改良 HPLC 方法分析大白鼠肝臟的能量積

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摘要

常見有關 adenosines 的 hplc 分析法，均僅附標準品於緩衝液或體外分析的圖譜，這些方法似乎不適用於分析肝細胞萃取液。我們萃取大白鼠肝細胞內的 adenosines，並在較少分解率的 pH 6.0 環境下以 chloroacetaldehyde 反應生成 1,N^6-ethenopurines 螢光衍生物，再用配備胺基分離管的 hplc 系統，定性定量分析肝萃取液內腺核苷三磷酸或其代謝物。本法所用胺基分離管的固定相，似乎具有逆相和離子交換的雙重特質。使用本 hplc 分析方法，大白鼠肝臟萃取液內的腺核苷三磷酸或其代謝物、或類同品可以被成功的單離和定性、定量分析，依此結果以 Atkinson 的方法計算得大白鼠肝臟能量績是 8.3 ± 0.4。此分析方法將有助於解析肝細胞凋亡和能量代謝的關係。

關鍵詞： 腺核苷三磷酸、 腺核苷二磷酸、腺核苷單磷酸、高壓液態層析儀、能量積

Analyze the Energy Charge of the Rats' Liver Using a Modified HPLC Method

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Abstract

Most reported methods for HPLC have shown chromatography of biological adenosines in clean buffers, indicating a defect in the method used. In the present research an amine column equipped-HPLC system together with a fluorescent derivatization of 1,N^6-ethenopurines using chloroacetaldehyde, and conditioned at pH 6.0 to decrease adenosines degradation, it were tested for the purpose of biological energy charge analysis in the liver. The fluorescent derivatives of ATP, adenosine metabolites, or its analogues taken from a rat's liver were prepared, and specifically resolved using HPLC. The amine column seems to have both the stationary merits of the reverse phase and ion exchange characteristics. This HPLC system enables us to analyze the energy charge in the liver that represents mitochondria functions. The achievement of this study may help us to interpret the relationships of energy charge on hepatocytes and its apoptosis.

Keywords: ATP, ADP, AMP, Adenosine, HPLC, Energy Charge

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I. Introduction

The mitochondria are known as the energy center of a cell which regulating the metabolism of adenosines including adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP). It has been reported that dysfunction of ATP metabolism with less ATP is one of the major reasons for triggering cellular apoptosis [1-4].

In 1968, Atkinson proposed an energy charge (EC) formula to represent the cellular energy status regulating energy related cellular physiological functions [5]. Therefore, many reports have shown that a decrement of EC caused dysfunction of membrane ions pumps and evoked a massive influx of calcium and sodium ions, deranging the intracellular integrity, thus leading to a deterioration of cellular functions [6,7]. It is also known that cellular swelling caused by the deterioration of membrane integrity by lower ATP was a typical process of apoptosis [3,4, 8-10]. Consequently, interpreting cellular energy status in assessing the process of cellular damage has become an important focus for us.

To determine the cellular energy status or the EC, it is necessary to measure qualitatively and quantitatively the cellular ATP, ADP and AMP respectively. Several methods have reported in measuring ATP by UV or luciferase methods [11-13]. However, due to the complicated biological compositions and many analogues interfering with the trace amount of adenosines; it is difficult to analyze cellular ATP and its metabolites without fractionating using previously methods. In order to solve such difficulties, a specific fluorescence derivative adducted by bromo- or chloroacetaldehyde to 1, N^6-etheno derivatives have been proposed to enhance the analytical efficacy of adenosines [14,15]. The properties of these1,N^6-etheno derivatives have been well characterized by Secrist et al. [16]. Consequently, several simultaneous determinations of fluorescent derivatives of ATP, ADP, and AMP using hplc equipped with either an ion exchange column, a phenyl column, or an ODS reverse phase column have been reported [14-18]. However, Kawamoto et al., has reported that above methods have a disadvantage of degradation of ATP-derivative [18]. Moreover, most of the above reported hplc methods showed the resolution of adenosines standard in clean buffers [15, 17-23]. Kawamoto has effectively used an ODS column to resolve purines and their derivative metabolites [18]. However, we could not have a similar experience using an ODS column to resolve ATP and its derivative metabolites in our biological samples. To get five adenosine analogues well-resoluted in one single chromatograph seemed remained difficult in fact. The reported methods seemed require modification to resolve the adenosines well from the other components in liver extract. On the other hand, Mikkola et al. had reported detailed kinetic studies of the reaction of adenosines and haloaldehydes , he analyzed the cellular excretion in the incubation solution, we had failed to replicate his method in the analysis of adenosines intra cellular [24]. It seems that the acid catalysis of adenosines degradation during the derivatization process remains controversial [17-23]. In order to prevent the 1, N^6-etheno derivative of adenosines from degradation during the processes in our experiment, an acidic catalysis degradation of fluorescent adenosines was tested. We modified an hplc system to measure the intracellular ATP, ADP, and AMP content in its fluorescent derivatives at higher pH by a propyl-amine column to test the efficacy of this derivatization processes. An example of the analysis of the liver EC on Sprague-Dawley (SD) rats using this developed hplc method was tested and reported.

