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SULPHATE CONJUGATION IN HUMAN LIVER

DIRECT MEASUREMENT OF *N*-ACETYLDOPAMINE-SULPHATE

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Abstract—The overall three-step sulphate conjugating activity of the human liver was determined by the direct measurement of *N*-acetyldopamine (NADA)-sulphate from ATP and inorganic sulphate. NADA-sulphate was separated from NADA and quantified by HPLC-ECD (high-pressure liquid chromatography–electrochemical detection). The overall sulphate conjugation of NADA showed a pH optimum of 8.0 with apparent K_m values for sodium sulphate and NADA of 103 μM and 4.8 μM, respectively. The optimum concentration of ATP was 5 mM and that for Mg^{2+} ions was 7 mM. The specific activities of 17 samples of human liver, measured by this HPLC–ECD procedure ranged from 940 to 23,128 pmol NADA-suphate/hr/mg protein. A comparison of these values with those determined by the radiometric method developed previously showed a direct correlation coefficient, $r = 0.89$. The rates of overall sulphate conjugation of NADA and dopamine measured in these samples by the radiometric assay procedure were also significantly correlated with $r = 0.82$.

The liver is an important site for sulphate and glucuronide conjugation which represent quantitatively two major phase II biotransformation processes. Sulphate conjugation, being a high affinity system compared to glucuronidation for the detoxification of phenolic compounds [1] would conceivably be more significant in the inactivation of endogenous neurotransmitter amines and hormones. In man, sulphate conjugation of the catecholamines and their metabolites appear to predominate [2]. Hitherto, studies on the sulphate conjugation of human liver [3, 4] had focused exclusively on the last of the three-step reactions involved in sulphate conjugation, namely the reaction catalysed by phenolsulphotransferase (PST,‡ EC 2.8.2.1). In this paper, the overall sulphate conjugation of *N*-acetyldopamine was examined as our previous studies demonstrated that PAPS generation was as important as PST in contributing to the overall sulphate conjugating capacity of the human platelets [5, 6]. The overall sulphate conjugation had been measured *in vitro* in various human tissues and in human liver cells in culture [7].

The radiometric assay for the overall sulphate conjugation of human platelets reported earlier [5, 6] required sodium ^{35}sulphate which, being radiolabelled could not be employed in saturating concentrations. In addition, a long chromatographic step was necessary to permit the specific measurement of the sulphate conjugate formed. Thus an alternative assay to overcome these inadequacies was deemed desirable, without compromising on the specificity and sensitivity of the radiometric assay. The HPLC–ECD method reported in this paper appeared to satisfy these criteria. A comparison between the two assay procedures was made in parallel measurements of several samples of human liver extracts. Part of the kinetic data on the sulphate conjugation of the human liver based on the radiometric assay had been reported [8].

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‡ Abbreviations: NADA, *N*-acetyldopamine; DTT, dithiothreitol; PST, phenolsulphotransferase; ECD, electrochemical detection.

MATERIALS AND METHODS

Chemicals

Five batches of sodium ^{35}sulphate of specific radioactivity: 717.39, 574.58, 626.32, 404.78 and 541.33 mCi/mmol purchased from New England Nuclear (Boston, MA, U.S.A.) were used in the development of the radiometric assay for the measurement of overall sulphate conjugation of human liver, as well as in the analysis and identification of NADA-^{35}sulphate. Methanol of HPLC grade was from JT Baker. All other chemicals of analytical grade were purchased from the usual commercial sources.

Preparation of enzyme extracts

Young Wistar rats of 150–200 g were killed by decapitation and the livers were homogenized in cold 0.15 M KCl containing 3 mM DTT solution to give a 20% (w/v) homogenate. The homogenate was centrifuged at 108,000 *g* for 30 min and the supernatant was used, without dilution, as the enzyme source.

