Biochemical Pharmacology, Vol. 31, No. 20, pp. 3251–3256, 1982.
Printed in Great Britain.

0006-2952/82/203251-06 $03.00/0

TETRAHYDROISOQUINOLINECARBOXYLIC ACIDS AND CATECHOLAMINE METABOLISM IN ADRENAL MEDULLA EXPLANTS

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(*Received* 17 *November* 1981; *accepted* 11 *March* 1982)

Abstract—In a study of the relationship of the tetrahydroisoquinolinecarboxylic acids (TIQCAs) to catecholamine metabolism, we have investigated their effects on cultured rat adrenal medulla explants. Medullae were incubated in medium containing norlaudanosolinecarboxylic acid (NLCA) or 3′,4′-deoxynorlaudanosolinecarboxylic acid (DNLCA) (0.5 mM) in the presence and absence of [^{3}H]tyrosine. By paired-ion reverse-phase high pressure liquid chromatography, tissue epinephrine (EPI), norepinephrine (NE), dopamine (DA) and TIQCA were resolved. Endogenous concentrations were measured with electrochemical detection, and radioactivity was assayed by collecting appropriate effluents. Tissue levels of the TIQCAs reached saturating levels of 0.36 mM by about 20 hr. DNLCA elicited a significant decrease (60%) in endogenous DA, NE and EPI at 40 hr, whereas only DA was depressed at 30 hr. NLCA had little effect after 30 or 40 hr. When tissues were maintained in the presence of α-methyltyrosine (0.5 mM) for 40 hr, catecholamine levels were depressed to an extent similar to that observed with DNLCA. Incubation with [^{3}H]tyrosine in the presence of TIQCAs revealed inhibition of tyrosine uptake and suggested a reduction in the rate of catecholamine synthesis. These results are consistent with previous data on the inhibition of tyrosine 3-monooxygenase by DNLCA *in vitro*.

A group of tetrahydroisoquinolinecarboxylic acids (TIQCAs)‡ that are condensation products of dopamine (DA) and hydroxylated phenylpyruvates has been implicated in the plant kingdom as precursors of tetrahydroisoquinoline (TIQ) alkaloids and morphine [1–3]. Recently, their occurrence in urine of Parkinsonian patients receiving L-DOPA ± a peripheral decarboxylase inhibitor [4] has been reported. In addition, Lasala and Coscia [5] noted the appearance of DNLCA, the condensation product of DA and phenylpyruvate, in urine of phenylketonuric children.

The occurrence of TIQCAs in humans raises the question of their effects on catecholamine metabolism. We discovered that TIQCAs inhibit both adrenal tyrosine 3-monooxygenase (EC 1.14.16.2), dopamine β-hydroxylase (EC 1.14.17.1), and catechol *O*-methyltransferase (EC 2.1.1.6) *in vitro* [6, 7]. A related group of TIQs, thought to be derived from DA and aldehydes under conditions of alcohol intoxication [8–10], also inhibit tyrosine 3-monooxygenase [11], catechol *O*-methyltransferase [12], and monoamine oxidase (EC 1.4.3.4) [13] *in vitro*. However, there are few data available on the action of TIQCAs on intact tissue containing a full complement of enzymes involved in catecholamine metabolism. Such a study would permit an assessment of the overall effect of TIQCAs as influenced by compartmentalization of relevant enzymes and compensatory regulatory mechanisms.

Organ cultures of rat adrenal medulla have been used to study aspects of the induction [14] and phosphorylation [15] of tyrosine 3-monooxygenase and the synthesis of dopamine β-hydroxylase [16]. Burke *et al.* [17] observed a diminution of phenylethanolamine *N*-methyltransferase (EC 2.1.1.28) activity and synthesis after the addition of epinephrine (EPI) to the medium, revealing that metabolism in the explants can be manipulated by exogenous catecholamines. The use of this system complements studies with whole animals and permits a systematic study of catecholamine metabolism under controlled conditions.