This experiment gave us a gate for the study of liver energy charge that represents the mitochondria functions; it in charge of triggering the hepatocytes apoptosis [4, 8, 10].

II. Materials and Methods

The hepatocytes adenosines, ATP, ADP, and AMP of the SD rats were measured, using the followed hplc

method. The average energy charge of the hepatocytes in rats was determined according to the method of Atkinson [5].

1. HPLC system

The hplc system consists of dual pumps (Gilson 302 model, Gilson, France), a fluorescence detector (L-7485, Hitachi, Tokyo), a computer software (Unipoint system software, Gilson, France), and a propyl-amine or an ODS column (Supelco, 5 μm particle size, 250 x 4.6 mm ID). The assay was achieved, using a mobile phase of 1mM KH_2PO_4 (pH 3.3) – acetonitrile - tetrabutylammonium bromide (98 : 2 : 0.02) (v : v : w) and a fluorescence detector (excitation 270 nm, emission 410 nm); the flow-rate was 1.0 ml/min.

2. Standard solutions and calibration graphs of the HPLC

Respectively, a working solution of ATP, ADP, or AMP (Sigma, St. Louise, MO) was prepared daily (1 mg/ml), using a 3 % phosphate buffered solution pH 6.0 as the solvent. The working solutions were further diluted with 4 % albumin (Sigma, St. Louise, MO)to give a series of standard solutions of ATP, ADP, or AMP ranging respectively from 0 to 800 ng/ml, using α, β-methylenadenosine -5'-diphosphate as a internal standard [Is]. The fluorescence derivative of each standard (ATP', ADP', AMP', and Is') was prepared respectively using 2 μ mol of chloroacetaldehyde (Fluka Chemie GmbH, Switzerland) incubated with the standards at 80 ℃ for 40 minutes, the reactions were stopped by ice-cooling. The biological unknown sample was fluorescent adducted as the processes of standards and measured by hplc. Stoichiometrics were achieved by measuring the eluted standards of ATP', ADP', or AMP' against the Is' using the linear regression lined calibration of standards versus peak-area ratios.

3. The Resolution and Specificity test of the HPLC

The specificity of ATP', ADP', and AMP' beside other analogues, such as adenine or adenosine (Sigma Co., St. Louise, MO)were prepared and co-eluted with the standards to ensure the specific analysis of ATP', ADP', and AMP', respectively. The molar absorptivity of each derivatives of fluorescent adenosines were obtained according to the Beer's rule [absorbance = molar absorptivity x path length (cm) x sample concentration (M)], and respectively compared [21]. The specific retention volume and the absorbance constant of each adenosines were monitored simultaneously, to ensure the specifically qualitative analysis of adenosines in our biological sample. The resolution factors (Rs) of each adenosine, were obtained against the internal standards and compared [24].

4. Recovery and detection of limit of the HPLC

The recoveries of each adenosine were tested, using the amount of standards detected against the amount of spiked standards in the preparations by hplc method. The detection limits of ATP', ADP', and AMP' on this hplc method were determined respectively under the basis of signal-to-noise ratios as in the International conference on Harmongation (ICH) regulated method: detection limit = [(3 x mean standard deviation of noise)/ slope] (26).

