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AN EVALUATION OF THE GAS CHROMATOGRAPHIC ANALYSIS OF PLASMA AMINO ACIDS*

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SUMMARY

The accuracy and reproducibility of gas–liquid chromatography of plasma amino acids as their *n*-butyl N-trifluoroacetyl esters are statistically evaluated. Plasma is deproteinized with picric acid and amino acids are purified by microcolumn chromatography on Dowex 50 W-X12. Routine analysis requires only 0.2 ml of plasma with recoveries of 75–90% for the protein precipitation–ion exchange steps as determined by tracer methods.

The ester derivatives are injected directly onto 0.7% EGA or 1.0% OV-17 columns. Variability due to the use of different GLC columns is minimized by careful calibration. Replicate analysis of the same sample indicates that the overall reproducibility is satisfactory for clinical usuage. The range of normal concentration for amino acids in our clinical population is within agreement with values obtained by other methods.

A complete analysis can be accomplished within 12 h, however grouping samples allows processing of 8–10 plasma samples in one working day, greatly facilitating biochemical investigations such as metabolic disorders in retarded children.

INTRODUCTION

The repertoire for amino acid analysis generally involves column, paper, and thin-layer chromatography as well as a variety of electrophoresis, spectrophotometric and ion-exchange techniques. Unfortunately, the elegant automated amino acid analyzers that are commercially available remain expensive and have relatively limited application to other problems.

Within the past decade, gas–liquid chromatography (GLC) has been applied to many arduous separation problems. It is an expedient analytical tool with capabilities of extremely high sensitivity and resolution, which provides the opportunity to obtain qualitative and quantitive data simultaneously. The relatively low cost

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of the instrument has allowed its extensive usage in biological studies. However, the technical difficulties involved in the preparation of volatile derivatives of amino acids has delayed its development in this area. A variety of suitable chemical subterfuges has been developed by GEHRKE AND STALLING[1] in which the amino, carboxyl and other functional groups are esterified whereby allowing gas chromatography. In addition, TALBOT *et al.*[2], have substantiated that standard amino acid mixtures can be quantitated accurately by GLC when columns are precisely calibrated.

The increasing need of an expedient method for quantitative analysis of plasma amino acids has prompted us to investigate further the potentiality of GLC. In previous studies, the purification, derivatization and resolution of each amino acid on a quantitative basis has not been adequately examined.

This report is a statistical evaluation of the accuracy and reproducibility for the quantification of plasma amino acids. Also, the results of the application of this technique is presented for determining the levels of individual plasma amino acids in retarded children who are suspected of carrying inborn errors of metabolism.

EXPERIMENTAL

Purification of amino acids from blood

Blood from retarded children was centrifuged to remove cells and a quantity of norleucine equivalent to 5 mg% was added as an internal standard. The purification of amino acids by ion-exchange is shown in Fig. 1. A volume of serum (0.2–0.5 ml)