In this paper, we report on the effects of two TIQCAs on catecholamine metabolism in adrenal medulla explants. NLCA, a condensation product of 3,4-dihydroxyphenylpyruvate and DA, and DNLCA were added to the culture media and their effects on catecholamine levels determined using HPLC and electrochemical detection (HPLC-EC). Also, tyrosine (TYR) uptake and metabolism were estimated by the addition of [^{3}H]tyrosine to the medium and subsequent localization of label in catecholamines and metabolites after resolution and isolation by HPLC. Our results support previous

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‡ Abbreviations used: α-MT, α-methyltyrosine; DA, dopamine; DNLCA, 3′,4′-deoxynorlaudanosolinecarboxylic acid; DOPA, 3,4-dihydroxyphenylalanine; EPI, epinephrine; HPLC-EC, high pressure liquid chromatography with electrochemical detection; NE, norepinephrine; NLCA, norlaudanosolinecarboxylic acid; TIQ, tetrahydroisoquinoline; TIQCA, tetrahydroisoquinolinecarboxylic acid; and TYR, tyrosine.

findings [6, 7] that the TIQCAs can inhibit the rate-limiting enzyme of catecholamine biosynthesis, tyrosine 3-monooxygenase.

MATERIALS AND METHODS

(±)DNLCA (1-carboxy-1-benzyl-6,7-dihydroxytetrahydroisoquinoline) and (±)NLCA [1-carboxy-1(3′,4′-dihydroxybenzyl)6,7-dihydroxytetrahydroisoquinoline) were synthesized in our laboratory [18] and were determined to be greater than 99.9% pure by HPLC. L-[2,6-^{3}H]TYR and L-[(U)^{14}C]leucine were obtained from the New England Nuclear Corp. (Boston, MA). Catecholamines and other chemicals were purchased from the Sigma Chemical Co. (St. Louis, MO). Heptanesulfonic acid was from MCB (Norwood, OH). Culture medium 199 and TYR-free medium were obtained from Gibco (Grand Island, NY). Separation of catecholamines was performed with a Waters Associated (Milford, MA) HPLC unit equipped with a model U6K injector, a model 6000A pump and a C_{18} reverse-phase column from Phase Sep (Hauppauge, NY) (25 × 4.6 cm, 5 μm) or Unimetrics (Anaheim, CA) (25 × 4.6 cm, 7 μm). The electrochemical detector was purchased from Bioanalytical Systems (Lafayette, IN, model LC-3).

Organ cultures. Male Holtzman rats were killed, and one adrenal was immediately removed from each and placed in a small amount of medium. Using a dissecting microscope, the adrenal was bisected with a sterile single-edge razor, and the medulla was dissected free of cortex. One section of the same medulla served as a matched control for the experimental piece. Initially, all explants were placed in small circular culture dishes containing 3 ml of medium 199 with modified Earle's salts supplemented with sodium ascorbate (6 mM), streptomycin (0.2 mg/ml), and penicillin (100 units/ml). After all medullae were removed from the animals, the medium was changed and replaced with medium 199 containing sodium ascorbate and penicillin (control) and TIQCA (0.5 mM except where noted). Routinely, tissue was maintained for approximately 40 hr at 37°, and the medium was changed once at 24 hr. In experiments where [^{3}H]TYR was used, the radioactivity was added at 30 hr, and tissue was removed for analysis at the times indicated. When [^{3}H]TYR uptake and metabolism were studied, the radioactive substrate was added in fresh medium devoid of unlabeled TYR (Gibco).

Catecholamine and TIQCA analyses. To terminate the incubation, medullae were removed from culture dishes and washed two to three times with fresh medium. Tissue was homogenized in 0.25 ml of 0.4 N $HClO_4$ (containing unlabeled catecholamine when radioactivity was studied) and then centrifuged in a Beckman microfuge. An aliquot of the supernatant fraction was subjected to HPLC to resolve catecholamines and metabolites [19]. Quantitation of endogenous catecholamines was performed with electrochemical detection (+0.74 V) whereas assessment of radioactivity was accomplished by collecting appropriate peak effluents monitored at 280 nm with subsequent liquid scintillation counting.

For routine HPLC separation of catecholamines, 5% aqueous ethanol, 175 mM acetic acid, 2 mM heptanesulfonic acid, and 1 mM EDTA at a pH of 3.8 was the preferred elution solvent. When resolution of TYR and Dopa was also desired, 5% aqueous methanol, 175 mM acetic acid, and 2 mM heptanesulfonic acid at a pH of 3.8 was used. TIQCAs were also resolved by HPLC with a solvent system consisting of 10% aqueous ethanol, 175 mM acetic acid, and 1 mM EDTA at a pH of 3.8. (The retention time of DNLCA was 17 min.)

All solvents were glass-distilled prior to HPLC use.

Protein and enzyme analyses. After precipitation of protein and centrifugation, the pellet was washed with H_2O and then dissolved in 0.6 ml of 2% Na_2CO_3 in 1 N NaOH. Protein concentration was measured using the method of Lowry *et al.* [20].