5. pH vs the degradation rate of 1,6-ethanopurines

The working solutions prepared as in the above procedures were diluted with 3％ phosphate buffer ranging from pH 3~7, and a series of standard solutions of ATP, ADP, or AMP (50, 200, 500 ng/ml) were prepared respectively, using β-methyleneadenosine -5'-diphosphate as the internal standard. Each fluorescence derivatives of standards and its degraded products ADP' or AMP' were measured and compared simultaneously.

6. Methodology precision test

Six lots of adenosines and its derivatives were prepared respectively within one day and analyzed, using the

hplc method. Consecutively, another single lot of adenosines standards and its derivatives were daily prepared and analyzed for six days. Parallel comparisons of the standards in these two assays were done as the within-day or the between-days test.

7. Analysis of liver energy charge

Eight male Sprague Dawley (SD) rats, 16 weeks old and weighing around 350-400 g were used in this study. The rats were cared according to the guidelines of the National Laboratory Animal Center in Taiwan and maintained under a 12-hour light/dark cycle in a 21℃ air-conditioned room. The rats were free to a standard laboratory rodent chow diet and clean water.

Before the test, the rats were fasted for 24 hours but free to water, and sacrificed by ether inhalation. The livers were harvested through a midline laparotomy on supine position, and frozen immediately in liquid N2. In order to prevent the mass variation from the liver vascular, about 100 mg of the edged tissues of each liver were collected and chopped into fine pieces. The protein content of the liver tissues were assayed according to Lowry et al., using bovine serum albumin as the standard [27]. The determination of the liver EC in each sample were done: First, the liver tissue was chopped into pieces of about 5 mm in diameter, mixed with 2 ml of 0.5N perchloric acid and sonicated for 2 min at 4℃ with 30% amplification to disrupt the cells using a Sonics Vibra Cell (Sonic and Material Co., CT). The slurries were filtered respectively through a stainless mesh of 200 and the filtrate was sonicated again for 2 min to ensure complete disruption of the cell organelles, and then centrifuged at 3000 g × 5 min to collect the supernatant. Second, the supernatants were adjusted by K_2HPO_4 buffer (3%) to pH 6.0, and were adducted with chloroacetaldehyde as in the standard procedures. Third, aliquot of 10 μl preparations were injected onto the hplc system respectively. The ATP’, ADP’, and AMP’ contents of each sample were calculated against the standards respectively. The average EC of the hepatocytes were calculated according to Atkinson’s method and compared [EC = (ATP + 0.5 ADP) / (ATP + ADP + AMP)][5].

8. Statistical methods

Each datum from above experiment was shown as mean $\pm$ SD. Respectively, the results were calculated and analyzed by the repeated ANOVA tests. Each result was compared respectively, and the differences associated with $p < 0.05$ were regarded as statistically significant.

III. Results

1. HPLC analysis

A typical chromatography from the amine or ODS column elution of the rat liver preparation is shown in figure 1 and figure 2 respectively. On figure1, ATP’, ADP’, AMP’, or the analogues of AND’ or the ADO’ separated well from the biological interferences using the hplc system with an amine column. It reveals that the chloroacetaldehyde adduct reaction was done and the derivatives of 1, N^6-etheno derivatives of adenine (AND’), adenosine (ADO’), or adenosines derivative of ATP’, ADP’, and AMP’ could be detected by the fluorescence detector at ex. 270 nm and em. 410 nm. Figure 2 shows chromatography of adenosines by an ODS column, and this pattern is similar to that of other reported methods [15]. Comparing the figures 1 and figure 2, the resolution of adenosines in biological samples with the amine column (figure 1) are superior to that of the ODS column (Figure. 2). Therefore, the amine column was used for the rest of the studies.

The linearity of each adenosine standards in chromatograpy were tested, the linearity was preserved up to 80 pmol of ATP’, as low as 5 pmol/ml of AMP’ could be analyzed. The traced reagent was low, and observed no significant interferences with the overall procedure.

