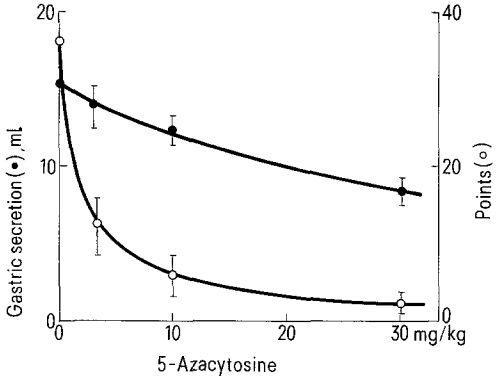

Depression of gastric ulcers and gastric secretion in rats by 5-azacytosine. Groups of 10 male animals received the drug i.p. immediately after pyloric ligation and 22 h later they were killed by cervical dislocation.

methyl-5-azacytosine the increase of its dosage to 50 or 100 mg/kg results in the inhibition of gastric secretion and gastric acidity but the action on the process of ulceration is less favorable. Hydrolytic products of 1-methyl-5-azacytosine and 5-azacytosine, N-amidino-N'-methyl urea and N-amidine methyl urea, respectively, are completely inactive.

In an earlier study we observed depression of experimental acute pancreatitis mediated in rats by 5% bile solution administered into the pancreas in vivo following 5-azacytidine or cycloheximide treatment[5]. Here we show that 5-azacytosine and its 1-methyl derivative also decrease the amount of abdominal fluid and prevent pathological changes in the pancreas (table 2) 6 h after the interstitial administration of natrium cholate. Other tested methyl derivatives of 5-azacytosine and 5-azacytidine were inactive in this respect.

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Specific determination of arylsulfatase A activity

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Summary. Arylsulfatase activities in biological materials are too low to be detected by the methods available hitherto. A sensitive and specific assay method for arylsulfatase A (AS-A) has been developed in the present study. Ascorbate-2-sulfate is known to be a specific natural substrate of AS-A; the ascorbic acid liberated by the action of AS-A was quantitatively determined using HPLC equipped with an amperometric detector. The method was used to analyze the activity of AS-A in biological materials.
Key words. Arylsulfatase A; natural substrate; ascorbate-2-sulfate; HPLC-amperometric detection.

Arylsulfatase [EC 3.1.6.1 arylsulfate sulfohydrolase] (AS) activities have been shown to be present in biological materials in connection with diseases. Three types of AS activities are known at present in mammalian tissues; AS-A, AS-B and AS-C. AS-A and AS-B are present in the lysosomal fraction of the cell. Since changes in AS-A and AS-B activities have been shown in disease states[1,2], AS activity determination could be useful in characterizing the nature of some diseases. For example, it has been documented that AS-A activity is absent in metachromatic leukodystrophy (MLD) and AS-B is absent in the Maroteaux-Lamy syndrome (MLS)[1,2].

Human leukocyte and rat brain arylsulfatase A activity with the natural substrate. HPLC conditions and assay procedure for arylsulfatase A activity; same as in the chart

Arylsulfatase A activity	Mean ± SD	Number
Human leukocytes	2.39 ± 0.90[a]	N = 8
Rat brain	112.90 ± 69.10[b]	N = 5

[a] Ascorbic acid nmoles/30 min/mg protein; [b] ascorbic acid nmoles/h/mg protein.

For the purpose of discriminative determination of AS-A and AS-B activities, specific inhibitors for each activity have been proposed[3,4]. However, the inhibitory effects of these compounds are not strictly specific. The enzyme activity was found to be variable among different animal species. Therefore, a universally applicable method would be valuable, but such a method has not been found so far.

There are natural substrates for AS-A and AS-B activities, and the enzyme activities are more specific for the natural substrates than for synthetic ones. UDP-N-Ac-galactosamine-4-sulfate is known to be a good and specific substrate for AS-B[5] and ascorbate-2-sulfate (AAS) for AS-A[6].

In the present investigation, AS-A activity was specifically assayed using AAS; this excludes the possibility of AS-B activity. The enzymatic reaction product, ascorbic acid (AA), could be separated from the unreacted substrate, sulfate and enzyme by HPLC.