In the protein synthesis experiment, 2.5 hr prior to termination of the incubation, 25 μCi of [^{14}C]leucine was added to the medium. Each medulla was subsequently homogenized in 0.75 ml of 0.4 N $HClO_4$. The protein was precipitated by centrifugation at 6000 *g* and the pellet was washed with 1 ml of 0.4 N $HClO_4$. The precipitate was taken up into 0.16 N NaOH and heated to 100° for 30 min, and a 100 μl aliquot was counted in PCS (Amersham, Arlington Heights, IL). The remainder was assayed for protein content.

Statistical analyses were done with Student's *t*-test for paired samples.

RESULTS

In conducting experiments in which adrenal medulla explants were cultured for as long as 2 days, it was important to determine both tissue viability and the stability of TIQCAs over this time interval. Previous histological examination of adrenal medulla explants by both light and electron microscopy showed them to be structurally intact over a 48 hr period [17]. The effect of 0.5 mM DNLCA on protein synthesis was also estimated as an additional criterion of tissue viability under the conditions of these experiments. DNLCA did not change the rate of incorporation of [^{3}H]leucine into adrenal medulla protein (1994 ± 248 vs 1904 ± 249 dpm per μg protein per hr) in tissue maintained for 38.5 hr in medium. In addition, the synthesis of ^{3}H-catecholamines from [^{3}H]TYR by tissues cultured for 30 hr also demonstrated viability (see below). Furthermore, the concentrations of DA, NE, or EPI in control tissue after 40 hr of incubation were not decreased in comparison to levels determined after 1 or 10 hr of incubation (data not shown).

The accumulation by the explants of 0.5 mM DNLCA from the medium was assessed by HPLC-EC (Fig. 1). After 20 hr in the medium, the level of DNLCA in the explants had reached a steady-state value of 6 ng/μg protein. Assuming no compartmentalization, this corresponds to a concentration of approximately 0.36 mM. The rate of uptake of DNLCA into tissue is comparable to that observed for EPI [17] or TYR (see below). NLCA tissue concentrations also reached steady-state levels, 7 ng/μg protein being observed after 30 and 40 hr of incubation in medium containing 0.5 mM NLCA.

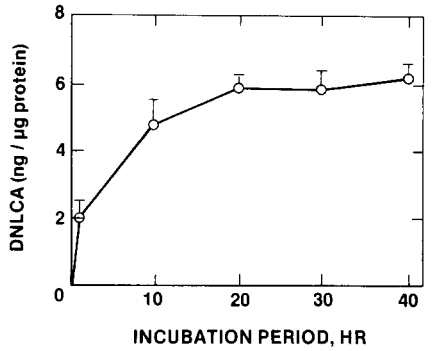

Fig. 1. Time course of DNLCA uptake into rat adrenal medulla explants. Immediately after killing the rat and dissection, medullae were incubated in 3 ml of culture medium containing 0.5 mM DNLCA. At the time intervals indicated, four halves were removed and each was homogenized in 0.25 ml of 0.4 N $HClO_4$. After protein removal, the fractions were subjected to HPLC-EC. See Materials and Methods for additional details.

Table 1. Dose dependence of the effect of DNLCA on endogenous catecholamine content of adrenal medulla explants*

DNLCA (µM)	N	DA	NE	EPI
		(% control ± S.E.M.)		
0	13	100 ± 16	100 ± 14	100 ± 17
50	3	92 ± 33	87 ± 2	102 ± 8
150	5	61 ± 25†	59 ± 15†	55 ± 17
500	5	31 ± 6‡	57 ± 11§	50 ± 13‖

* See Figs. 1 and 2 and Materials and Methods for details. All incubations were conducted for 40 hr. Statistical significance was determined by comparing experimental values to those obtained in matched control medullae.
† $P < 0.05$.
‡ $P < 0.005$.
§ $P < 0.01$.
‖ $P < 0.025$.

Determination of medium DNLCA by HPLC-EC revealed no evidence of breakdown. Within experimental error, 100% of added DNLCA could be accounted for. As in earlier studies wherein 0.5 to 1.0 mM EPI was maintained in the medium for the same length of time, the addition of 6 mM ascorbate was essential to prevent oxidative degradation [17].