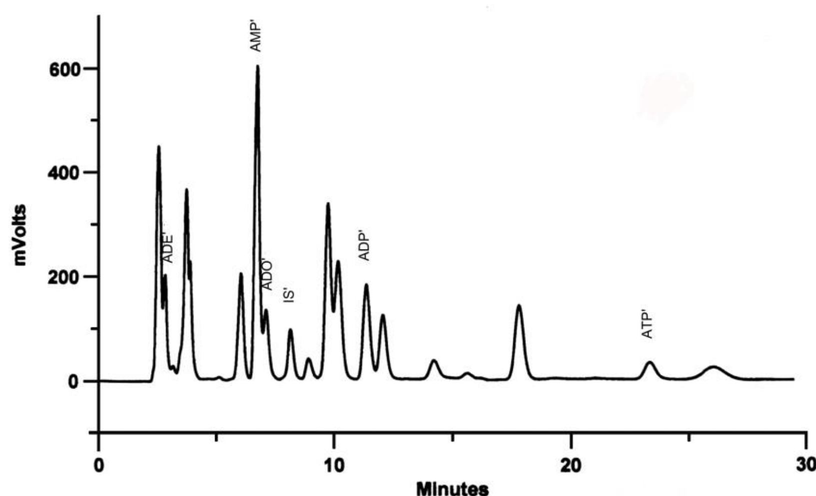

Figure1 Analysis of biological adenosines on the amine column equipped hplc

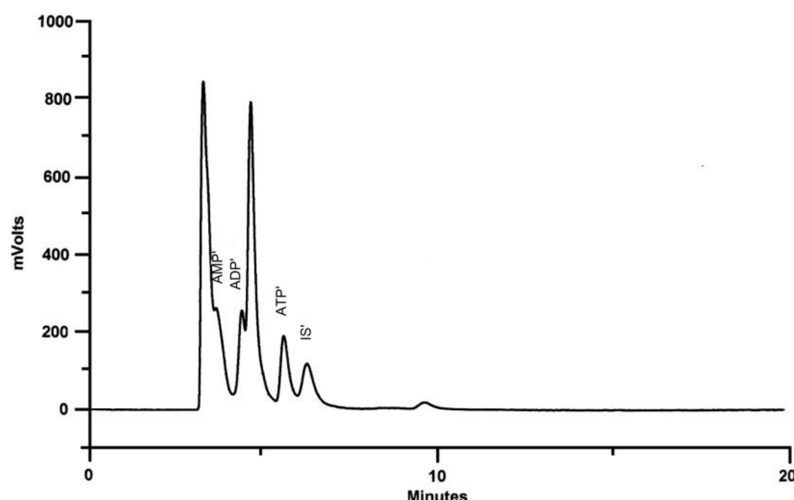

Figure2 Analysis of biological adenosines on the ODS column equipped hplc

2. Detection limit and recovery rate in HPLC analysis

The standard curve for ATP' was y = 542.488 x – 19.814 (R2 = 0.998), for ADP' y = 300.365 x + 14.057 (R2 = 0.996), for AMP' y = 136.648x – 2.348 (R2 = 0.998) (y = peak area ratios of the adenosines in liver extract to standards, x = concentrations of the adenosine). According to figure 1 and the standard curves of ATP', ADP', and AMP', the detection limits, calculated as the ICH regulated guidelines, were 37, 15, and 5 pmol/ml. The absolute recoveries of ATP, ADP, and AMP were more than 90%. This hplc system with pico mole detecting ability enables us to analyze the adenosines at the cellular level.

3. Resolution and Specificity of the HPLC analysis

The specific Rs obtained from the chromatography of ATP', ADP', and AMP' against the internal standard were 24.1, 2.6, and 3.0, respectively. Compared with other reported chromatogram with ODS column [18], it seems that our hplc method with amine column has the merit of 6.3-fold higher Rs for ATP', 1.4-fold higher Rs for ADP', and 1.2-fold higher Rs for AMP', respectively. In addition, for each hplc eluted adenosine, the Beer's

rule derived molar absorptivity for ATP’ was 287,300 ± 3,700, for ADP’ was 412,200 ± 10,900, and for AMP’ was 947,000 ± 28,000, respectively. The molar absorptivities of each adenosine derivative by hplc analysis were compared in each run; they showed that the error between each run was less than 9 CV%. These results gave further evidence of successful qualitative and quantitative analysis of adenosines by its 1, N6-ethenopurine derivative not only by its retention volume but also by its specific molar absorptivity.

