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Spartaco Crescenzi, Giuseppe Prota, Adriana Iaccheo, Maria Assunta Santacroce, Alfredo Ruffo

A new method for the determination of 5-S-cysteinyldopa in the urine from patients with malignant melanoma

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Biochimica. — *A new method for the determination of* 5-S-cysteinyldopa *in the urine from patients with malignant melanoma.* Nota di SPARTACO CRESCENZI (*), GIUSEPPE PROTA (*), ADRIANA IACCHEO (*), MARIA ASSUNTA SANTACROCE (**) e ALFREDO RUFFO (***), presentata (****) dal Corrisp. A. RUFFO.

RIASSUNTO. — Viene descritto un procedimento analitico che consente di effettuare una rapida valutazione dei livelli di escrezione della 5–S–cisteinildopa direttamente sui campioni di urina deproteinizzata con un comune analizzatore automatico di amminoacidi.

Nelle condizioni di eluizione messe a punto, la 5–S–cisteinyldopa viene separata dai comuni amminoacidi presenti nell'urina e può essere dosata fino ad un limite inferiore di circa 250 μg/litro, sufficiente per valutare e seguire il grado di evoluzione del melanoma e di ogni altra alterazione nel metabolismo del sistema pigmentario.

Per concentrazioni inferiori a questo limite, l'analisi viene effettuata su campioni di urina concentrata dieci volte mediante cromatografia di adsorbimento su colonna di resina e scambio cationico e successiva eluizione selettiva della 5–S–cisteinildopa e di altri metaboliti analoghi con acidi di opportuna molarità.

INTRODUCTION

In the last few years research on the metabolism of dopa in patients with melanoma has been focused on the biochemical and clinical significance of urinary excretion of 5-S-cysteinyldopa (cysdopa) and of other biogenetically correlated metabolites [1–3]. The results of these studies have shown that in the urine of patients with melanoma the concentration of cysdopa and of other metabolites connected with the biosynthesis of melanins appears to increase with respect to physiological levels (50–300 μg/24 h) and can assume particularly high values (up to 100–150 mg/24 h) in the case of diffused metastases. These data, together with other biochemical observations, show that the determination of this particular amino acid in the urine represents a valuable means for characterizing the metabolic activity of normal and pathological melanocytes [4, 5].

The methods used so far for the analysis of cysdopa, structurally similar to catecholamines, involve pre-purification of the urine by absorption on activated alumina followed by elution with acids of cysdopa which is then measured

(*) Istituto di Chimica Organica e Biologica, Facoltà di Scienze, Università di Napoli, Via Mezzocannone 16, Napoli.

(**) Istituto Tumori « Fondazione Pascale », Via M. Semmola, Napoli.

(***) Istituto di Biorganica, Facoltà di Farmacia, Università di Napoli, Via L. Rodinò 22, Napoli.

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either by spectrofluorimetric [6] or by liquid chromatography on proper phases [7].

Though highly sensitive, these analytical procedures are somewhat difficult to handle in ordinary laboratories and have the usual drawbacks connected with the use of alumina including a low and variable yield (30–35 %) of recovery of absorbed cysdopa.

In the present paper we report on an alternative analytical procedure [8] which permits determination of cysdopa directly on samples of deproteinized urine by means of an ordinary amino acid analyser.

MATERIALS AND METHODS

24 h urine samples were collected in plastic bottles containing 750 mg of sodium metabisulphite and 25 ml of glacial acetic acid and frozen until use. The urine aliquot necessary for the determination was deproteinized by addition of perchloric acid up to a concentration of 0.4 N, vigorous shaking on an oscillating mixer for 5 min and centrifugation at 30,000 × *g* and 10 °C for 10 min. The clear supernatant was then subdivided into analytic samples and stored in the refrigerator or frozen. Cysdopa, used as a reference, was synthesized using the procedure described by Ito and Prota [9].

The analyses were carried out on a Beckman amino acid autoanalyser mod. 120 C, with ninhydrin detector sensibility 0.1 u.a.f.s., equipped with columns filled with cationic resin AA-15, of appropriate lengths. Elution was carried out with sodium citrate/HCl buffers at the indicated ionic strength and pH.