The inclusion of 0.5 mM DNLCA in the medium had pronounced effects on the levels of DA, NE, and EPI after 40 hr of incubation (Fig. 2) in comparison to the values obtained in matched control medullae. DNLCA produced a decrease in the dopamine concentration after 30 hr of incubation (69 ± 11% control, N = 4, $P < 0.025$) but no changes were observed for NE and EPI levels at this time. There was no apparent effect on catecholamine levels at earlier periods. Dose-dependency studies revealed that 50 µM DNLCA did not change catecholamine levels but upon elevation to 150 µM the effect of DNLCA became apparent (Table 1). NLCA had no significant effect on catecholamine pools (Fig. 2).

The decrement in catecholamine levels may also have arisen from an increased activity of degradative enzymes. When monoamine oxidase activity was determined in dialyzed homogenates of tissue exposed to 0.5 mM DNLCA for 40 hr, there was no difference compared to control tissue [21]. The same phenomenon was observed for catechol *O*-methyltransferase activity [21]. A loss of catecholamines in the explants could also have arisen if the extragranular pool had been elevated enough to cause an inhibition of tyrosine 3-monooxygenase and/or to make these amines more accessible to degradative enzymes. To determine whether vesicular transport was being affected, the uptake or release of [^{3}H]EPI was measured in isolated bovine chromaffin granules in the presence of 0.2 mM DNLCA. No apparent effect was observed.* Thus, it appears that catecholamine levels were not influenced through these mechanisms.

Since DNLCA can inhibit rat adrenal tyrosine 3-monooxygenase *in vitro* [6, 7], its effects were compared to those of α-methyltyrosine (α-MT) which has also been shown to block catecholamine metabolism at this enzymatic reaction. As seen in Fig. 2, α-MT lowered the levels of all three catacholamines comparably at 40 hr and, like DNLCA, decreased only DA levels at 30 hr (data not shown).

The lowered content produced by DNLCA may also have been due to an alteration of TYR accumulation by the explant. To test this hypothesis, medullae were incubated with 0.5 mM DNLCA for 30 hr, after which [2,6-^{3}H]TYR was added to the medium. Tissue was removed at various intervals after the introduction of labeled TYR, and total tissue radioactivity was determined. DNLCA had a

* J. C. Bush, J. Ebel, S. Willman and M. P. Galloway, unpublished observations.

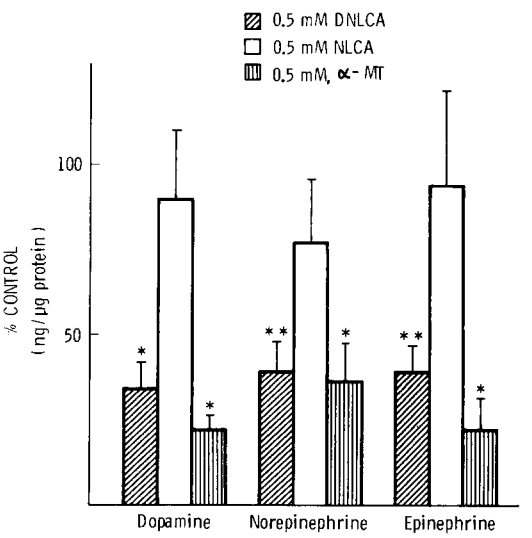

Fig. 2. Endogenous catecholamine levels in explants treated with DNLCA, NLCA or α-MT for 40 hr. The protocol outlined in Fig. 1 and Materials and Methods was followed. Control explants were matched halves of treated medullae; the medium contained the same additives except the drug. Basal catecholamine levels at 40 hr were: DA = 2.0, NE = 11.9, EPI = 57.0 ng/µg protein (N = 10). Each value represents the average obtained from four explants. Key: * $P < 0.005$, and (**) $P < 0.01$.

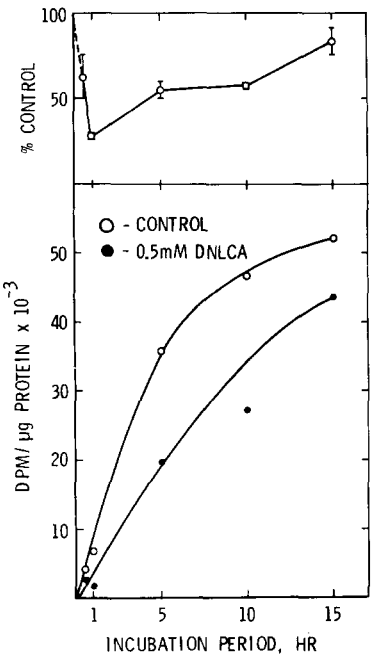

Fig. 3. Accumulation of [^{3}H]TYR by matched rat adrenal medulla explants in the presence and absence of DNLCA. Medullae were cultured in medium ± 0.5 mM DNLCA for 30 hr whereupon they were transferred to medium which was devoid of unlabeled TYR but contained 100 µCi of [2,6-^{3}H]TYR. At the time intervals indicated, tissue was removed and treated with 0.4 N $HClO_4$. Total acid-soluble radioactivity was measured; N = 4 explants per point. Top panel shows the experimental values as a percentage of the control.