The human liver homogenate was prepared from a frozen sample which had been stored at −80° since 1983. The condition of collection was as reported earlier [9]. The 108,000 *g* cytosolic fraction, prepared as described above, was used in the preliminary development of the assay procedure. Similar cytosolic fractions were prepared from 17 samples of human liver obtained from the human liver bank collected over a period of 2 years. They were biopsies

from patients suspected of cancer of the GI tract. All biopsy samples were collected during surgical operation. The liver extracts were stored in small aliquots of 200 μL at −80° until subsequent assay. All, except for one Malay and one Indian, were Chinese, aged between 48–83 years. Five were males and seven were females and the sex of the remaining were not recorded. Four of them were diagnosed as hepatitis B positive, three were diabetic and two hypertensive. The common pre-operative drugs were pethidine and phenergan and the medications of the diabetic and hypertensive patients were recorded to be daonil (or glibenciamide), nifedipine and 5-fluorouracil.

Protein determination

The protein contents of the extracts were measured by the method of Lowry *et al.* [10] with bovine serum albumin as standard.

Formation of NADA-sulphate in vitro

The 108,000 *g* cytosolic extract of rat liver was used to generate NADA-sulphate in sufficient quantity for the purpose of isolation, separation, identification and quantification by TLC, paper chromatography, HPLC–ECD and HPLC–radioisotopic techniques. The incubating mixture contained the following chemicals with their final concentrations in parentheses: sodium sulphate (7 mM) or sodium ^{35}sulphate (0.8 or 1.24 mM), ATP (7 mM), NADA (50 μM) and Mg^{2+} (7 mM) in DTT (3 mM), made up to a final volume of 450 μL with 50 mM phosphate–NaOH buffer of pH 8.0. The reaction was started by the addition of 50 μL of rat liver supernatant containing 450–500 μg protein. The reaction was stopped at 1 hr by precipitation with 50 μL each of 5% zinc sulphate and 0.3 M barium hydroxide.

Isolation and identification of NADA-sulphate/NADA-^{35}sulphate

(a) *Thin-layer chromatography*. NADA-sulphate was separated on TLC plates (20 cm by 20 cm) coated with silica gel (Merck, Darmstadt, F.R.G.) and developed in 1-butanol : acetic acid : water (3 : 1 : 1, by vol) for 4 hr. NADA-sulphate and unreacted NADA were visualized by spraying with 1 N Folin phenol reagent [11] followed by 10% sodium carbonate. The material contained in the spot with an R_f of 0.68 corresponding to NADA-sulphate was scraped and eluted in 2 mL of water for analysis by HPLC–ECD. Two hundred μL of the eluate was hydrolysed in the presence of 0.6 M perchloric acid and 3 mM DTT in boiling water for 20 min to release NADA which was subsequently analysed by HPLC–ECD.

(b) *Paper chromatography–liquid scintillation counting*. NADA-^{35}sulphate was isolated on Whatman No. 1 paper developed in isopropanol : ammonia : water (8 : 1 : 1, by vol.) following the procedure as described [5, 6]. It was eluted with 2 mL of water from sections of the paper chromatograms corresponding to an R_f of 0.73. Subsequent analysis was by HPLC–liquid scintillation counting as described [5, 6]. NADA-^{35}sulphate was identified by HPLC using a radioactive flow detector (Radiomatic Instruments & Chemical Co., Inc., Tampa, FL U.S.A.) to which was attached a 250-μL solid flow-cell.

(c) *High-pressure liquid chromatography–electrochemical detection*. Two mobile systems were used in this study: mobile phase A contained 20 mM phosphate, 1 mM EDTA at pH 7.0 and 5% methanol. At a flow rate of 0.5 mL/min, NADA-sulphate could be separated from NADA, both of which were detected by ECD at a working potential of 0.7 V. However, with this solvent system, there was a decrease in detector response with multiple injections in contrast to mobile phase B which contained 1.2% acetic acid, 1 mM EDTA at pH 4.4 and 2% methanol. Even at a working potential of 0.8 V, there was no observable deterioration of detector response with repetitive injections. Using a flow rate of 0.6 mL/min of mobile solvent B, NADA-sulphate was readily separable from NADA.

Optimization of overall sulphate conjugating assay

The overall sulphate conjugating activity of the human liver was assayed at incubation time of 15, 30 and 60 min and at an enzyme concentration of 106–414 μg protein content in a total reaction volume of 200 μL. Subsequently, an incubation time of 30 min and enzyme content of 212 μg were employed in determining the optimum conditions at a temperature of 37°. The pH optimum was studied using 50 mM phosphate buffer from pH 6.0–8.0 and 50 mM glycine–NaOH buffer from pH 8.4–9.2. The variation of sodium sulphate, ATP and Mg^{2+} ion concentrations ranged from 50 μM to 10 mM and NADA concentration was from 1 μM to 100 μM.