Analytical procedures:

a) Direct analysis of urine. A sample of deproteinized urine (0.1–0.5 ml) is loaded on a column (15 × 0.9 cm) of Beckman AA-15 resin, kept at 70 °C. After washing with 1 ml of 0.2 N buffer at pH 2.2, the column is eluted first with 0.14 N buffer pH 3.25 for 80 min and then with the same buffer at pH 3.90. Under these conditions, elution of cysdopa takes place at about 127 ml.

b) Analysis of samples enriched with cysdopa: a sample of deproteinized urine (25 ml) is loaded onto a column (7 × 1 cm) of Bio Rad AG50 W-X2 resin (100–200 mesh, H^+ form), equilibrated with 0.5 N HCl. After successive washings with 0.5 N HCl (50 ml) and 1 N HCl (30 ml), the cysteinyldopas adsorbed on the resin are eluted with 25 ml of 4 N HCl discharging the first 2.5 ml. The eluate is then concentrated to a small volume at 35 °C under reduced pressure, and is brought to an exact volume (2.5 ml) with citrate buffer.

An aliquot of the enriched sample, corresponding to 1–10 ml of the original urine, is loaded onto a column (56 × 0.9 cm) of Beckman AA-15 resin. Elution is carried out at 55 °C first with 0.2 N buffer pH 3.25 (90 min) and subsequently with 0.2 N buffer pH 4.00. The elution volume of cysdopa is 198 ml.

Results

In exploratory experiments the chromatographic behaviour of cysdopa under the conditions commonly used for the analysis of acid and neutral amino acids was examined. As can be seen from the elutogram reported in Fig. 1, cysdopa gives a well-defined peak with a retention volume close to that of phenylalanine. In the case of comparable amounts of these two amino acids, a condition which rarely takes place in physiological samples, it is possible to determine the concentration of cysdopa with a reasonable degree of accuracy. However, since only minute amounts of this amino acid are found in the urine, the relative peak may either be covered or appear as a shoulder of the phenylalanine peak.

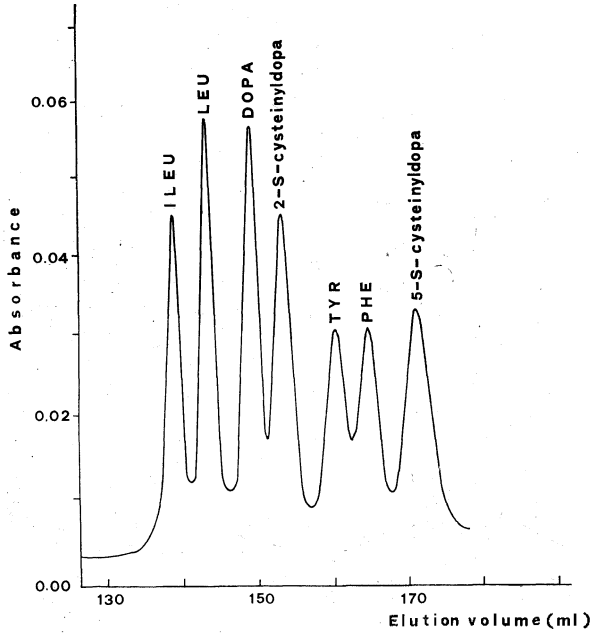

Fig. 1. – Elutogram of a synthetic mixture of amino acids containing 2-5- and 5-S-cysteinyldopa, obtained under the standard conditions used for the analysis of amino acids in physiological fluids. Column: Resin AA-15 Beckman (56×0.9 cm). Eluents: 0.2 N citrate buffers at pH 3.25 and pH 4.25 (change time: 90 min). Temp. 55 °C.

By carrying out systematic experiments with various types of resin (AA-15, PA-35 and M-81 Beckman, 3 AR/2/A55 Erba, Aminex 50 W-X 4 Bio Rad) it was found that the relationship between the pH of the buffer and the elution volume of cysdopa (Fig. 2) differs from the relationship valid for common proteic amino acids with comparable elution volumes such as tyrosine and phenylalanine (Fig 3). Subsequent experiments on the effect of other parameters (temperature, ionic strength, *etc.*) allowed us to define a number

of equivalent working conditions for elution of cysdopa in a given region of the elutogram. Some data illustrating the results of these studies are reported in Table I. They show that an increase of the temperature or of the ionic