marked effect on tyrosine uptake at 1 hr after introduction of the label (Fig. 3). Thus, a reduction in the TYR uptake can contribute to the catecholamine deficit. In the presence of 0.5 mM NLCA, TYR uptake from the medium was reduced considerably after 10 hr (38 ± 8% control, N = 6, $P < 0.01$).

Inhibition of tyrosine 3-monooxygenase should cause an increase in TYR and a concomitant decrease in DA levels. Since the amount of TYR in these tissues was below the minimal amount necessary for accurate fluorometric or electrochemical detection, specific activity of tissue TYR could not be measured in these tracer experiments. Thus, the amounts of radioactivity associated with TYR and various catecholamines were determined by adding carrier metabolites to tissue extracts to permit monitoring by ultraviolet absorbance and fractionation by HPLC. Noteworthy in Fig. 4 are the relatively similar levels of tritium associated with TYR in tissue, irrespective of the presence of DNLCA and time. Apparently the reduction in TYR uptake was offset by inhibition of tyrosine 3-monooxygenase. On the basis of uptake, one might have expected less label in the TYR fraction of the DNLCA-treated tissue. This was not observed. In contrast, tyrosine 3-monooxygenase inhibition by DNLCA should lead to an increase in label in TYR as compared to control. Indeed, if one expressed the radioactivity associated with TYR as a percentage of the total radioactivity present, then [^{3}H]TYR in DNLCA-treated tissue was 300% greater than that in untreated tissue.

Finally, there was a time-dependent increase in radioactivity associated with the catecholamine fraction for both control and treated tissue (Fig. 4). At

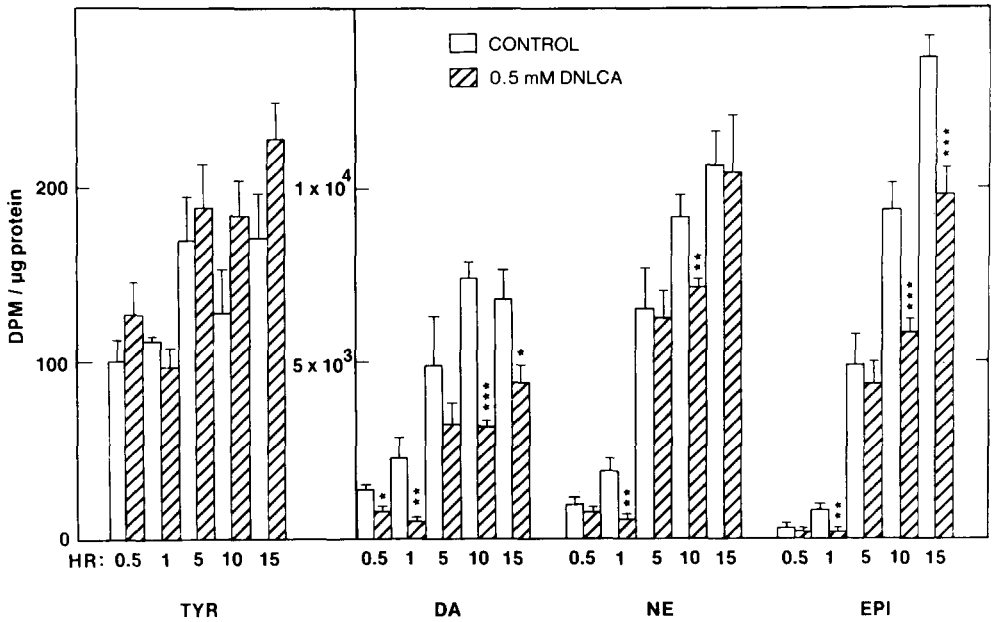

Fig. 4. Effect of DNLCA on radioactivity associated with specific catecholamines of rat adrenal medulla explants treated with [^{3}H]TYR. Conditions were identical to Fig. 3. Carrier metabolites were added to the acid-soluble radioactivity and aliquots were subjected to HPLC with monitoring by ultraviolet absorbance. The radioactivity associated with each resolved metabolite was determined by collection and liquid scintillation counting. Little tritium was found under peaks of L-dopa or acidic catecholamine metabolites. N = 6. Key: (*) $P < 0.05$, (**) $P < 0.01$, and (***) $P < 0.005$.

most time points, DNLCA-treated tissue contained less radioactivity in DA, NE and EPI than controls, consistent with the data on catecholamine levels (Fig. 2).