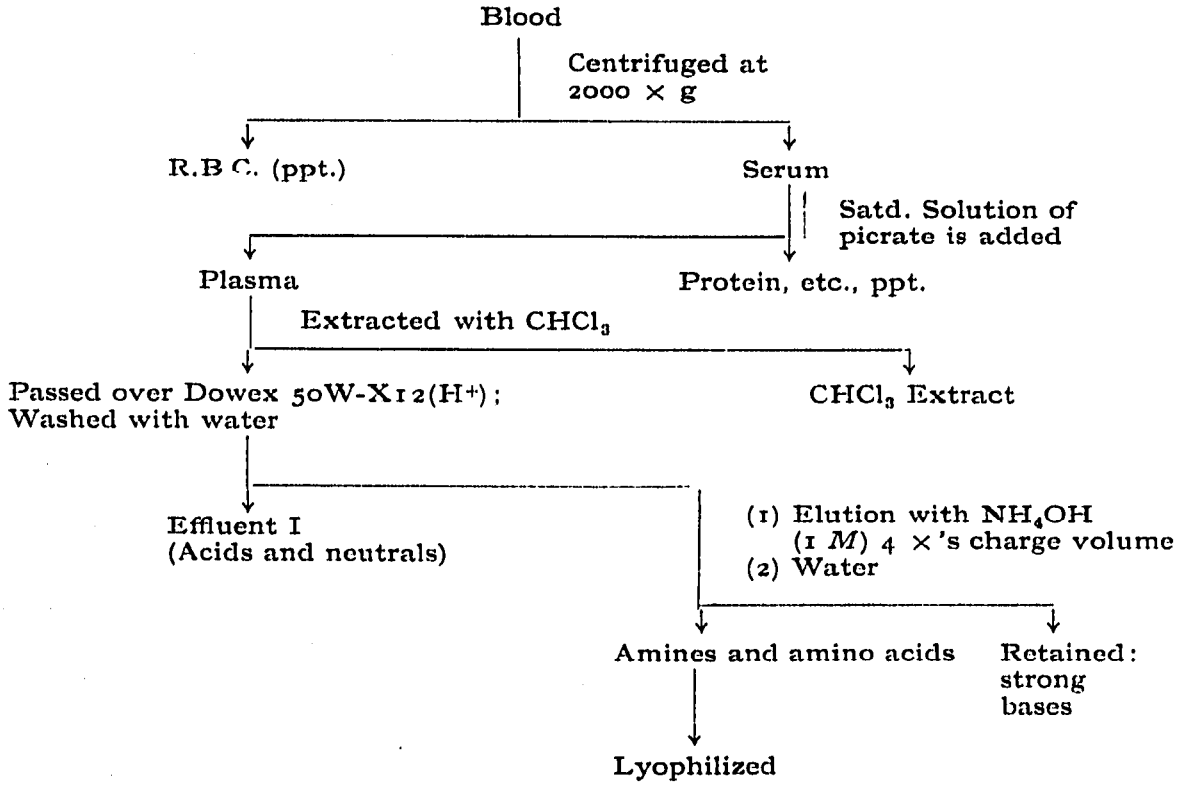

Fig. 1. Schematic diagram for the extraction of amino acids from blood.

was added to precipitate proteins and the precipitate was washed three times with fresh picric acid solution. The washings were combined and plasma was extracted three times with one-half the volume of $CHCl_3$. The aqueous extract was passed through a 6–9 ml bed-volume of Dowex 50 W-X12 (H^+) resin (50–100 mesh); followed by ten times the charge-volume with water; four times the bed-volume with 1 *M* NH_4OH; and again with water. The fraction containing amino acids and weak bases were lyophilized.

The percent recovery for each amino acid for the purification procedure was determined by adding 0.1 μCi of a uniformly labeled [^{14}C]amino acid to the serum and an aliquot from the final elution fraction was assayed for radioactivity with a Nuclear Chicago liquid-scintillation spectrometer. Samples were dissolved in 15 ml of Triton scintillation fluid and counted until a standard error of 0.2% was attained. The Triton scintillation fluid consisted of one part Triton X-100, two parts toluene scintillator solution containing 8.25 g PPO and 0.25 g Me_2POPOP per liter. Observed radioactivity was corrected for quenching by the channels ratio method. The percent recoveries were based on the added disintegrations per min.

Preparation of derivatives and gas–liquid chromatography

The method of GEHRKE AND STALLING[1] was modified to prepare the *n*-butyl N-trifluoroacetyl esters of the amino acids for GLC. Methyl esters were prepared by treating the lyophilized residue with 2.0 ml of dry methanol–HCl (1.25 *N*) solution for 30 min at ambient temperature. Excess solvent was removed under a nitrogen stream; the residue interesterified with 1.0 ml of *n*-butanol–HCl (1.25 *N*) for 2.5 h at 100° and the excess solvent removed. Trifluoroacetic anhydride (1.0 ml) was added to the *n*-butyl esters and heated to 100° for 1 h. Prior to analysis the remaining reagent was evaporated and the derivatives were resuspended in 0.5 ml of CH_2Cl_2.

Analyses were performed on a Microtek 220 and Barber-Coleman instruments equipped with flame ionization detectors. Two columns, 6 ft. × 4 mm (I.D.) were employed consisting of unsilanized glass tubes packed with 100–200 mesh heat-treated Chromosorb G (Supelco) coated with 1.0% OV-17 (phenyl, methylsilicone polymer) or 0.68% EGA (ethylene glycol adipate). The conditions for analyses were identical for both columns. The flash heater and the detector were maintained at 220° respectively. The columns were programmed from 100° to 220° at 5°/min. The carrier gas, nitrogen, hydrogen and air flow rates were 60, 55, and 480 ml/min respectively.