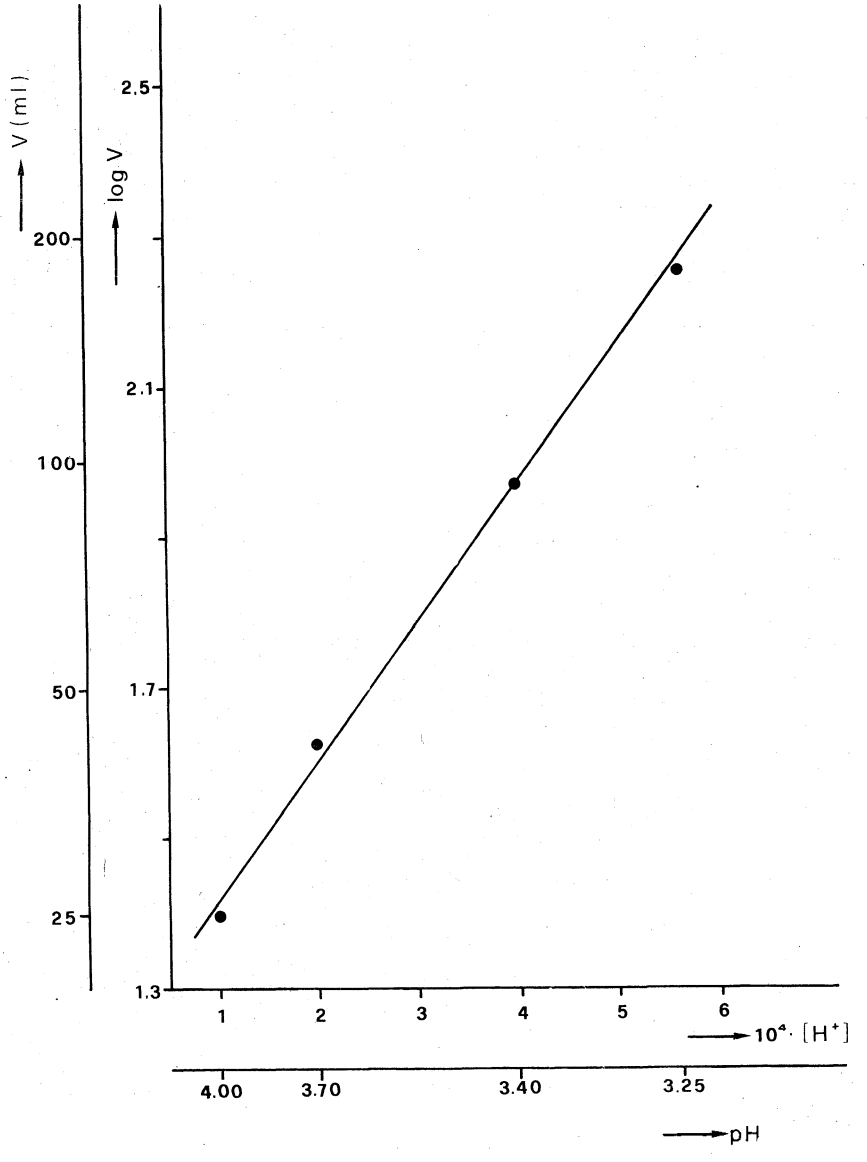

Fig. 2. – Correlation between the elution volume of 5-S-cysteinyldopa and $[H^+]$ of the eluent on Beckman type PA-35 resin (7×0.9 cm). Eluent: 0.2 N citrate buffer; temp. 55 °C.

strength of the buffer produces effects on the elution volume which are equivalent to those caused by an increase of pH with significant changes of the sharpness of the cysdopa peak.