DISCUSSION

In previous studies, we have found that DNLCA is an inhibitor of the enzyme tyrosine 3-monooxygenase *in vitro* [6, 7]. In addition, chronic administration of DNLCA to rat neonates resulted in a reduction in the rate of cerebral DA synthesis [6]. The present data indicate that the effects of DNLCA on adrenal medulla explants are not only consistent with the notion that reduction in the rates of synthesis of three catecholamines occur but, also, that levels of these catecholamines are decreased in a dose- and time-dependent manner.

Maximum effects occurred 40 hr after initiation of the culture and at a time when overall synthesis of catecholamines from [^{3}H]TYR in both control and treated explants was still demonstrable. Futhermore, protein synthesis at this interval was unchanged from zero time.

In the explant experiments, the decline in the dopamine levels occurred before subsequent reduction of all three catecholamine pools. In addition to the slow uptake of DNLCA (Fig. 1), there is another reason for this relatively slow response. In light of the high endogenous concentrations of the catecholamines, especially NE and EPI, it would be anticipated that some time would elapse before these relatively large pool sizes would be affected. Consistent with this hypothesis are the comparable effects elicited by α-MT (Fig. 2), a previously demonstrated inhibitor of tyrosine 3-monooxygenase.

In contrast to DNLCA, NLCA did not significantly reduce catecholamine levels (Fig. 2). This may be attributable to several factors. NLCA is not as potent an inhibitor of tyrosine 3-monooxygenase from rat adrenal [6] or striatum [7] as DNLCA *in vitro*. In addition, its mechanism of inhibition is competitive with respect to cofactor, thereby differing from DNLCA, which is a noncompetitive inhibitor. NLCA also proved to be a poorer inhibitor of this enzyme when intact synaptosomes were used [7]. Finally, NLCA is a potent substrate-competitive inhibitor of catechol *O*-methyltransferase (K_i = 56 μM) [6]. Inhibition of this enzyme could reverse a decline in catecholamine levels by reducing their methylation. At the same time, methylated NLCAs, which are formed from NLCA *in vivo* [6], are even less inhibitory toward tyrosine 3-monooxygenase than NLCA itself [7].

As seen in Fig. 3, DNLCA depressed the initial uptake of labeled TYR into explants and this may have contributed to the overall depletion of catecholamines. However, NLCA also blocked TYR uptake, but did not deplete catecholamine pools appreciably. Thus, it appears that the effect on TYR uptake alone cannot account for the catecholamine deficits observed. Little effect on TYR incorporation into protein was observed in the experiments reported in Figs. 3 and 4, despite the reduction in uptake. Finally, tyrosine degradation would not be expected to be influenced by TIQCAs. Tyrosine transaminase is present in adrenal medulla [17], but neither NLCA nor its methylated derivative inhibited the rat liver enzyme *in vitro* [6].

The possibility exists that the decrease in endogenous catecholamine levels observed in this study was partially a result of a release of catecholamines into the medium. Our preliminary studies on catecholamine release from vesicles do not obviate this possibility. Determination of medium catecholamines is complicated by the fact that they are present in low concentrations in a complex mixture of nutrients. In this case HPLC-EC must be preceded by several prefractionation steps, hereby requiring internal standards. Experiments are in progress to determine whether DNLCA can elicit an increase in net release of catecholamine from the explants into medium.

A catecholamine deficit has been reported in brain, plasma and urine of phenylketonurics [22, 23 and references cited therein]. The etiology of this secondary effect remains to be elucidated, but evidence exists to suggest that tyrosine monooxygenase may be involved [24]. Previously, we reported evidence for the occurrence of DNLCA in human phenylketonuria [5]. The results described here provide further support for the working hypothesis that, if DNLCA is formed in sufficient amounts in discrete cellular loci, it may contribute to the catecholamine deficit observed in this amino acidopathy.

Acknowledgements—The constructive criticism of Bryan Roth is greatly appreciated. This work was supported by Grant NIH-NS-12342. We thank Dr. J. W. Davis for the use of his explant incubation equipment.

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