., Rauchs, E., Rummel, W., *Naunyn Schmiedeberg's Arch. Exp. Path. Pharmak.*, 1962, v243, 366.

Received July 23, 1962. P.S.E.B.M., 1962, v111.

Estradiol-Cellular Interaction in Tissue Culture.* (27926)

ROBERT J. KUCHLER, NANCY J. ARNOLD, AND ROBERT C. GRAUER
Department of Microbiology, William H. Singer Memorial Research Laboratory, Allegheny General Hospital, Pittsburgh, Pa.

Estrogens are inhibitors of mammalian cell growth *in vitro*. This activity is believed to be associated with the fact that these steroids physically block the uptake of essential metabolites(1). Substances as unlike as amino acids, monosaccharides, and inorganic phosphate are hindered in their normal passage into cells by free and sulfated estrogens. Included are inactive mediations, active transports and one apparently unmediated migration(2). LeFevre and Marshall have shown that glucose transport is inhibited when stilbesterol is fixed to superficial sites on the human red cell surface (3). In a previous report we presented evidence in a tissue culture system indicating that substrate utilization and cell growth were inhibited when estrogens were bound at the cell membrane(1). In this report we have attempted to clarify some aspects of the cell-estradiol interaction since this compound is not metabolized by L strain fibroblasts.

Materials and methods. Large numbers of stationary bottle cultures of L strain mouse fibroblasts were set up in a replicate series in 150-ml milk dilution bottles in medium 199 supplemented with 0.5% bacto-peptone (199P). After 48 hours the cells on the bottles were in the exponential phase of the growth cycle. At this time the medium was replaced with fresh 199P containing estrone-16-C^{14}† or estradiol-16-C^{14}† at tracer levels which did not inhibit cell growth. These radioactive steroids were solubilized in propylene glycol, sterilized by autoclaving and added to the tissue culture medium at a final concentration of 1 μg/ml. The cultures were harvested at 24 hour intervals for the next 72 hours. When harvested, the media were decanted and the fibroblasts attached to the glass were washed with BSS,

* This work was supported in part by grants from U. S. Public Health Service and from the Health Research and Services Foundation, United Fund of Allegheny County, Pa.

† 1 mg/2.7 μc, Charles E. Frosst and Co., Montreal, Canada.