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SIMULTANEOUS MEASUREMENT OF 5-HYDROXYTRYPTOPHAN AND L-DIHYDROXYPHENYLALANINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

MEASUREMENT OF SEROTONIN AND CATECHOLAMINE TURNOVER IN DISCRETE BRAIN REGIONS

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SUMMARY

A sensitive method, using reversed-phase high-performance liquid chromatography with electrochemical detection, which allows the measurement of both 5-hydroxytryptophan and L-dihydroxyphenylalanine is described. This method, used in conjunction with the administration in vivo of an L-aromatic amino acid decarboxylase inhibitor, allows the simultaneous measurement of serotonin and catecholamine synthesis rates in small discrete brain regions.

INTRODUCTION

Serotonin, norepinephrine and dopamine are established as important brain neurotransmitters. Alterations in the synthesis or metabolism of these monoamines appear to be reasonable biochemical reflections of altered function in their respective neurons. A number of approaches to the estimation of turnover or synthesis rates of these monoamines have been suggested. These are based on certain assumptions and are open to a number of criticisms [1]. A method described by Carlsson [2], in which the accumulation of 5-hydroxytryptophan (5HTP) or L-dihydroxyphenylalanine (DOPA) is measured following inhibition of aromatic-L-amino acid decarboxylase, addresses the rate-limiting step in monoamine biosynthesis more directly than other turnover methods. Application of this method has been limited by the relative lack of sensitivity and specificity of the fluorimetric methods employed to measure these monoamine precursors. Radioenzymatic assays [3—6] are specific and sensitive but also laborious.

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Recently, high-performance liquid chromatographic (HPLC) methods for the measurement of either 5HTP [7—10] or DOPA [11, 12] have been described. In this paper, we describe modifications which allow the simultaneous measurement of picogram quantities of both 5HTP and DOPA in discrete brain regions. Furthermore, we have used this method to measure synthesis rates of serotonin and the catecholamines in brain following decarboxylase inhibition.

MATERIALS AND METHODS

Standards and reagents

m-Hydroxybenzylhydrazine dihydrochloride (NSD 1015; Aldrich, Milwaukee, WI, U.S.A.), 5HTP and DOPA (Sigma, St. Louis, MO, U.S.A.) were used.

Analytical reagent grade chemicals were used without further purification. All solutions were prepared from deionized and glass-distilled water.

Standards were dissolved in 0.2 *N* acetic acid.

HPLC apparatus

The high-performance liquid chromatograph system consisted of a single-piston pump (Altex 110A) equipped with a solvent reservoir filter (20—30 μm), an additional in-line solvent filter (7 μm sintered stainless steel; Nupro), a 48 MPa (7000 p.s.i.) injection valve (Valco), a guard column (3 cm × 4.6 mm I.D.) dry-packed with Perisorb RB-18, particle size 30—40 μm (Merck, Darmstadt, G.F.R.), a high-performance reversed-phase column (Beckman Ultrasphere-ODS, Beckman, Fullerton, CA, U.S.A.; particle size 5 μm, column size 25 cm × 4.6 mm I.D.), an LC-4 electrochemical detector (Bioanalytical) with a carbon paste in oil electrode and a Ag/AgCl reference electrode, and a linear strip-chart recorder.

Sample preparation

Samples were homogenized in 8 volumes of 0.1 *N* perchloric acid containing 2 mg EDTA and 0.5 mg sodium bisulphite per ml using either a Polytron homogenizer or a motor-driven pestle fashioned out of resin. After incubation at 4°C for 30 min, homogenates were centrifuged at 32,000 *g* for 30 min. The supernatant was separated and either directly analyzed or stored at —70°C for assay later.

Chromatography

Supernatant was injected into the system in volumes of 20—50 μl. Internal standards consisted of various amounts of 5HTP and DOPA added to supernatant of brain homogenate from untreated animals; these standards were injected at intervals of 4—6 samples.

System I. The mobile phase (pH 4.2), a modification of that described by Koch and Kissinger [13] consisted of 0.1 *M* citric acid—0.2 *M* disodium hydrogen phosphate—methanol (5.4:3.6:1). Flow-rate was set at 0.8 ml/min; detector potential was 720 mV.