Quantification of amino acids

Each gas chromatographic column employed for quantification of amino acids was calibrated with the assistance of a Fortran IV program and IBM 7094 computer as described by TALBOT *et al.*[2]. Standard curves were generated for each amino acid with 95% confidence limits using the internal standardization method. The relative response ratio was calculated and plotted *versus* the concentration of each amino acid.

The area of each chromatographic peak was determined by triangulation (height × width at 1/2 peak height); the relative response ratio calculated and the unknown concentration of each amino acid was determined.

Quantitative data for plasma amino acids from replicate analysis and population sampling was statistically evaluated. Standard deviations, standard errors of the mean, and their confidence limits were calculated by computer means.

TABLE I

PERCENT RECOVERY OF AMINO ACIDS FROM HUMAN PLASMA

The data represents an average of four replications plus their standard deviations. To [^{14}C]Norleucine, a non-protein amino acid, carrier was added to give an equivalent concentration of 5 mg% when added to serum.

Amino acid	*% of added D.P.M. ± S.D.*	*Amino acid*	*% of added D.P.M. ± S.D.*
Norleu	77 ± 3.0	Glu	90 ± 2.0
Ala	87 ± 1.0	Asp	77 ± 3.0
Val	82 ± 1.0	Phe	89 ± 1.0
Gly	81 ± 7.0	Tyr	75 ± 10.0
Ile	83 ± 1.0	Lys	78 ± 3.0
Leu	90 ± 3.0	Arg	60 ± 10.0
Thr	89 ± 1.0	His	95 ± 3.0
Ser	86 ± 2.0	Try	73 ± 7.0
Pro	83 ± 1.0	Cys	61 ± 2.0