Assay of overall sulphate conjugating activity of human liver

The standard assay mixture for measuring sulphate conjugating activities of human liver extracts contained the following chemicals; the final concentrations are given in parentheses: sodium sulphate (7 mM) was used in the HPLC–ECD procedure and sodium ^{35}sulphate (0.924 mM) in the radiometric procedure; ATP (5 mM), NADA or dopamine (50 μM) and Mg^{2+} ions (7 mM) in DTT (3 mM). The final volume of the incubate was 200 μL and 50 μL respectively for the HPLC–ECD and radiometric assays. When dopamine was the acceptor substrate, *trans*-2-phenylcyclopropylamine at a final concentration of 10^{-3} M was preincubated with the enzyme extract to inhibit MAO activity [12]. Dopamine ^{35}sulphate formed was assayed by the radiometric procedure under conditions which were identical to those established for the sulphation of NADA. This was intended to provide a basis for comparison of the kinetic data obtained from these two amine substrates. The reaction was started with the addition of human liver supernatant containing 11–212 μg protein per 50 μL assay mixture. A proportionate concentration of protein was employed in each of the two assay incubates. This wide range in protein variation was unavoidable as different zones of the human liver were excised during collection and normalization to a more equable concentration range was difficult because of the limited sample size.

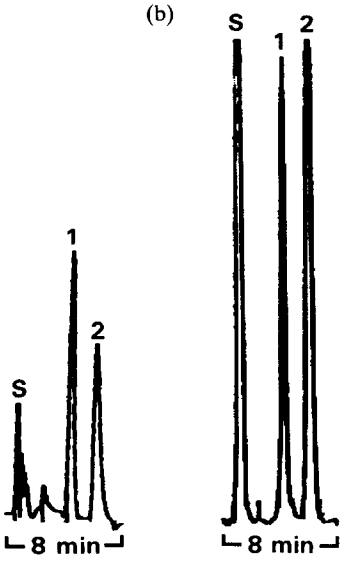

Fig. 1. Separation and detection of NADA-sulphate (peak 1) and NADA (peak 2) by HPLC–ECD in (a) mobile phase A: 20 mM phosphate, 1 mM EDTA at pH 7.0 and 5% methanol; (b) mobile phase B: 1.2% acetic acid, 1 mM EDTA at pH 4.4 and 2% methanol. S = solvent front.

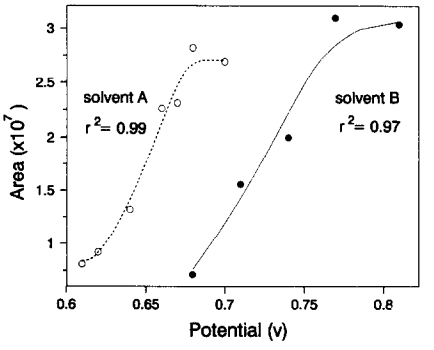

Fig. 2. The plots of applied oxidation potential against the detector response of NADA-sulphate in mobile solvent A (O---O) and B (●——●).

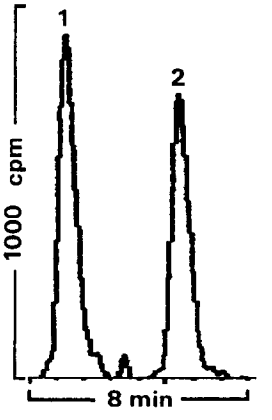

Fig. 3. Separation and quantification of sodium ^{35}sulphate (peak 1) and NADA-^{35}sulphate (peak 2) by HPLC-radioactive flow detection in mobile phase A (see Fig. 1a).