System II. The mobile phase (pH 4.1), a modification of that described

by Mefford and Barchas [9] consisted of 0.1 *M* sodium acetate—0.1 *M* citric acid—methanol (6.3:3.2:0.5). Flow-rate was set at 0.6 ml/min; detector potential was 720 mV.

Animal experiments

Adult, male Sprague-Dawley rats (Charles River CD) weighing 200—225 g were housed in an environmentally controlled room at 20°C and 40% relative humidity with light—dark cycles of 09.00—21.00 h light for at least seven days before use. Food was removed 12 h before an experiment in order to remove variability among animals of tryptophan and tyrosine intake. The decarboxylase inhibitor, *m*-hydroxybenzylhydrazine, 100 mg/kg in saline vehicle, was administered intraperitoneally [14]. Rats were sacrificed by decapitation at various intervals following injection; brains were quickly removed and brain parts dissected as described previously [15, 16] on a Petri dish cooled by dry ice. In one study median eminence was dissected with fine scissors and the aid of a dissecting microscope. Brain parts were stored at —70°C until assay. The possible effect of an additional handling and injection stress was examined in an experiment in which animals were injected with saline 20 min after the initial stress of injection of *m*-hydroxybenzylhydrazine and 10 min before sacrifice.

RESULTS

System I

A clearly separated peak representing 5HTP was seen 5.5 min after injection of 5HTP standard in 0.1 *N* perchloric acid (Fig. 1B). No comparable peak was observed following injection of brain homogenate from untreated rats (Fig. 1C). Neither the retention time nor the sensitivity were altered by injecting 5HTP standard in this brain homogenate. Hypothalamic homogenate from rats injected in vivo with *m*-hydroxybenzylhydrazine showed a similar, isolated peak consistent with 5HTP (Fig. 1E). The assay of 5HTP was linear ($r = 0.989$, $p < 0.001$) over a range of 4—7500 pg. The coefficient of variation was 4.1%.

This method was employed to measure accumulation of 5HTP in specific brain areas following decarboxylase inhibition in vivo. Following *m*-hydroxybenzylhydrazine administration, accumulation of 5HTP was linear for at least 30 min in both hypothalamus (Fig. 2A) and brain stem (Fig. 2B). Reproducibility of this accumulation is illustrated in Fig. 2A.

System II

A second chromatographic system employing changes in mobile phase and flow-rate allowed the simultaneous determination of 5HTP and DOPA. Clearly separated peaks representing DOPA and 5HTP were seen at 4.0 and 13.5 min respectively after injections of these standards in 0.1 *N* perchloric acid (Fig. 3B—D). The retention time for 5HTP using this system was 2.5 times that seen with System I. The presence of brain homogenate did not influence these results (Fig. 3F). Hypothalamic homogenate from rats injected in vivo with *m*-hydroxybenzylhydrazine showed similar, isolated peaks

