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## Summary

The rapid and accurate method of calcium determination in whole blood in connection with Sephadex gel filtration has been studied. This method has an advantage in complete separation of calcium from protein.

## THE AMINO ACIDS IN OPIUM II

## Quantitative Determination\*

Sadaichi MIYAMOTO and Einar Brochmann-Hanssen

In a qualitative study of the amino acid composition of opium, the presence of a large number of free amino acids was reported (1). Reasons were presented why the amino acids might be useful as a basis for origin determination. For such purpose, it is necessary to have a quantitative method of determination which gives reproducible results and is suitable for routine operation. Various paper chromatographic methods were explored, but in our hands were not satisfactory for the large number of amino acids in question. We found the most accurate and reproducible method to be the ion exchange chromatographic procedure of Moore and Stein (2, 3). Although time-consuming and hardly suitable for routine analyses in its original form, equipment is now commercially available or may be built from available parts (4, 5) that will make the method almost entirely automatic. Considerable progress is also being made in the field of gas chromatography of amino acids.

This paper reports the results of quantitative determination of free amino acids in a limited number of opium samples of different origin.

## EXPERIMENTAL

A total of fifteen authenticated opium samples were analyzed, representing seven different countries and three opium-producing regions in India.

**Preparation of the Sample.**--Two hundred mg. of finely powdered opium was triturated in a glass mortar with 1 ml. of ice-cold water. Gradually, 10 ml. of cold water was added while stirring. The stirring was continued for five minutes, and the aqueous extract was decanted into an extraction tube (1) containing 1 g. of a strongly acidic, cationic exchange resin (Dowex® 50-X<sub>2</sub>, 50-100 mesh), previously activated with 4 N hydrochloric acid. The residue was extracted twice more with 10 ml. of ice-cold water each time and the extracts added to the extraction tube. The opium residue was then washed quantitatively into the extraction tube with enough cold water to give a volume of 40 to 50 ml. The tube was shaken mechanically for fifteen minutes. The liquid was allowed to drain slowly from the extraction tube through an ion exchange column of Dowex® 50-X<sub>2</sub> (H<sup>+</sup>), 1 cm. diameter and 25 cm. high. The resin in the extraction tube was washed with about 100 ml. of cold water and the washing allowed to pass through the ion exchange column. The extraction tube and the column were washed separately with 50 ml. of 80% ethanol followed by distilled water and eluted as follows:

The extraction tube was eluted with 4 N ammonia, and the eluate evaporated to dryness

\* United Nations' Secretariat, Document ST/SOA.K/109 に発表

under reduced pressure at a temperature not exceeding 50°. A rotating vacuum evaporator was used for this purpose.

The ion exchange column was eluted with 2*N* ammonia until the effluent became basic. Elution was then continued with about 150 ml. of water. This eluate was combined with the residue from the previously evaporated eluate and concentrated to a small volume under reduced pressure and at a temperature below 50°. The alkaloids were removed by repeated extraction with a mixture of chloroform and isopropyl alcohol (3:1). The combined extracts were washed twice with 10-ml. portions of distilled water, the washings being combined with the aqueous phase and evaporated to dryness under reduced pressure. The dried sample was stored in a desiccator over concentrated sulfuric acid overnight in order to remove free ammonia. A known mixture of eighteen amino acids found in opium was subjected to the treatment described above for opium. The product obtained after ion exchange treatment had the same leucine equivalent as the same amounts of amino acids evaporated to dryness in the presence of ammonia.

When opium was extracted with and without addition of known amino acids, the recovery of added amino acids was likewise quantitative.

**Determination of Amino Acids...**The amino acids were analyzed essentially by the method described by Moore and Stein (3). The dried sample of amino acids from opium was dissolved in 5 ml. of pH 2.2 citrate buffer, the solution was centrifuged, and 2 ml. of the clear supernatant liquid applied to the ion exchange column.

Fractions of 1 ml. each were collected in test tubes by means of a fraction collector actuated by a drop counter. The fractions were analyzed by means of a ninhydrin reagent as described by Rosen (6). To the test tubes containing the eluate fractions were added 0.5 ml. of a cyanide-acetate buffer and 0.5 ml. of ninhydrin solution, and the mixture was heated in a test-tube heating unit at 120°. After eight minutes, the tubes were removed from the heat, 5 ml. of isopropanol and water (1:1) was added and the mixture shaken vigorously. The solution was allowed to cool to room temperature and the absorbance read in a Bausch and Lomb colorimeter at 570 m $\mu$  (440 m $\mu$  for proline). Water was used as a blank. A good grade of ninhydrin is necessary for best results.