The quantification of NADA-sulphate and NADA-^{35}sulphate was carried out in parallel studies by the following two procedures:

(a) *HPLC–ECD*. The reaction was stopped by boiling the assay incubate for 1 min after 1 hr of incubation and the reacted mixture was adjusted to pH 8.6 by the addition of 50 μL of 2 M Tris–HCl buffer of pH 8.6. Unreacted NADA was extracted twice by adsorption onto 10 mg activated alumina according to the procedure of Anton and Sayre [13]. The sample effluent which contained NADA-sulphate was then filtered through a membrane of 0.45 μ pore size and analysed by HPLC–ECD, using mobile phase B. The chromatographic system consisting of the Hewlett-Packard 1090 M model was connected to a microbore column (100 mm × 2.1 mm) packed with Hypersil-ODS of 5 μ particle size. The ECD (Waters model 460) was set at a sensitivity of 2 nA and operated at a working potential of 0.8 V.

(b) *Radiometric method*. At incubation time of 15, 30 and 60 min, 10 μL of the reacted incubate was spotted on Whatman No. 1 paper. The strips were developed in isopropanol:ammonia:water (8:1:1, by vol.) for 16 hr and the radioactivity present in the sections of the chromatograms corresponding to NADA-^{35}sulphate and dopamine ^{35}sulphate was measured by liquid scintillation counting as described [5, 6].

RESULTS

Isolation and identification of NADA-sulphate/ NADA-^{35}sulphate

On TLC plates developed in 1-butanol:acetic acid:water (3:1:1, by vol.), the R_f values of NADA and NADA-sulphate were respectively 0.88 and 0.68. By using the 108,000 *g* cytosolic fraction of rat liver, it was possible to isolate a substantial quantity of NADA-sulphate in this manner for subsequent identification. Likewise, NADA-^{35}sulphate with an R_f value of 0.73 was isolated from paper chromatograms developed in isopropanol:ammonia:water (8:1:1, by vol.) for identification by HPLC–ECD and HPLC-radioactive flow detection. Sodium ^{35}sulphate remained close to the origin in this solvent system.

NADA-sulphate isolated by TLC, when analysed by HPLC–ECD showed a retention time of 5 min in mobile phase A and 4.3 min in mobile phase B. It was well separated from NADA which registered a retention time of 7.2 min and 6.4 min in the respective solvent systems (Fig. 1a and 1b). The plots of applied oxidation potential against the detector response of NADA-sulphate in mobile phase A and B are shown in Fig. 2, from which the respective operating potentials of 0.7 V and 0.8 V were chosen for its measurement.

NADA-^{35}sulphate, isolated by paper chromatography appeared as a peak with a retention time of 5.6 min in mobile phase A on analysis by HPLC-radiometric flow detection while that of sodium ^{35}sulphate was 1.5 min. (Fig. 3). NADA-^{35}sulphate

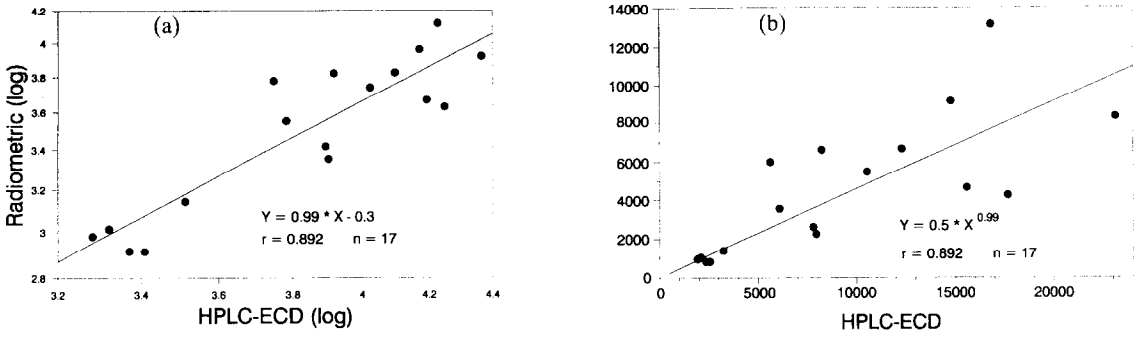

Fig. 4. Correlation between the rate of overall sulphate conjugation of NADA expressed in pmol NADA sulphate/hr/mg protein measured by the HPLC–ECD and radiometric procedures. Analysis is by power regression, giving the regression equation $y = 0.5x^{0.99}$, which on logarithm (base 10) transformation becomes linearised as $y = 0.99x - 0.3$. The correlation coefficient (r) is 0.892 with P = 1.566 exp(−6). Even without log transformation, the plotted regression line $y = 0.5x^{0.99}$ is practically linear and approximated by $y = x/2$ (see Fig. 4b).

synthesized *in vitro* using human and rat liver extracts was identified by both ECD and radioactive flow detection, while NADA, released by hydrolysis of NADA-sulphate was identified by ECD.