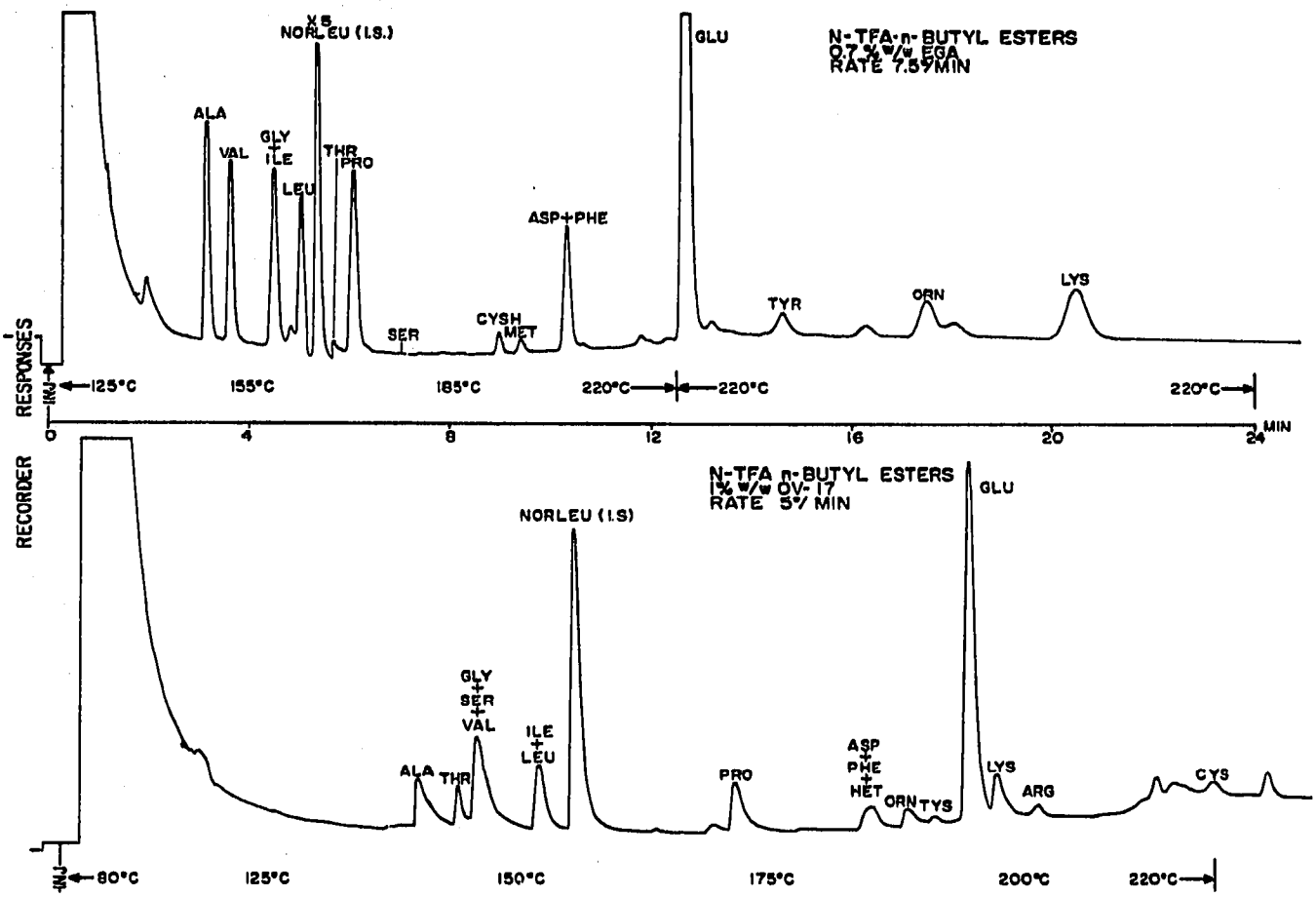

Fig. 2. Gas–liquid chromatograms for amino acids purified from human blood.

RESULTS AND DISCUSSION

The percent recoveries including standard deviations for each amino acid from plasma was determined using individually labeled compounds and these results are shown in Table I. Recoveries reported here are in agreement with other studies[3]. Arginine, tryptophan, and tyrosine were not as reproducible as the other amino acids studied. Tryptophan and possibly cystine may have been partially destroyed during acid precipitation of protein. Coprecipitation of basic amino acids such as arginine with proteins could not be alleviated by successive washing, an observation also noted by others[3] who employ acid denaturation techniques. The actual extent of amino acid loss has been shown to depend on the relative protein concentration, precipitating agent and is not greatly altered by variation in modes of precipitation. The lost amino acids are firmly bound to the precipitate and were not recovered by repeated washing.

Depicted in Fig. 2 are the gas–liquid chromatograms of the ester derivatives of amino acids from human plasma. The employment of two chromatographic columns containing EGA and OV-17 stationary phases provided satisfactory resolution and peak symmetry for their quantification by the triangulation method. However, the EGA coated support provides better separation and by judiciously selecting the gas chromatographic parameters, it is possible to isolate all of the amino acids in a relatively short period of 25 min. Since the *n*-butyl N-trifluoroacetyl ester derivative of arginine, histidine and cystine do not elute quantitatively from the EGA column, an OV-17 column is employed for their quantification. Limited use of the OV-17 to these amino acids is recommended since peak tailing does occur for some amino acids and this reduces the accuracy of peak area measurement.

The variability of amino acid analysis on several gas chromatographic columns employing the same stationary phase was investigated. Columns prepared and calibrated as described by TALBOT *et al.*[2] were used in this study. Amino acids were purified from a sample of plasma; derivatives were prepared and then injected onto two EGA and two OV-17 columns. The concentration (mg%) of each amino acid

TABLE II

VARIABILITY OF AMINO ACID ANALYSIS ON DIFFERENT GAS–LIQUID CHROMATOGRAPHIC COLUMNS
The concentration (mg%) of each amino acid from one sample of plasma was determined on different calibrated GLC columns. Standard deviation (S.D.) for the two EGA and OV-17 columns are given respectively.