Table I. --Recovery test of synthetic mixture of amino acids.

Amino Acids	Recovery % <sup>a</sup>	Amino Acids	Recovery % <sup>a</sup>
Alanine	98.5 ± 2	Lysine	98.8 ± 3
$\beta$ -Alanine	96.6 ± 3	Methionine	82.2 ± 5
$\gamma$ -Aminobutyric acid	96.4 ± 2	Phenylalanine	100.0 ± 3
Arginine	99.0 ± 3	Phenylalanine + $\gamma$ -aminobutyric acid	98.5 ± 3
Aspartic acid	99.1 ± 3	Pipecolic acid	100.0 ± 2
Glutamic acid	100.2 ± 3	Proline	101.5 ± 1
Glycine	96.6 ± 2	Serine	98.4 ± 3
Homoserine	78.0 ± 5	Threonine	95.5 ± 3
Histidine	98.6 ± 3	Tyrosine	95.9 ± 2
Isoleucine	99.9 ± 1	Valine	96.2 ± 3
Leucine	102.0 ± 2	Ammonia	97.2 ± 2

<sup>a</sup>Mean values based on four runs.

The readings were expressed in terms of leucine equivalents by means of a standard curve prepared for leucine. A separate standard curve was needed for proline which with ninhydrin gives a yellow instead of the usual blue-violet color.

In checking the separation and recovery of amino acids in the chromatographic procedure, a synthetic test mixture of amino acids corresponding to an opium extract was used. A sample of solution containing 0.1 to 0.2 mg. of each amino acid and of ammonium bicarbonate in 1 ml. buffer solution (pH 2.2) was chromatographed as described above. The values obtained are given in Table I. The accuracy as well as the precision of the method are good. Of twenty amino acids in the test mixture, only homoserine and methionine showed recoveries of less than 95%. These results agree well with the recoveries reported by Moore and Stein (3) and by Bender et al. (7).

In the calculation of the content of amino acids in opium, the results of the individual acids are corrected on the basis of the recoveries listed in Table I.

## RESULTS AND DISCUSSION

The amino acid composition in the authenticated opium samples analyzed are recorded in Table II. The total amino acid content was found to vary over a wide range from about 0.77 to about 6.6%, with most samples falling between 1.2 and 2.1%. There is also a considerable variation in the relative composition of amino acids in different opium samples. In addition to the amino acids identified by paper chromatography in our preliminary report, we can now report the presence of leucine, pipercolic acid and lysine. Leucine is not present in all samples, at least not to the extent that it is detected by the present method. Proline is observed by the yellow color produced in the fractions where it is known to be eluted, but the color is too weak to permit a quantitative estimation. Like leucine,  $\beta$ -alanine, homoserine, glycine, lysine and tyrosine could not be found in every sample analyzed. In our experiments,  $\gamma$ -aminobutyric acid, which had previously been identified by paper chromatography (1), was eluted together with phenylalanine.

The eluate fractions corresponding to ammonia gave results which could not readily be related to the amounts of acidic amino acids. The reason for this is not yet quite clear. It would seem, however, that free ammonia was not completely removed by storage in desiccator over sulfuric acid.

Although the number of samples from each country is too small to permit definite conclusions, it would appear that the amino acid composition might be a suitable basis for origin determination. Figure 1 illustrates one of the many different types of scatter diagrams that may be drawn to indicate origin. From this, one can clearly differentiate between opium from Japan, Madhya Bharat and Yugoslavia, Less distinct, but still reasonably good separation is observed for samples from Turkey and Iran.

Elution diagrams may also be used as a means of identification. Thus, Fig. 2, 3, 4 and 5, representing Iran and Turkey, U.S.S.R. and India (Uttar Pradesh),

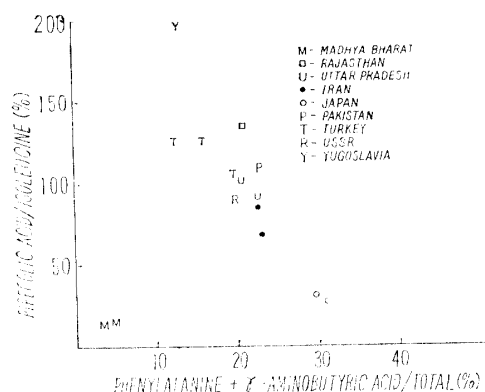


Fig. 1—Scatter diagram based on molar ratios of amino acids in opium.