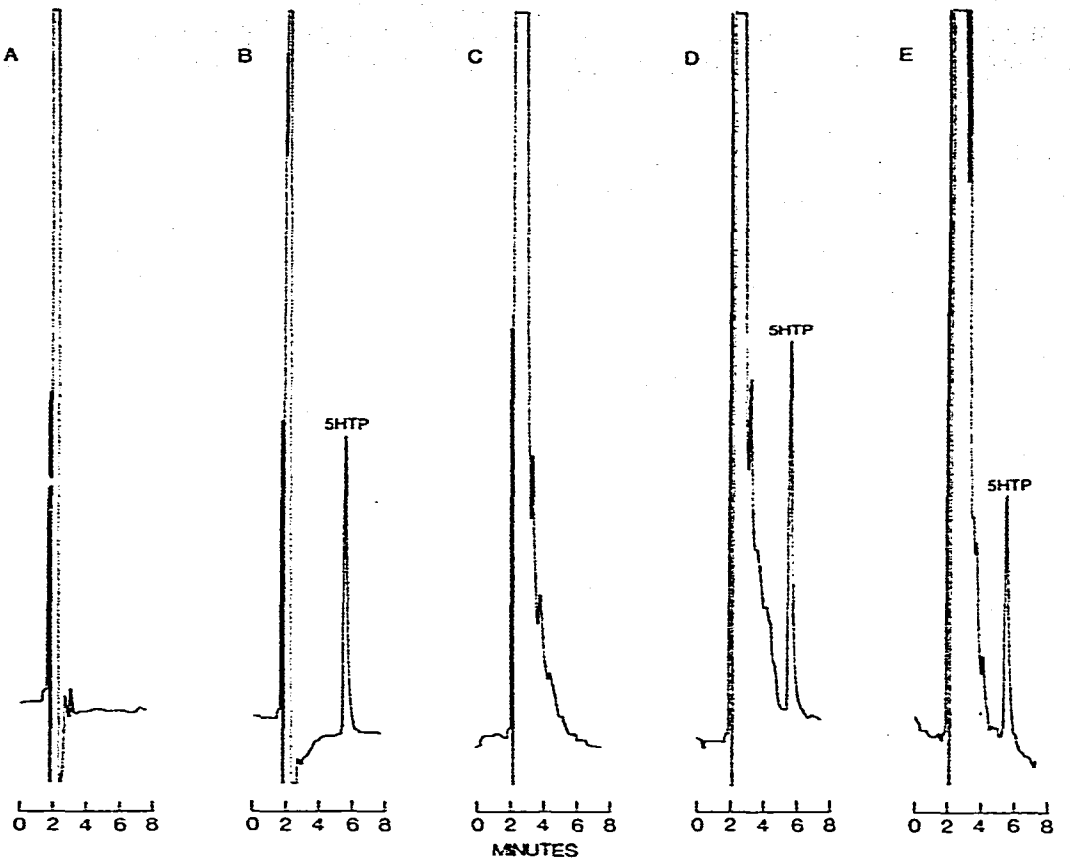

Fig. 1. Representative chromatograms of 5HTP standards and brain tissue using system I (see Methods). (A) 20 μl 0.1 *N* perchloric acid vehicle; (B) 200 pg of 5HTP standard in 0.1 *N* perchloric acid; (C) 2 mg equivalents in 20 μl of homogenate of rest of brain; (D) 200 pg of 5HTP standard in 2 mg equivalents of rest of brain homogenate; (E) 2 mg equivalents of hypothalamic homogenate from rats injected with *m*-hydroxybenzylhydrazine 30 min earlier.

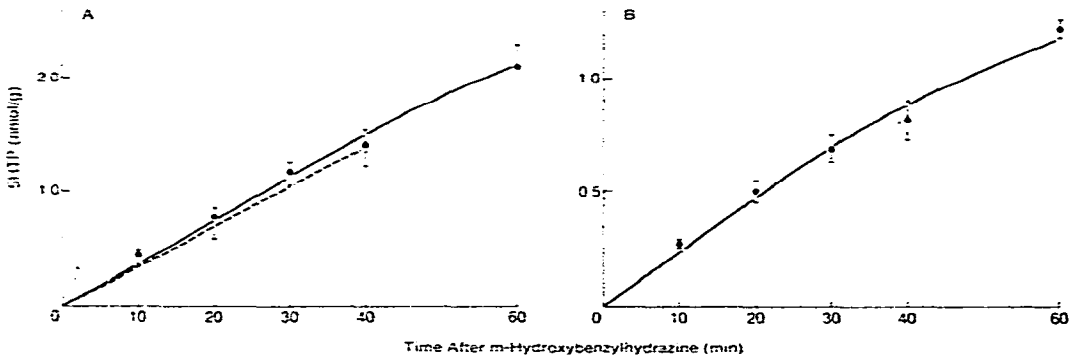

Fig. 2. Accumulation of 5HTP in rat brain after decarboxylase inhibition with *m*-hydroxybenzylhydrazine. (A) Replicability of accumulation in hypothalamus; (B) accumulation in brain stem. •———•, one experiment; ○— — —○, another experiment. The vertical bars around the data points represent standard errors of the means of 4—5 determinations.

