A SIMPLE METHOD FOR DESALTING BIOLOGICAL FLUIDS FOR CHROMATOGRAPHY

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Received October 30, 1954

Summary

A simplified procedure for desalting biological fluids, the amino acid composition of which is to be determined by chromatographic technique, has been developed. Recovery experiments carried out have given results which are within the usual experimental error. The chromatograms from desalted samples are by far better than the ones obtained without desalting. The method has been compared with the electrolytic desalting procedure.

The advent of paper chromatographic technique has made it possible to carry out the amino acid analysis of protein hydrolysates, biological fluids and similar other materials with comparative ease and rapidity. Microbiological assays are time-consuming and require fairly rigorous conditions, besides the costly amino acids involved in the preparation of the media. Chemical methods also require a great deal of time and, for routine work involving estimation of the amino acid contents of a number of samples everyday, these methods could not be considered suitable from the practical standpoint. Chromatographic procedures and particularly the circular paper chromatographic technique developed in this laboratory could be used for both qualitative and quantitative estimation and a large number of samples could be analysed in the course of a day (Giri, Radhakrishnan and Vaidyanathan, 1952; Giri and Rao, 1952 *a* and *b*).

While carrying out the estimation of amino acids excreted in urine under a variety of conditions by the circular paper chromatographic technique, it was found that in some cases the amino acid bands did not separate in a well-defined manner. This was due to the salt and other interfering substances present in the urine samples.

Consden, Gordon and Martin (1947) devised a simple apparatus for desalting solutions to be chromatographed. The procedure consisted in electrolysing the fluid between a constantly renewable surface of mercury as cathode and a dilute solution of sulphuric acid in a cellophane bag as anode. In carrying out quantitative chromatography of silk hydrolysates containing salts, Polson (1947) obtained satisfactory results by applying the hydrolysates in a series of dilutions as drops and by setting up composite standards in which the known amino acid mixture was also applied at a series of dilutions. The amount of amino acid in the unknown was determined by finding spots of equal intensity and area on the

standard. Berry and Cain (1949) suggested the superposition of a known amount of amino acid to a spot to which urine had been added. This reproduced the same conditions of migration in the presence of the constituents of the particular urine for the known amount of amino acids as for the unknown amount of amino acids in the sample. This procedure was found to be simpler than desalting employing the desalting apparatus developed by Consden *et al.* (*loc. cit.*). In the case of sugars which are slow in movement, a persistent interference between salt and sugars causing a continuous elongation of the spots was observed by Baar and Bull (1953). They suggested that sugar chromatograms which it might be impracticable to desalt should be run in a neutral medium such as N-propanal-ethyl acetate-water mixtures. Removal of the urea by using urease was found to be a feasible way of getting rid of urea from the sample. Some ion-exchange resins were also used for desalting fluids intended for chromatographic analysis (Phillips and Pollard, 1953). In this communication, a simple method of desalting is described. It consists in evaporating the biological fluid nearly to dryness and extracting with acidified 95% alcohol whereby the amino acids are extracted leaving the inorganic salts behind.

PROCEDURE

The required amount of urine, hydrolysed or unhydrolysed, was evaporated at reduced pressure and at a low temperature. To the residue 1 or 2 ml. of alcohol (95% with 0·5% v/v HCl) was added and stirred. The alcohol was allowed to stand with the residue for 30 minutes during which time the mixture was stirred a few times. The clear supernatant was filtered and the residue was washed three or four times using 2 ml. of alcohol each time. The strength of the alcohol should be maintained at 95% concentration as otherwise salt was dissolved and carried into the filtrate. In order to prevent the evaporation of the alcohol thereby weakening the concentration of the alcohol, the dish containing the residue and alcohol was kept in a bell jar in which was also kept a dish containing alcohol by which the atmosphere in the bell jar was saturated with vapours of alcohol. This prevented the evaporation of the extracting alcohol.

The filtrate was evaporated as before and was taken up in a small volume of 0·5% v/v hydrochloric acid. This facilitated the solution of glutamic acid and cystine. This solution was used for spotting on the circular paper.

Recovery experiments were carried out by adding known amounts of amino acids to urine and desalting by the procedure outlined above. Recoveries of amino acids added to 1% solution of sodium chloride were also made. The procedure was found to give satisfactory results, the recoveries being of the order of 100 ± 10%. A typical set of results are presented in Tables I and II. In Table III are given the results obtained by adopting the same circular paper chromatographic technique, the desalting being carried out by the method of Consden *et al.* (*loc. cit.*), using Shandon's desalting apparatus.