Amino acid	*EGA*		±*S.D.*	*OV-17*		±*S.D.*
	I	*II*		*I*	*II*	
Ala	2.0	1.7	±0.15	0.9	1.3	±0.20
Pro	1.3	1.1	±0.10	1.2	1.1	±0.05
Lys	1.3	1.4	±0.05	1.2	1.4	±0.10
Val	7.6	7.0	±0.30	—	—	—
Gly + Ile	3.7	3.2	±0.25	—	—	—
Leu	3.5	3.2	±0.15	—	—	—
Thr	0.4	0.6	±0.10	—	—	—
Asp + Phe	1.7	1.3	±0.20	—	—	—
Glu	—	10.8	—	9.9	9.1	±0.40

TABLE III

REPRODUCIBILITY OF AMINO ACID ANALYSIS BY GAS–LIQUID CHROMATOGRAPHY

Amino acid	*Average + C.L.*[a]
Ala	2.07 ± 0.39
Val	3.44 ± 0.58
Leu	1.31 ± 0.29
Thr	1.66 ± 0.42
Pro	1.88 ± 0.34
Ser	1.49 ± 0.65
Glu + Gln	7.28 ± 0.80
Gly	0.52 ± 0.08
Ile	4.44 ± 1.35
Phe	1.52 ± 0.60
Asp	1.27 ± 0.38
Orn	1.16 ± 0.47
Lys	3.76 ± 1.56

[a] Values are in mg% and include standard error at the 95% confidence limits for five determinations.

was calculated and the mean of two determinations including their standard deviation are listed in Table II. These data indicate that careful calibration of gas chromatographic columns by the computer method[2] will provide reliable values when analyses are performed on different columns. The observed standard deviations can possibly be further diminished by employing more accurate techniques for peak area measurements, for example, disc, electronic or computer integration.

A study on the over-all reproducibility of amino acid analysis by employing ion-exchange purification, derivatization and gas chromatography was initiated. A single plasma sample was divided into five 0.1 ml aliquots and the amino acids recovered by ion-exchange. Derivatives were prepared in the usual manner and each sample analyzed by gas chromatography. The average concentration (mg%) for each amino acid plus the standard error at the 95% confidence limit is shown in Table III for the five determinations. These data indicate that analysis of a single sample of plasma by the procedure described here allows the determination of the levels of amino acids with a high degree of reproducibility.

The gas chromatographic and amino acid analyzer methods were compared as to their accuracy for the determination of amino acid concentrations in a synthetic mixture. The average values obtained from six determinations are depicted in Table IV. In most cases, the accuracy of the amino acid analyzer is higher, as reflected by the standard error of the mean and the calculated concentration of amino acid. However, the gas chromatographic method is a relatively accurate method for quantitation since the standard errors of the mean were low.

A comparative study was initiated to discern whether the concentrations of amino acids determined by GLC are in agreement with those reported by the conventional method. Several children with normal levels of amino acids in plasma were used to establish mean values with standard deviations. The results are given in Table V. Comparison of the average plasma values obtained by gas chromatography and the amino acid analyzer technique reveals a rather good correlation between

TABLE IV

COMPARATIVE ANALYSIS OF A SYNTHETIC AMINO ACID MIXTURE

Values are given in mg %.

Amino acid	*GLC*	*Calculated*	*Amino acid analyzer*
	Average S.E.		*Average S.E.*
Ala	1.62 ± 0.26	2.02	2.17 ± 0.06
Val	2.13 ± 0.36	2.81	3.15 ± 0.18
Leu	2.04 ± 0.20	2.10	2.21 ± 0.12
Thr	1.32 ± 0.13	1.50	1.51 ± 0.06
Pro	1.78 ± 0.27	2.31	3.05 ± 0.39
Ser	2.25 ± 0.30	2.01	1.96 ± 0.09
Glu	10.41 ± 0.66	8.51	7.94 ± 0.31
Gly	1.36 ± 0.11	1.51	1.55 ± 0.05
Phe + Asp	3.21 ± 0.31	2.41	2.56 ± 0.09
Lys	2.66 ± 0.36	2.40	2.33 ± 0.14
Tyr + His	7.83 ± 0.76	6.10	6.53 ± 0.18
Arg	2.23 ± 0.29	2.00	2.04 ± 0.07
Met	0.64 ± 0.09	0.50	0.53 ± 0.01
Orn	2.07 ± 0.29	1.20	1.36 ± 0.12
Ile	1.29 ± 0.20	1.51	0.67 ± 0.03
Cys	1.17 ± 0.16	1.00	—