Materials and methods. All chemicals used were of analytical grade or reagent grade, and obtained from Sigma Chem. Co., St. Louis, MO (USA) and from Wako Pure Chem. Co., Tokyo (Japan). HPLC column is LiChrosorb RP-18, 7 μm, of Merck (FRG).

1) Optimal conditions for HPLC. AA liberated after the enzymatic reaction was separated from the substrate, AAS, by HPLC using a reversed phase column, LiChrosorb RP-18 (7 µm, 3.9 × 300 mm). The mobile phase was 0.05 M acetate buffer, pH 4.5, containing 30 mM tetra-butylammonium hydroxide (TBA). The flow rate of the eluent was 1.0 ml/min at 25 °C. AA was detected with an amperometric detector, at the oxidation electrode and potential +0.7 V vs Ag/AgCl, and also detection was performed with a UV detector, at $A_{254\,nm}$.

2) Enzyme source. Rat brain AS-A and AS-B activities were obtained by the method developed by the authors[7]: that is, homogenate of the extracted rat brain, with 0.25 M surcrose solution, was centrifuged at 157,000 × g for 60 min at 4 °C, was subjected to DE-52 column chromatography (0.6 × 1.0 cm), and AS-B was eluted with 0.01 M Tris-HCl buffer, pH 8.0, containing 0.1 M NaCl and AS-A with 0.01 M Tris-HCl buffer, pH 8.0, containing 0.2 M NaCl.

Human leukocytes were obtained by the method described by Boyun[8] using the dextran sedimentation method, and fractionation of the AS-A and AS-B activities was performed according to the method reported by Humbel[9]: that is, the leukocyte fraction was treated by ultrasonication for 30 s, and centrifuged at 2850 × g (about 3000 rpm) for 10 min at 4 °C. The supernatant was subjected to DE-52 column (0.6 × 1.0 cm), and AS-B was eluted with 0.01 M Tris-HCl buffer, pH 8.0, and AS-A with 0.2 M NaCl added 0.01 M Tris-HCl buffer, pH 8.0.

3) Assay of arylsulfatase A activity. AS-A was determined by the method shown in the chart. The enzymatic reaction was stopped by the addition of 10% phosphotungstic acid. Protein, which would disturb the following chromatographic procedures was removed by precipitation with 10% phosphotungstic acid. After centrifugation for 10 min at 2850 × g, 20 µl of the supernatant was used for separation by HPLC.

The pH optimum for AS-A activity was investigated using 0.3 M sodium acetate buffer, at pH value 3.5, 4.0, 4.5, 5.0, 5.5 and 6.0. Kinetic study of AS-A activity was carried out using various substrate concentrations: 3.3, 6.7, 10.0, 13.3 and 16.7 mM AAS at the final concentration. The optimum time of incubation was also examined.

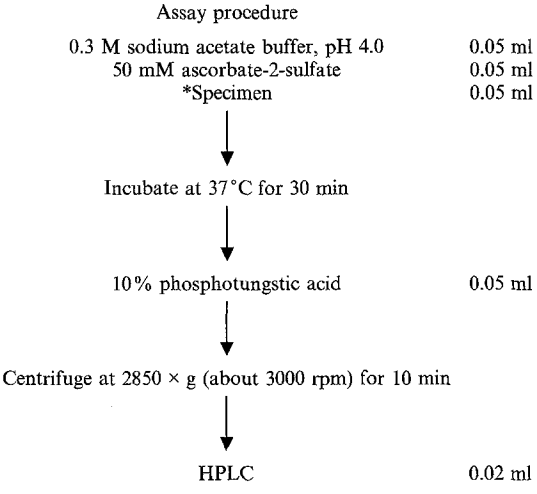
Results and discussion. 1) Separation of ascorbic acid from the substrate (ascorbate-2-sulfate) by HPLC. AA and AAS were separated on the reversed phase HPLC column with 0.05 M AcOH or 0.05 M AcOH containing TBA of various concentration used as the mobile phase. AA was not sufficiently retained on the LiChrosorb RP-18 column when the mobile phase was composed only of 0.05 M AcOH. Therefore, TBA was added to the mobile phase as the counter ion. It was found that 0.05 M acetate buffer containing 30 mM TBA, pH 4.5, was the best solvent for the present purpose: that is, addition of 30 mM TBA to 0.05 M AcOH gave the pH of the mixture 4.5.