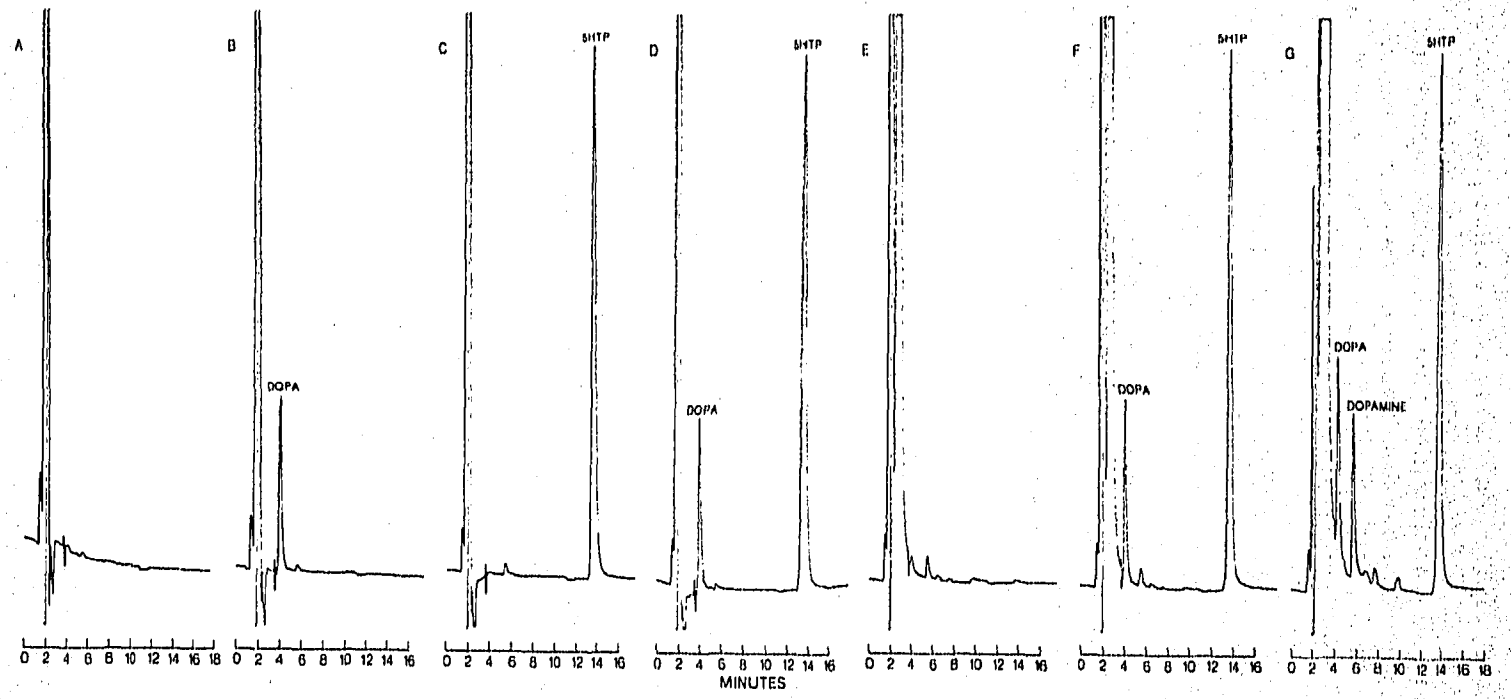

Fig. 3. Representative chromatograms of 5HTP and DOPA standards and brain tissue using system II (see Methods). (A) 20 μl of 0.1 *N* perchloric acid vehicle; (B) 200 pg of DOPA standard in 0.1 *N* perchloric acid; (C) 200 pg of 5HTP standard in 0.1 *N* perchloric acid; (D) 200 pg of each of DOPA and 5HTP standards in 0.1 *N* perchloric acid; (E) 2 mg equivalents in 20 μl of homogenate of rest of brain; (F) 200 pg of each of DOPA and 5HTP standards added to 2 mg equivalents of rest of brain homogenate; (G) 2 mg equivalents of hypothalamic homogenate from rats injected with *m*-hydroxybenzylhydrazine 30 min earlier.

consistent with DOPA and 5HTP (Fig. 3G). A prominent peak at 5 min, clearly separated from the DOPA peak, was evident following injection of hypothalamic homogenate. We determined that this peak represented dopamine. A much smaller peak representing dopamine was seen with injections of rest of brain (whole brain with hypothalamus and brain stem removed) (Fig. 3F), and a much larger peak representing dopamine was seen with injections of striatum.