Kinetic parameters

The pH optimum of the overall sulphate conjugation of NADA by the human liver extract was 8.0, measured by the HPLC–ECD and radiometric assay procedures. The apparent K_m values of sodium sulphate and NADA measured by the HPLC–ECD method were 103 μM and 4.8 μM, respectively; these values were generated by the ENZPACK software [14]. Although these values were obtained from multi-step enzymatic reactions, sodium sulphate and NADA were substrates of only ATP sulfurylase and PST, respectively.

Specific activities of human liver extracts

The overall sulphate conjugation of 17 human liver extracts, determined by the HPLC–ECD method ranged from 940 to 23,128 pmol NADA-sulphate/hr/mg protein (mean ± SD = 8871 ± 6557). As NADA-sulphate was not commerically available, these values were calculated by comparing with the ECD response of varying amounts of preformed NADA-^{35}sulphate, generated under identical conditions to the standard assay. The latter was in turn quantified from a standard of inorganic ^{35}sulphate, as described [5]. The linear range of NADA-sulphate measured by this HPLC–ECD procedure was 10 to 100 pmol. The corresponding values of specific activities, measured in these same samples by the radiometric assay were lower as suboptimal concentrations of sodium ^{35}sulphate were employed (see Materials and Methods); the range was 818 to 13,125 pmol NADA-^{35}sulphate/hr/mg protein (mean ± SD = 4568 ± 3481). A correlation coefficient (r) of 0.892 was obtained for the two sets of specific activities (Fig. 4). The coefficient of determination (r^2) is 0.8. The percentage of deviation is 33.394%, which means that 66.06% of the original variation is explained by the regression equation $y = 0.99x - 0.3$ (± 0.158 at 95% confidence limits) in log scale (see Fig. 4a). The correlation is very highly significant, P = 1.566 exp(−6). In linear scale, the regression equation is $y = 0.5x^{0.99}$, and the plotted regression line is shown in Fig. 4b and can be seen to be practically linear.

Parallel measurements of the overall sulphate conjugation of NADA and dopamine were also carried out by the radiometric assays. In contrast to NADA which showed predominantly only one radioactive peak corresponding to NADA-^{35}sulphate, two peaks, presumably the 3-*O*- and 4-*O*-^{35}sulphates of dopamine were observed, with R_f values of 0.46 and 0.75. The relative proportions of these two conjugates varied in the different hepatic samples. The sum of the two peaks was chosen to represent the magnitude of total dopamine sulphate conjugation as it is difficult at this point of time to attribute one or the other or both for comparison with NADA sulphation. There is evidence that NADA, at micromolar concentration, serves as a substrate of both the "P" and "M" forms of PST [17] although dopamine at micromolar concentration is a substrate of the "M"-PST form. Of the 17 samples, dopamine ^{35}sulphate could not be detected in seven. These were also samples with very low NADA-sulphating activity. This confirmed our earlier studies that dopamine was not as good a substrate of PST as NADA [5, 15–17]. A correlation coefficient of 0.82 was established for the rates of overall sulphate conjugation of NADA and dopamine in the remaining 11 samples, as analysed by orthogonal linear regression. The regression of x on y is $x = 9.91y - 2697.25$ (±2867.65) and y on x is $y = 272.31 + 0.1x$(±349.91). The two regression lines were almost overlapping (Fig. 5) suggesting that the prediction of x values from y values is as good as the prediction of y values from x values.