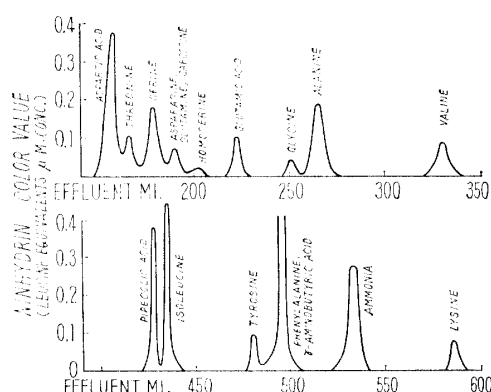


Fig. 2—Elution diagram of amino acids in opium from Iran (UNE 506)

Table II.—Amino Acid composition of opium

Amino Acid (mg./100 g.)	INCIA					IRAN		JAPAN	
	UN 174 Madhya Bharat	UNE 625 Madhya Bharat	UNE 605 Uttar Pradesh	UNE 609 Uttar Pradesh	UNE 661 Rajas- than	UN 52 Neha- rand	UNE 506 Arak	UNE 527 Aichi	UNE 530 Hiro shima
Alanine	52.9	50.9	78.1	61.4	62.1	200.4	152.8	41.7	76.5
$\epsilon$ -Alanine	3.0	69.0	—	—	49.6	—	—	—	31.9
Asparagine, Glutamine, Sarcosine	212.4	458.1	159.6	191.3	166.5	—	60.5	266.4	215.9
Aspartic Acid	99.9	2901.1	320.9	326.9	144.3	402.6	380.4	177.6	130.4
Glutamic Acid	90.8	46.1	170.7	221.1	202.6	221.8	80.9	90.8	67.4
Glycine	11.7	46.4	11.3	11.7	8.0	24.1	19.5	—	6.8
Homoserine	223.7	216.7	—	—	—	—	15.8	—	53.2
Isoleucine	454.0	85.0	125.2	147.0	90.6	178.0	198.0	27.5	15.9
Leucine	—	62.4	10.9	25.4	—	—	—	—	—
Lysine	28.9	31.2	56.4	—	31.6	27.9	76.4	—	30.8
Phenylalanine, $\gamma$ -Aminebutyric Acid	73.3	401.0	452.8	445.7	373.6	585.8	620.3	449.7	521.8
Pipecolic Acid	66.0	15.7	124.0	132.0	120.0	146.2	131.0	83.7	45.9
Serine	102.1	177.8	99.1	52.4	77.7	89.8	154.7	83.9	85.3
Threonine	44.9	1832.4	64.4	42.0	51.8	74.7	91.0	23.1	22.6
Tyrosine	61.5	152.5	48.4	49.3	16.6	78.6	97.9	42.5	76.2
Valine	45.4	102.8	80.2	93.6	65.2	66.1	90.6	65.5	Fraction lost
Total, mg./100 g.	1570.5	6649.1	1802.0	1799.8	1460.2	2096.0	2169.8	1352.4	1380.6

respectively, show many points of difference, qualitatively as well as quantitatively.

### SUMMARY

The amino acids in opium have been determined quantitatively by an ion exchange chromatographic procedure. The amino acid composition is subject to regional variation. Attempts have been made to use the amino acids in opium as

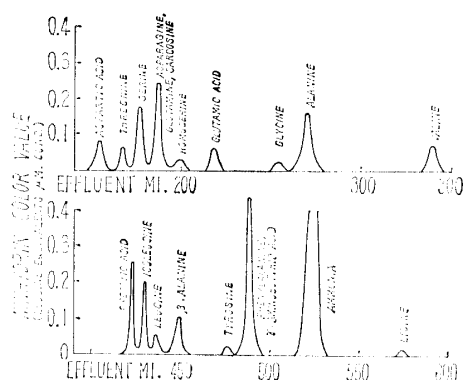


Fig. 3—Elution diagram of amino acids in opium from Turkey (UNE 690)