The assays of both 5HTP and DOPA were linear (for 5HTP, $r = 0.997$, $P < 0.001$; for DOPA, $r = 0.942$, $p < 0.001$) over a range of 4—7500 pg (Fig. 4). Coefficients of variation were 3.2 and 4.5% for 5HTP and DOPA, respectively.

Following *m*-hydroxybenzylhydrazine administration, accumulation of 5HTP was linear for at least 30 min in hypothalamus (Fig. 5), striatum (Fig. 6A) and brain stem (data not shown). The results for 5HTP accumulation using either System I or System II were identical (Fig. 5).

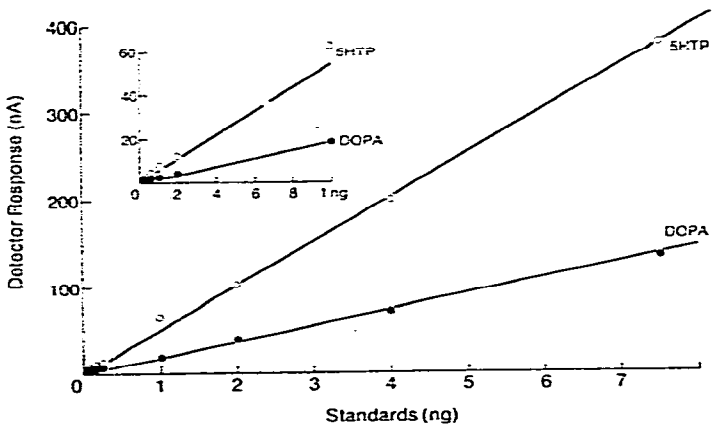

Fig. 4. Linearity of detector responses to 5HTP and DOPA standards in 0.1 *N* perchloric acid. The plots were derived by linear regression.

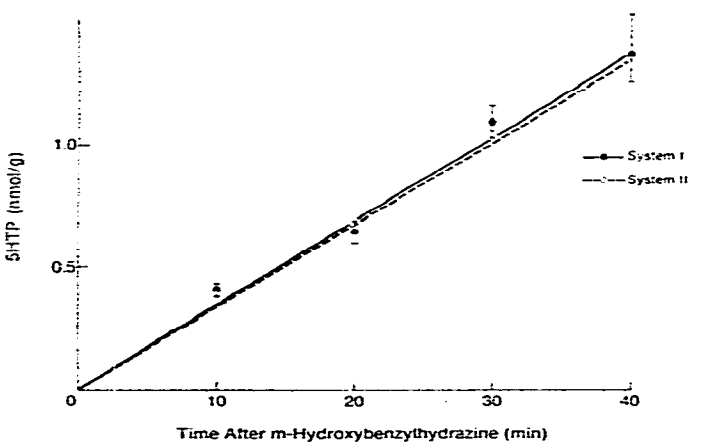

Fig. 5. Accumulation of 5HTP in rat hypothalamus following decarboxylase inhibition with *m*-hydroxybenzylhydrazine: comparison of assays using mobile phase systems I and II (see Methods). Data points represent means ± S.E.M. of 3—5 determinations.

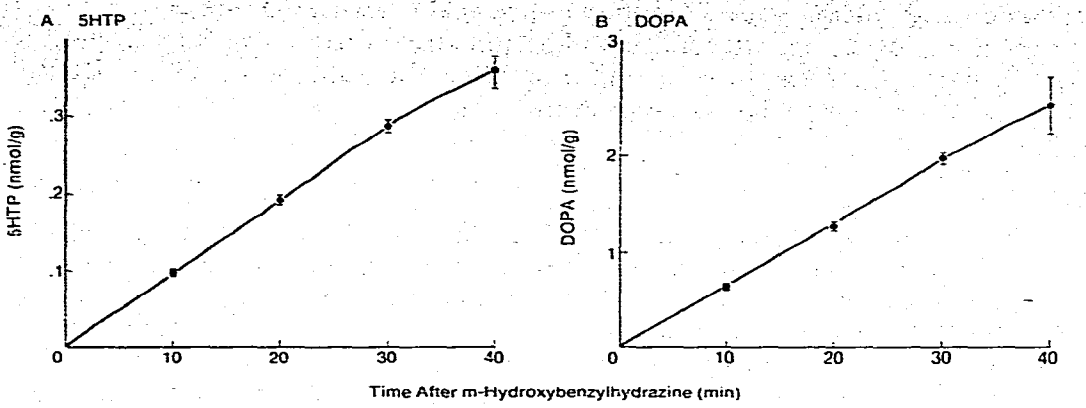

Fig. 6. Accumulation of (A) 5HTP and (B) DOPA in striatum following decarboxylase inhibition with *m*-hydroxybenzylhydrazine. Data points represent means ± S.E.M. of 4—6 determinations.