TABLE V

COMPARISON OF GAS–LIQUID CHROMATOGRAPHIC AND AMINO ACID ANALYZER METHODS

Amino acid	*GLC*	*Amino acid analyzer*
	Average ± S.D.[a]	*Average ± S.D.*[b]
Ala	1.74 ± 0.60	2.42 ± 0.32
Val	2.63 ± 0.93	2.12 ± 0.24
Leu	1.92 ± 0.84	1.19 ± 0.17
Thr	1.59 ± 0.90	1.73 ± 0.19
Pro	1.21 ± 0.85	2.05 ± 0.38
Ser	1.29 ± 0.37	1.27 ± 0.15
Glu + Gln	7.87 ± 3.48	— —
Lys	0.84 ± 0.75	1.91 ± 0.29
Tyr	— —	0.83 ± 0.15
Arg	1.92 ± 0.59	1.49 ± 0.27
Met	0.47 ± 0.30	0.24 ± 0.05
Orn	1.25 ± 0.86	0.61 ± 0.11
Gly + Ile	2.91 ± 1.49	1.23 ± 0.33
Phe + Asp	1.39 ± 1.57	0.99 ± 0.13

[a] Plasma values (mg%) with standard deviations for 12 children who demonstrated normal levels when also analyzed by the amino acid analyzer method.

[b] Data taken from BRODEHL AND GELLISSEN[5] on a population of 12 normal children (ages 3–13 years).

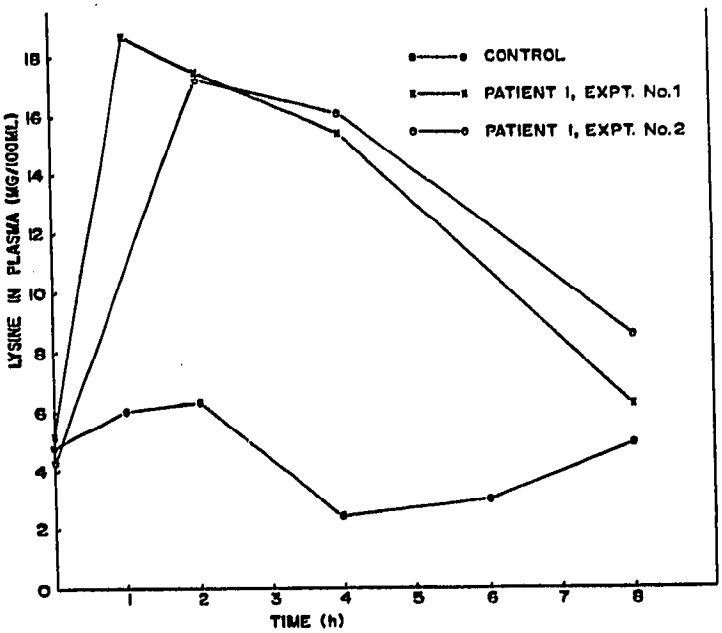

Fig. 3. Lysine concentration in plasma after oral administration.

the two methods since the population of individuals used in both cases was rather small.

As an example of the application of this method to a specific problem, at retarded patient with hyperlysinuria and hyperlysinemia[4] was chosen. The subject was given an oral lysine tolerance test and the results indicate the ability of this method to differentiate abnormal tolerance levels of lysine in the plasma (Fig. 3).

Studies of amino acid metabolism are facilitated by reliable ultramicro quantitative analytical methods. The gas chromatographic method described here is particularly attractive from this point of view since it allows for the analyses of amino acids in small volumes of plasma (0.1–0.5 ml). The preparation of the *n*-butyl N-trifluoroacetyl esters which have a high electron capture coefficient allows the possibility of extending the sensitivity even by an additional several orders of magnitude through the employment of electron capture detection. This is particularly important in clinical studies involving infants which restricts the investigator to extremely small quantities of plasma for analyses.

In order that an analytical procedure be acceptable for routine laboratory usage, it is imperative to demonstrate its accuracy and reproducibility. The variability of the gas chromatographic method due to employment of different columns can be minimized by careful calibration. The analytical method described here was statistically analyzed for its overall reproducibility and the results indicated that it is satisfactory for clinical usage. In addition, the range of normal concentrations in our clinical population is within agreement with values obtained by other methods.

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