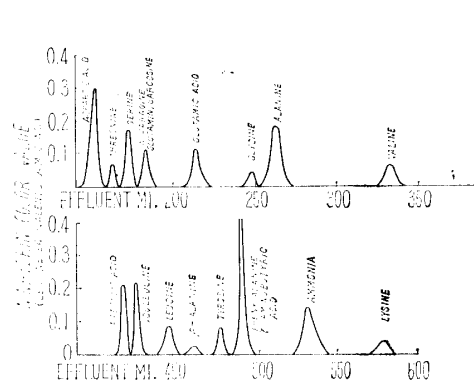


Fig. 4—Elution diagram of amino acids in opium from U.S.S.R. (UNE 701)

Table II. (continued)

Amino Acid (mg./100 g.)	PAKI STAN	TURKEY			U.S.S.R.	YUGO SLAVIA
	UN 100 Lahore	UN 15	UN136D Zile	UNE 690 Acipauam	UNE 701 Kirghiz S.S.R.	UN 38 J
Alanine	82.5	31.9	63.4	105.8	141.1	74.6
β-Alanine	—	63.9	44.1	40.6	14.1	11.3
Asparagine, Glutamine, Sarcosine	34.9	—	—	182.6	100.5	—
Aspartic Acid	348.1	190.7	315.8	62.5	243.2	369.3
Glutamic Acid	38.7	58.7	116.3	50.0	114.0	76.3
Glycine	14.7	3.0	10.0	10.9	17.3	4.7
Homoserine	144.2	—	—	30.0	—	—
Isoleucine	119.0	66.5	89.2	85.5	125.9	83.9
Leucine	30.8	—	57.6	37.5	102.8	37.2
Lysine	47.7	35.5	33.2	16.9	50.3	54.1
Phenylalanine, γ-Aminobutyric Acid	461.9	159.4	215.2	322.3	451.8	214.2
Pipecolic Acid	129.0	82.5	111.8	89.6	111.3	156.0
Serine	51.6	24.4	78.2	100.5	111.3	11.8
Threonine	40.3	15.6	125.9	40.8	49.7	64.7
Tyrosine	66.7	—	17.2	24.0	93.3	16.6
Valine	76.9	42.3	75.7	59.5	84.7	76.4
Total, mg./100 g.	1687.0	774.4	1353.6	1259.0	1811.3	1251.1

a basis for origin determination.

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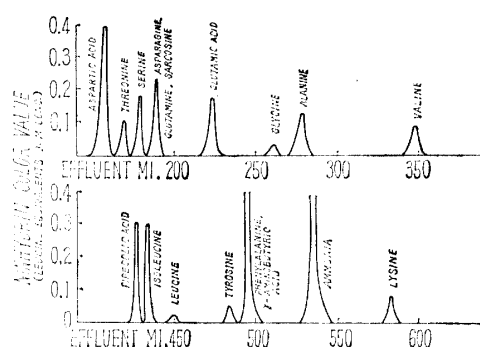


Fig. 5-Elution diagram of amino acids in opium from India (Uttar Pradesh-UNE 605)

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## DETERMINATION OF THE ORIGIN OF OPIUM BASED ON ITS CONTENT OF MECONIC ACID, SULFURIC ACID AND TOTAL ACID\*

Sadaichi MIYAMOTO and Einar Brochmann-Hanssen

Meconic acid is an acid characteristic of opium, and the same perhaps also may be said of sulfuric acid. Together, these two acids account for about 60 to 80 per cent of the total acid content. The meconic acid content of opium is subject to considerable variation, and use is made of this fact in two of the most promising methods developed so far for the determination of the origin of opium (1, 2). It would seem reasonable to expect the content of sulfate and of total acid to be subject to the same regional variations as other constituents of opium.

Rapid and reproducible methods have been developed for quantitative determination of meconic acid, sulfuric acid and total acid in the same sample of opium. It should be noted, however, that the "total acid," as obtained in the present procedure, does not include all of the amino acids, which to a great extent are adsorbed on the ion exchange resin (3),

### EXPERIMENTAL

A total of 104 authentic opium samples were studied, representing 12 different countries. Included in this number are 45 samples from five opium-producing provinces in India.

#### Determination of Meconic Acid

Methods for quantitative determination of meconic acid have been described by Witte (4),

\* United Nations' Secretariat, Document ST/SOA/SER-K/106 に発表