4. Optimal conditions for the derivatives

The relationship between buffer pH and the amount of 1, N6-ethanopurine end products are shown in figure 3. Compared those end products at pH 3~7,AMP’ showed less sensitive to acid catalyzed degradation. The degradation rates of ADP’, andAMP’ in buffer (pH6.0)were 7.1%, 6.9%, and 0.3%, respectively. The degradation rates for ATP’ at pH 3~7 were 13.4%, 9.8%, 7.8%, and 10.2%, respectively. It showed thatATP’ appeared to be less degraded at pH 6.0. We selected pH 6.0 for the rest of studies.

5. Methodology precision test

Table 1 shows the within-day and between-day analysis of methodology variations of the hplc for ATP (data of ADP, AMP and adenosine, resolved with less retention volumes that showed fewer errors, are not shown). Some detail factors are compared and listed on Table2. In general, the analysis error of AMP, ADP, and ATP detections of within-day were less than 14.7 CV%. Analysis of the between-day variations of adenosines was less than 18.1 CV% in general, an exception was observed of ATP (10 ng/ml) as 22.1 CV%. For any lots of this analysis, the linear regression correlated factors in this study were more than 0.93.

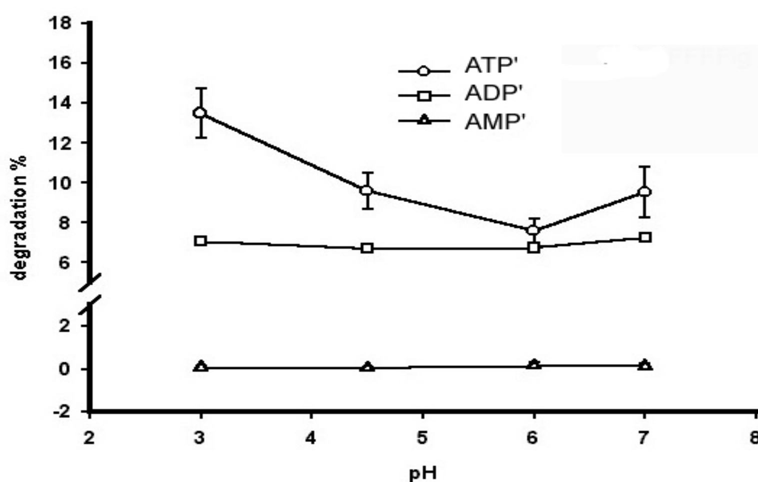

Figure3 The degradation of adenosines catalyzed by acids

Table1 The precision test of adenosines analysis on the amine column equipped hplc.

ATP(ng/ml)	Within-day	CV%	Between-day	CV%
10	11.0±1.6	14.6	13.3±2.9	21.8
20	22.3±1.7	7.7	22.2±4.0	18.1
40	37.4±2.4	6.4	43.3±1.9	4.4
60	63.3±3.4	5.3	60.1±5.4	9.0
80	77.8±1.9	2.4	78.2±3.0	3.8
100	104.5±6.4	6.1	109.6±8.5	7.8

(datum= mean ± SD, N=6)

Table2 The efficiency comparison of the amine to ODS column.

	Resolutions		Capacity factors		Theoretical plates	
	Amine	ODS	Amine	ODS	Amine	ODS
ATP'	24	3.8	0.32	0.81	1156	18
ADP'	2.6	1.9	0.61	0.79	538	19
AMP'	3.1	2.5	0.75	0.69	219	28

6. Energy charge of the liver

The average energy charge of the liver, with the hplc measured adenosines against its protein content, by the Atkinson's method was 8.3 ± 0.4. This modified hplc method enables us to achieve not only the stoichiometry of adenosines but also the test of EC of rats' liver.

IV. Discussion and conclusion

This study shows that our modified hplc method could simultaneously analyze the ATP, ADP, and AMP in biological samples successfully. An example of qualitative and quantitative analysis of trace adenosines in rats liver was done, which gave a direct evidence for the clinical application of this hplc method on EC analysis. It could be the first report of an hplc method for biological analysis of adenosines in a liver extract.