With this background available it was possible to define the optimum conditions for elution of cysdopa from the AA-15 Beckman type resin in a short time in a region of the elutogram free of other urinary ninhydrinpositive

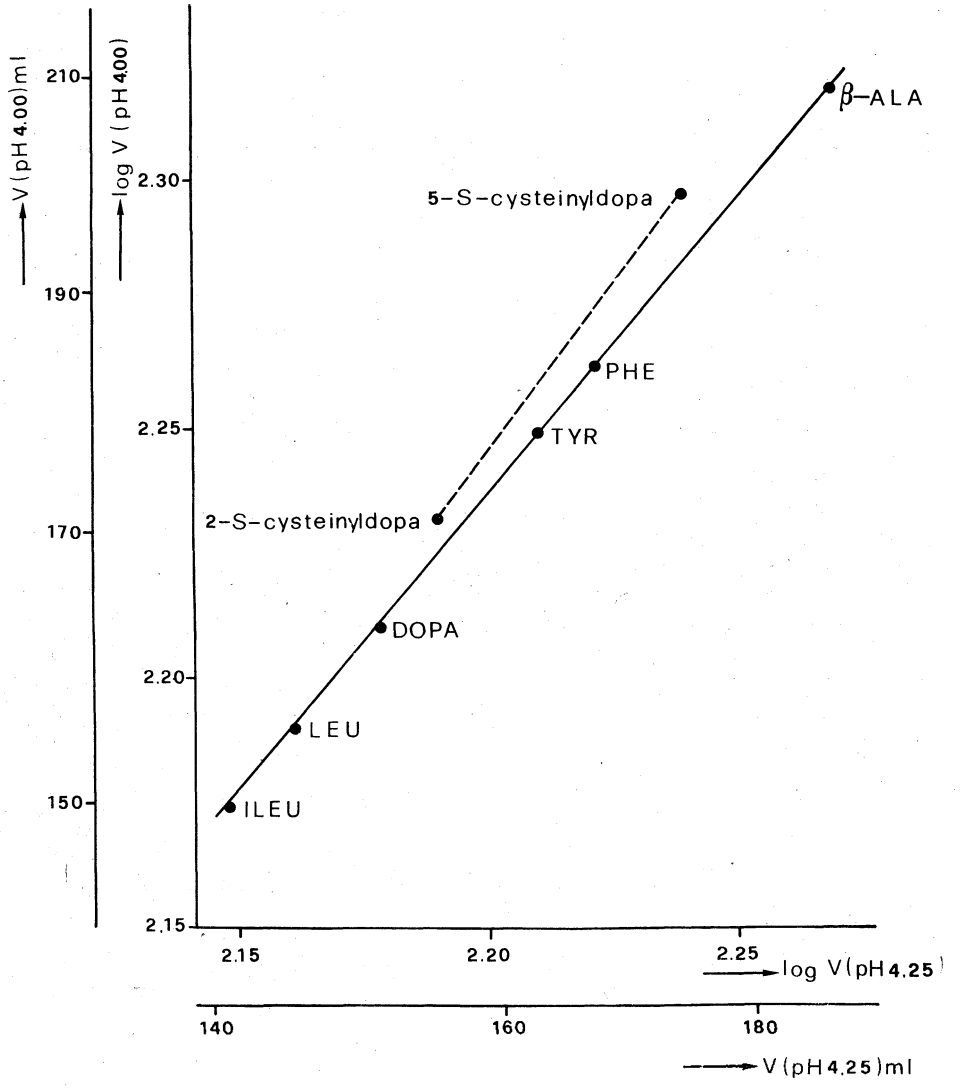

Fig. 3. – Correlation between the elution volumes of cysteinyldopas and some common amino acids at different pHs on Beckman type AA-15 resin (56×0.9 cm). The elution was carried out first with 0.2 N citrate buffer at pH 3.25 (90 min) and then with the same buffer at pH 4.25 (abscissae) or pH 4.00 (ordinate). Temp. 55 °C.

metabolites. In Fig. 4 are reported the elutograms of two samples of urine from patients with melanoma metastases, showing increased excretion of cysdopa. These results provide evidence that the method is of diagnostic

TABLE I.

Variation of the elution volume of 5-S-*cysteinyldopa with ionic strength*, pH, *and temperature of the eluent.*

Ionic strength, N	0.20	*0.33*	0.20	0.20
pH	3.25	3.25	*3.37*	3.25
Temperature, °C	55	55	55	*70*
Elution volume, ml	179	107		
Band width, ml	8.2	7.2	5.5	4.7
Relative column efficiency	1	0.46	0.79	1.07

Column: Resin PA-35 Beckman (7×0,9 cm). Eluent: citrate buffer.