Accumulation of DOPA following *m*-hydroxybenzylhydrazine was linear for 30 min in both hypothalamus (Fig. 7) and striatum (Fig. 6B). This method can be applied to the measurement of serotonin and catecholamine synthesis rates in very small discrete brain regions. For example, accumulation of 5HTP and DOPA in median eminence (ca. 0.5 mg) was 1.02 ± 0.12 and 12.25 ± 0.78 pmole/median eminence/h, respectively.

Interference of the 5HTP and DOPA peaks by norepinephrine, epinephrine, dopamine, homovanillic acid, 3,4-dihydroxyphenylacetic acid, methoxytyramine, normetanephrine, metanephrine, serotonin, 5-hydroxyindoleacetic acid, tyrosine, tryptophan, and γ-aminobutyric acid was excluded.

The possibility of improving further the sensitivity of the assay was explored using a variety of adsorption (Florisil; Amberlite CG50) and extraction (diethyl ether; chloroform; acidic butanol; toluene; toluene—isoamyl alcohol; ethyl acetate—acetone) techniques. None of these treatments improved the background sufficiently to justify their use for assays of brain tissue. However, it should be noted that the assay of DOPA in plasma required prior adsorption

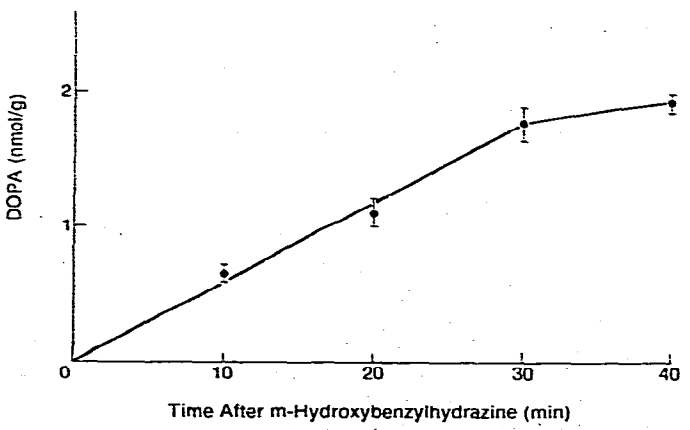

Fig. 7. Accumulation of DOPA in hypothalamus following decarboxylase inhibition with *m*-hydroxybenzylhydrazine. Data points represent means ± S.E.M. of 3—5 determinations.

by alumina and elution with 0.1 *N* perchloric acid (data not shown).

Application of a second handling/injection stress during the 30-min period after *m*-hydroxybenzylhydrazine did not alter the rate of 5HTP or DOPA accumulation in hypothalamus or striatum.

DISCUSSION

In this paper, we describe a reversed-phase HPLC method which allows the simultaneous measurements of 5HTP and DOPA with sensitivities which compare favourably with those of radioenzymatic methods. These monoamine precursors were measured without interference from related compounds such as the monoamines or their metabolites.

It is generally assumed that the rate-limiting steps for serotonin and catecholamine synthesis are the hydroxylation of tryptophan and tyrosine, respectively. Thus, measurement of the hydroxylated amino acids, 5HTP and DOPA, in brain should provide a valid functional index of monoaminergic neuronal activity [2]. In the present study, we have described a method for the measurement of these monoamine precursors which, used in conjunction with the administration in vivo of a decarboxylase inhibitor, allows the reliable measurement of serotonin and catecholamine synthesis rates in small discrete brain regions. It is clear that considerable advantage accrues from the simultaneous measurements of 5HTP and DOPA, and this was accomplished using our chromatographic system II. However, in studies in which the measurement of 5HTP alone is desired, system I decreases assay time considerably.

ACKNOWLEDGEMENT

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