The derivatives of 1, N^6-ethenopurine adenosines for stoichiometry purposes have been well discussed, and several haloacetaldehydes have been tested for adenine derivative purpose. Yoshioka et al. showed that the reactivity of bromoacetaldehyde was a stronger reagent than the other haloacetaldehydes for adenine derivatives at pH 5.0 [14]. Meanwhile, the reaction of adenine and adenosine with chloroacetaldehyde forming 1, N^6-ethenopurine derivatives had been described with detection sensitivity up to 0.5 pmol/ml [15]. It seems that chloroacetaldehyde has the merit of lower cost and higher sensitivity for the derivatization of 1, N^6-ethenopurine. On the other hand, many factors affected the product of 1, N^6-ethenopurine derivatives; including pH, temperature, and the reaction duration were well discussed [18, 27, 28]. Mikkola et al. had reported that haloacetaldehyde adducted derivatives were cleaved under either acidic or basic conditions, Barrio et al. reported the reasonable reaction condition should be at pH 6.0 [28]. Mikkola et al. also shown that the longer the duration of the derivatization process the higher the degradation of 1, N^6-ethenopurine, his suggestion was to process the reaction at 60 ℃ for 60 minutes. Barrio et al. have also reported to achieve the derivatization at 37℃ or 100℃ [27]. However, in this study, the 1, N^6 ethenopurine was processed at 80 ℃ for 40 minutes as most reported condition [18, 19, 27]. Our result of more than 90% recovery of 1, N^6- ethenopurine implicates with the completion of the derivatization process, correlates well with those reports [18, 19, 27]. We also showed the degradation of ATP' at pH 6.0 was 7.8%; it is 26% less degraded than the preparation at pH 4.5. However, the degradation of our ADP' preparation at pH 6.0 is 7.1%, showing little difference with the preparation at pH 4.5; this result is in agreement with other reports [21, 29]. The AMP' is quite stable and the degradation is insensitive to acid in this study (see figure 3).

Several columns had been used to separate 1, N^6-ethenopurine derivatives including ion exchange, phenyl, and ODS columns, respectively [14-18]. However, the ion-exchange column as Yoshioka et al. used has the disadvantage of being expensive and having a short life span. In addition, most of the reported ODS columns showed resolution of the pure 1, N^6-ethenopurines in a buffered clean solution only. We also tested other columns including cyanamide, and normal phase silica columns [data not shown], these columns could not resolve our

biological samples well as compared with the amine column used in this study. The reason for the ODS column could not well resolving our biological sample well seems to require further discussion (see figure 2). We tested the analysis of EC by an ODS column on our liver extracts, the EC is 8.0 ± 1.2, showing 4.2 folds of higher error then the one from amine column. In addition, the EC tested by ODS column showed insensitive to the amount variation of ADP', and AMP', which made the clinical comparison of EC becoming difficult and the result was insignificant [data not shown]. The detailed reason account for these errors are unknown, it may due to some unknown substances eluted together with the adenosines on the ODS column. However, it might be due to the dual properties of the stationary phase of amine column including the alkyl property of propylamine and the weak anion-exchange property of amine moiety [26,30]. The amine column seems to have both the merits of ODS and ion-exchange column; it enables the separation of fluorescence derivative of adenosines well from the extract. Through this finding, we successfully use the hplc system with an amine column to analyze the EC on rat's liver extract.

Together with the above benefits, we used only a one-step extraction of adenosines and ethanopurines, allowing us to analyze ATP', ADP', and AMP' within 30 minutes using the automated hplc system. An example of our hplc system was tested in the analysis of trace amounts of biological adenosines in rats' liver (figure 1). It showed that not only the ATP metabolites but also the other analogues could be well resolved. Precision test of this method showed that even the last identified ATP' peak (retention volume 25.3 ml), the within-day and between-day analysis of precision error were less then 22.1 % in general. It showed that this modified HPLC method could meet the qualitative and quantitative purposes of adenosines analysis of the liver extract. The obtained result of EC of rats' liver gave a direct evidence of those benefits received from our modified HPLC method with the amine column and the fluorescence derivatization of adenosines at pH 6.0.

In conclusion, our modified condition for 1, N^6-ethenopurine derivatives at pH 6.0 has the merit of a lower degradation rate and the modified hplc method allows us to determinine the biological ATP and its metabolites simultaneously in one chromatography. Using this modified hplc method the energy charge of rats' live could be successfully determined. Further study of this modified hplc analysis and application are expected.

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