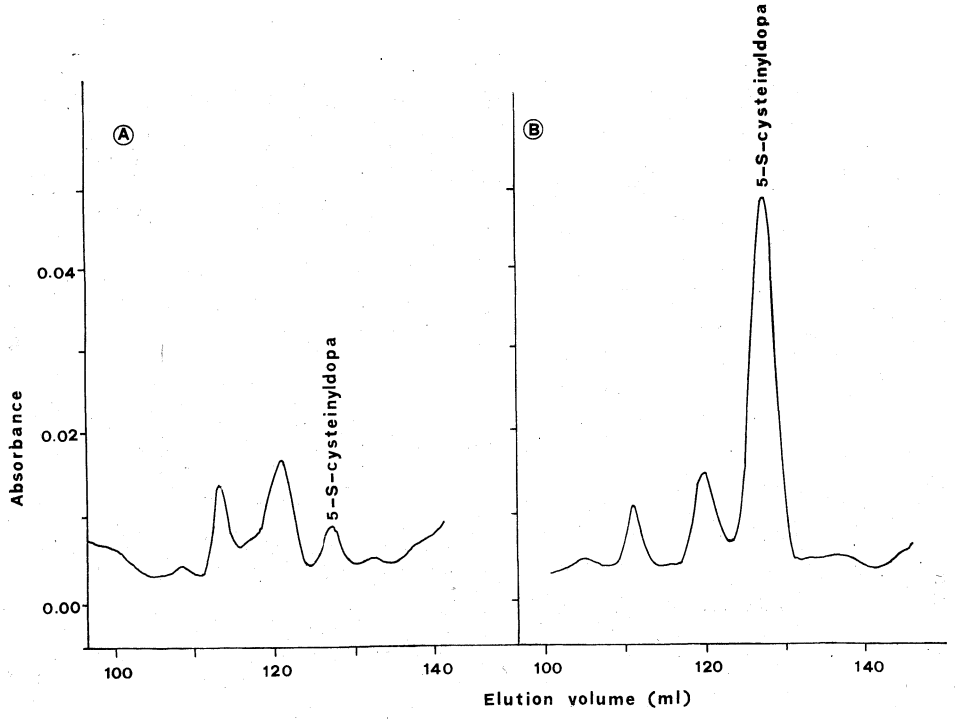

Fig. 4. – Elutograms of deproteinized samples (A and B) of urine (0.3 ml) from two melanoma patients showing peaks of 5-S-cysteinyldopa equivalent to: A – 1.86 mg/l; B – 25.9 mg/l. Conditions: after application of the sample on the AA-15 Beckman resin (15×0.9 cm), elution was carried out with 0.14 N citrate buffer first at pH 3.25 (80 min) and then at pH 3.90. Temp. 70 °C. Column: Resin AA-14 Beckman (15×0.9 cm).

value, even if the sensitivity of the ninhydrin detections is not great enough for quantitative determinations of cysdopa lower than 250 μg/liter, as in the case of physiological urine. A variant of the method has been therefore developed which involves an initial concentration and purification of the urine