DISCUSSION

Measurement of sulphated amines usually involved acid or enzymatic hydrolysis to release the parent compounds which were then determined radioenzymatically or by HPLC with electrochemical detection [18, 19]. Direct determination of sulphate

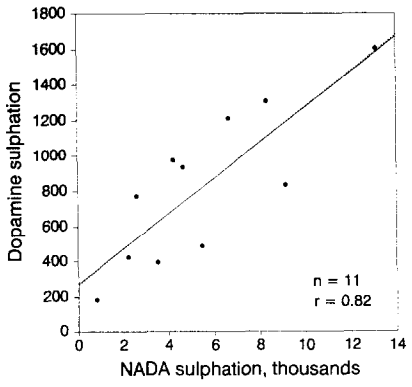

Fig. 5. Correlation between the specific activities (expressed in pmol/hr/mg protein) of the overall sulphate conjugation of NADA and dopamine measured radiometrically. Analysis was by orthogonal linear regression giving a correlation coefficient of $r = 0.82$. The regression of x on y (dotted line) is $x = 9.91y - 2697.25$ (± 2867.65) and y on x (solid line) is $y = 272.31 + 0.1x$ (± 349.91).

conjugates of dopamine has been reported using HPLC with spectrophotometric detection [20] or with an ECD equipped with dual electrode system [21, 22]. The direct determination of dopamine sulphate by ECD was reported to be difficult as an oxidation potential higher than 1.4 V was required [19]. However, in our study, NADA-sulphate showed a relatively steady ECD response at 0.7 V and 0.8 V in two different solvent systems (Fig. 3). An advantage of the procedure was the direct measurement of the sulphate-conjugate produced. Secondly, NADA is a biogenic amine [23] and it is a good substrate of PST of human liver, with an apparent K_m of 4.6 μM, comparable to that of dopamine of 27 μM [3]. As the rates of overall sulphate conjugation of NADA and dopamine were positively correlated (Fig. 5), this sensitive HPLC–ECD assay measured with NADA as the amine substrate should conceivably reflect the sulphate conjugation of dopamine.

Sulphate conjugation is an important detoxication process in man as shown by the high percentage of conjugated catecholamines in human plasma: 70% of noradrenaline and adrenaline and 99% of dopamine [18]. Two likely sites of sulpho-conjugation of the catecholamines in man are the platelets and the liver. Studies on human subjects showed that neither the platelets [24, 25] nor the liver [26] was indispensable for the sulpho-conjugation of dopamine in the human circulation. The red blood cells, previously shown to possess PST activity [27] were proposed to be a probable alternative site [28]. We had observed that the erythrocytes had negligible overall sulphate conjugating activity (unpublished observation).

This study was an attempt to assess the relative contribution of the human liver and platelets to the overall sulphate conjugation by measuring and comparing their sulphate conjugating activities *in vitro*. This overall sulphation would reflect more closely, compared to the PST activity, the situation *in vivo*. Under similar conditions of measurement by the radiometric procedure, the mean specific activity ± SD of the overall sulphate conjugation of the human platelets was 1202 ± 933 pmol NADA-sulphate/hr/mg protein [5]; the corresponding values for the liver was 4568 ± 3481 pmol/hr/mg protein (Fig. 5). A wide inter-individual variation was observed in both sets of values. Based on an approximation of 10–70 μg per 10 μL platelet preparation processed from 0.1 mL blood and an average volume of 5 L of blood in an individual, the total sulphate conjugating capacity of the circulatory system would be estimated to be 0.6 to 4.2 μmol/hr. Likewise, values obtained in this study showed that 10 μL of the various human liver cytosolic fractions contained 11–212 μg of protein, which was derived from 2 mg wet wt of hepatic tissue. Based on an average weight of human liver of 1500 g [29], the total sulphate conjugating capacity of the human liver would approximate 38 to 726 mmol/hr. It would appear from this simplistic comparison that the sulphate conjugating capacity of the liver far exceeded that of the platelets by several orders of magnitude. Undoubtedly, higher hepatic PAPS-generation and PST activity may be contributing factors. Caution however must be exercised in the interpretation of these data which were derived from platelets of normal human subjects and hepatic biopsy specimens of patients of different clinical conditions and who had been treated with different drugs (see Materials and Methods). Besides, the blood flow through the whole organ may be rate-limiting and different zones of the liver may possess different sulphate-conjugating ability. Thus our computation based on the net weight of the whole liver may be an overestimation. On the other hand, the more encompassing distribution of the platelets in the human body via the circulatory system together with the greater availability of the neurotransmitter amine substrates which are released directly into the blood and subsequently concentrated in the platelets [30] may confer on the platelets a more strategic location for sulphate conjugation of neurotransmitter amines in the plasma.

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