2) Detection of ascorbic acid after the separation by HPLC. AA and AAS could both be detected by a UV detector, but only AA was oxidized electrically, so, AA could be detected by an amperometric detector but AAS was not. Therefore, for the present purpose of quantitative detection of AA after the separation by HPLC, an amperometric detector was found to be more satisfactory and sensitive.

3) Calibration of the ascorbic acid levels by HPLC. Using the HPLC conditions described above, a linear calibration for AA was obtained from 2.0 to 20.0 ng using 32 nAFS of detection range, with the value of coefficient of variation (CV), ±6.8% at 5.0 ng, ±11.4% at 10.0 ng, ±3.3% at 15.0 ng and ±3.7% at 20.0 ng. CV values were calculated from the peak height of HPLC chromatograms in five determinations.

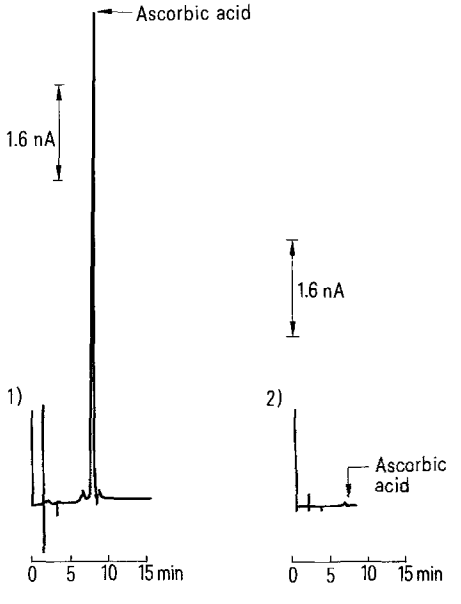
4) Optimum conditions for the arylsulfatase A activity assay. There are two pH optima for AS-A activity, at pH 4.0 and pH 5.0, in 0.3 M sodium acetate buffer. This fact was also documented by Roy[10]. The enzymatic reaction proceeded linearly up to 40 min of incubation. Therefore, the conditions for AS-A activity determination were set as shown in the chart. K_m value of AS-A activity was approximately 1.0 mM with AAS used as the substrate. The present result was comparable in sensitivity to the result reported by Roy, who obtained it by a method using pH-stat, and to that of Stevens et al.[11,12], who used ^{35}S-labeled substrate. However, using ^{35}S-labeled substrate, Stevens et al. reported that spontaneous and nonenzymatic radioactive decomposition products yielded high background radioactivity levels in the control mixtures, and because of this high background, the determination of low levels of enzyme activity in biological materials was interfered. Therefore Stevens et al. re-

Assay procedure and HPLC conditions



*Specimen: 1) human leukocyte homogenate; 2) rat brain homogenate

HPLC condition
Column: LiChrosorb RP-18 (3.9 × 300 mm, 7 µm).
Mobile phase: 0.05 M acetate buffer, pH 4.5, containing 30 mM tetra-butylammonium hydroxide.
Flow rate: 1.0 ml/min, temp.: ambient.
Detection: + 0.7 V vs Ag/AgCl by amperometry (AMD).
Range: 16, 32 or 80 nAFS.
Injection volume: 20 µl loop injection.



HPLC chromatogram of arylsulfatase A activity in human leukocyte. HPLC conditions and assay procedure for arylsulfatase A activity; same as in the chart. *1* Sample; *2* blank (without sample).

ported that the problem of high-background have minimized this difficulty by extensively diluting the reaction mixtures before precipitation. However, Stevens et al. used the sediment by Ba^{2+} in which most ^{35}S-compounds were present, while in our method, phosphotungstic acid was used, and phosphotungstic acid did not precipitate the AAS of substrate. Therefore, AA in the supernatant, after deproteinization by phosphotungstic acid, was used for HPLC, and the procedure was found to be more sensitive than that reported by Stevens et al. In the present study, with HPLC-amperometric detection, such co-precipitation and high-background problems could be excluded: that is, disturbing protein was removed by precipitation after the enzymatic reaction, and only the product of enzyme activity (AA) was detected electrochemically.