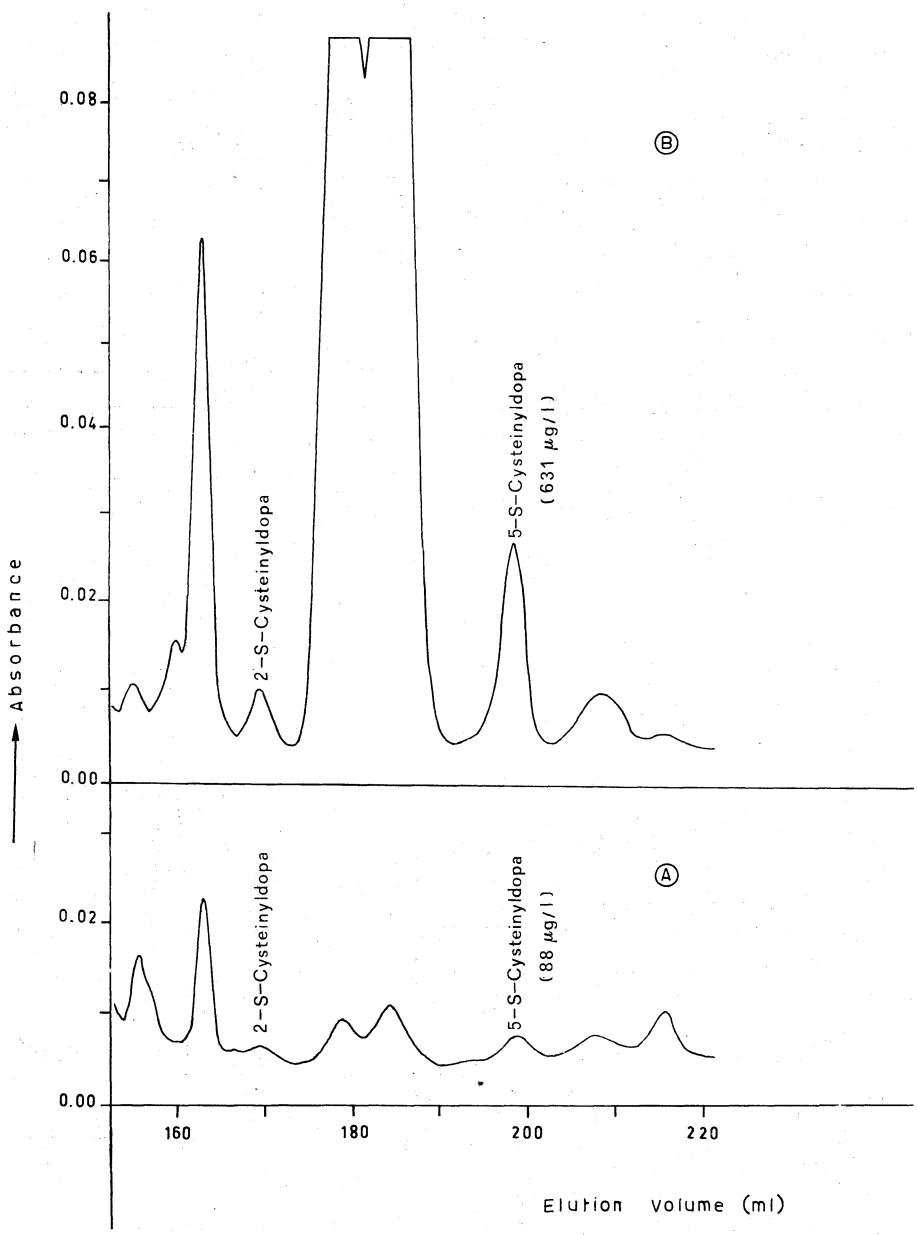

Fig. 5. – Examples of chromatograms of urine from normal subjects (A) and from melanoma patients with low levels of cysteinyldopas (B) after concentration on AG 50 W–X 2 resin as described in Materials and Methods – proc. (b). This procedure permits also the detection of 2-S-cysteinyldopa, present in a smaller amount than 5-S-cysteinyldopa.

by absorption on a small Dowex AG-50 W-X 2, column followed by washings with dilute acid to remove most of the common amino acids. Elution of cysdopa adsorbed on the resin is then achieved with 4 N HCl and the eluate so obtained is concentrated *in vacuo* to a volume ten times lower than the original urine sample before being examined on the amino acid analyser. Two typical examples of cysdopa analysis with this procedure are illustrated in Fig. 5.

As shown in separate experiments there is a recovery of cysdopa after the pre-purification step of about 95 % that is much higher than that obtainable with alumina (about 35 %).

DISCUSSION

The results reported in this paper show that under appropriate experimental conditions it is possible to utilize a common amino acid autoanalyzer for the estimation of the levels of the urinary excretion of cysdopa.

In the defined elution conditions, cysdopa is separated from the amino acids present in the urine and can be measured up to a limit of about 250 μg/l, high enough for a rapid determination of pathological levels of excretion.

The application of this method to the urine of numerous patients with pigmented melanomas has permitted confirmation of the correlation, first shown by Rorsman *et al* [4, 5], between the level of urinary excretion of cysdopa and advanced stages of melanoma.

The results obtained after surgical extirpation were also significant: it was in fact verified that the urinary rate of cysdopa decreases to normal values after the extirpation of the melanoma and emptying of the lymph nodes. Further studies are in progress in order to gather more statistical data and for the perfection of direct analytic procedures by chromatography of cysdopa in physiological liquids.

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