5) Application of the method for the detection of arylsulfatase A activity in biological materials. The method was used for the measurement of AS-A activity in human leukocytes. Using the natural substrate for AS-A activity, AS-A activity could be specifically determined, as shown in the figure. In preliminary trials, attempts were made to determine AS-A activity in rat brain, and it was found that even when a different tissue and animal species were used, the present method could be satisfactorily applied for the determination of AS-A activity (table). The present method may be applicable for the discriminative determination of AS-A and AS-B in connection with diseases, particularly related to allergy. Such investigations are presently being undertaken and the results will be reported in future.

Conclusion. It was possible to apply the present method of AS-A activity determination to crude biological materials containing low enzyme activity, because of 1) the specific nature of the substrate and 2) the method of detection of the enzymatic reaction product. In addition, the time of analysis was shortened considerably because only the product, AA, is determined by HPLC-amperometric detection. In addition, the substrate, AAS, did not disturb the chromatographic process and detection. That is, the retention time of AAS was long (approx. 40–50 min). Thus, high sensitivity could be achieved with a much shorter time of analysis.

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Failure of coffee to inhibit the pharmacodynamic activity of morphine in vivo

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Summary. High doses of caffeine-containing as well as decaffeinated instant coffee neither inhibited morphine-induced analgesia in mice nor the morphine-induced fall of blood pressure, heart rate and respiratory rate in rats. On the contrary, caffeine-containing coffee even enhanced the analgesic effects of morphine in mice. Coffee thus does not exhibit opiate-antagonizing activity in the whole organism in vivo. The very weak morphine-antagonistic efficacy of coffee powder in the myenteric plexus-longitudinal muscle preparation from the guinea pig ileum is of no practical importance.
Key words. Coffee; caffeine; morphine; naloxone; analgesia.

Boublik and coworkers[1] recently reported the startling discovery that solutions of instant coffee powder, both regular and decaffeinated, are able to inhibit the in vitro binding of ^3H-labeled naloxone and other ligands to opiate receptor binding sites of rat brain membranes. Furthermore, a partially purified material from instant coffee powder antagonized the effects of morphine on the guinea pig ileum in vitro. From their results they estimated that one cup of coffee contains the equivalent of one-third of an ampoule of naloxone, and raised the possibility that drinking coffee might be followed by opiate receptor blockade not only in the gastrointestinal tract but also in the central and peripheral nervous system. We report here that instant coffee even in very high doses does not inhibit the pharmacodynamic activities of morphine in mice and rats in vivo. On the contrary, caffeine-containing coffee powder even enhanced the analgesic effects of morphine in mice.

The analgesic activity of morphine was assessed in male NMRI mice weighing 18–20 g by the well-known tail flick response test to radiant heat using the analgesia-meter of Joh. Friedrich Bundschuh (Griesheim, FRG). The reaction time was measured once before treatment with coffee and morphine and thereafter at 15-min intervals for the 1st, and at 30-min intervals for the 2nd hour. For each animal, the differences between the reaction times measured after morphine and the initial value were calculated. A commercially available instant coffee powder, both regular and decaffeinated (Jacobs Cronat Gold and Jacobs Nacht & Tag, caffeine content as measured gaschromatographically amounting to 20.2 mg/g and 2.6 mg/g, respectively), was dissolved in distilled water for oral administration and in saline for i.v. administration. The volume administered of these solutions was kept constant at 10 ml/kg b.wt. Morphine was dissolved in saline (10 ml/kg b.wt) and injected i.p. 15 min after coffee administration. Control animals treated with vehicle instead of coffee were studied simultaneously with the coffee-treated mice.

The mean reaction time of mice to radiant heat initially ranged between 5.6 and 7.7 s and did not change consistently upon treatment with saline or coffee (table 1). Morphine (10 mg/kg i.p.) doubled the reaction time in most experiments, with the analgesic effect reaching its maximum 45 min after the treatment