TABLE I

Recovery of Added Amino Acids from Urine by the Alcohol Desalting Procedure

No.	Amino acids	Photoelectric colorimeter reading				Recovery %
		Added	Urinary	Reading for added +Urinary	Recovered	
1	Leucine ..	75	40	117	77	102·7
2	Methionine ..	57	54	112	58	101·7
3	Serine ..	59	200	260	60	101·7
4	Arginine ..	29	28	58	30	103·4
5	Cystine ..	26	16	43	27	103·7
6	Phenylalanine ..	45	..	44	44	97·8
7	Glutamic acid ..	68	104	171	67	98·5
8	Glycine ..	52	200	254	54	103·8
9	Histidine ..	42	75	116	41	97·6
10	Valine ..	103	54	158	104	100·9
11	Threonine ..	50	104	155	51	102·0
12	Aspartic acid ..	52	200	256	56	107·6
13	Lysine ..	30	75	104	29	97·6

Amino acids 1 to 5, 6 to 9, and 10 to 13 were taken up as separate solutions containing the different concentrations of amino acids as shown in the table. These were then added to urine samples and desalted by the alcohol method and chromatographed. Figures in column 5 constitute the exact photoelectric colorimeter reading from which the corresponding value for urinary content was deducted to work out the recovery.

In the cases of amino acids serine, glycine and aspartic acid, the values given in column 4 represent the values of the combined band as these always appear together in the chromatogram. The same is the case with methionine-valine, glutamic acid-threonine and lysine-histidine.

TABLE II. *Recovery of Added Amino Acids from Sodium Chloride Solution by the Alcohol Desalting Procedure*

No.	Amino acids	Photoelectric colorimeter reading		Recovery %
		Added	Reading after desalting	
1	Leucine	43	42	98·0
2	Methionine ..	25	24	96·0
3	Serine	55	54	98·0
4	Arginine	37	36	97·0
5	Cystine	9	9	100·0
6	Phenylalanine ..	18	18	100·0
7	Glutamic acid ..	36	35	97·0
8	Glycine	36	36	100·0
9	Histidine	18	18	100·0
10	Valine	33	33	100·0
11	Threonine ..	34	35	100·0
12	Aspartic acid ..	17	18	102·6
13	Lysine	30	30	105·0
				100·0

TABLE III. *Recovery of Added Amino Acids from Sodium Chloride Solution by the Electrolytic Desalting Procedure*

No.	Amino acids	Photoelectric colorimeter reading		Recovery %
		Added	Reading after desalting	
1	Leucine	43	42	98·0
2	Methionine ..	25	24	96·0
3	Serine	55	32	58·0
4	Arginine	37	17	46·0
5	Cystine	9	9	100·0
6	Phenylalanine ..	18	17	94·4
7	Glutamic acid ..	36	35	97·0
8	Glycine	36	35	97·0
9	Histidine	18	16	89·0
10	Valine	33	34	103·0
11	Threonine ..	34	30	88·0
12	Aspartic acid ..	17	18	105·0
13	Lysine	30	16	53·0

DISCUSSION

The desalting procedure outlined by Consden *et al.* (*loc. cit.*) was not found to be quite satisfactory. Recoveries of certain amino acids, such as arginine, lysine and histidine, were rather poor. Stein and Moore (1951) reported large losses of arginine and slight losses of some other amino acids during the electrolytic desalting procedure of Consden *et al.* (*loc. cit.*). The low recovery of arginine was found to be due to the conversion of a major part of it to ornithine during the process of desalting.

Results presented in Table III also show low recovery (46%) of arginine. Recovery of lysine is also poor (53%). A comparison of the data obtained by the desalting procedure reported in this communication with the electrolytic desalting procedure of Consden *et al.* (*loc. cit.*) would show that the former procedure has yielded better results. That the alcohol desalting procedure gives very clear chromatograms and that in the electrolytic method, destruction of certain amino acids takes place could be seen from Fig. 1. The method as may be seen is quite simple and does not involve costly equipment and could be adopted in any laboratory.

It was found that copper could not be removed by this method. It formed an intense red band in the ninhydrin stained chromatogram just above the glutamic acid-threonine band. By cutting out this band and ashing the paper, the red band was identified as copper by the usual reaction with rubianic acid.

It was, however, found that the presence of copper did not interfere with the development of the chromatogram and identification of the different bands. Perhaps this is due to the fact that copper salts also migrate and occupy a specific position on the paper.

REFERENCES

1. Baar, S. and Bull, J. P. .. *Nature*, 1953, **172**, 414.
2. Berry, H. K. and Cain, L. .. *Arch. Biochem.*, 1949, **24**, 179.
3. Consden, R., Gordon, A. H. and Martin, A. J. P. *Biochem. J.*, 1947, **41**, 590.
4. Giri, K. V., Radhakrishanan, A. N. and Vaidyanathan, C. S. *Anal. Chem.*, 1952, **24**, 1677.
5. ——— and Rao, N. A. N. .. *Nature*, 1952 *a*, **169**, 923.
6. ——— ——— .. *J. Ind. Inst. Sci.*, 1952 *b*, **34**, 95.
7. Phillips, J. D. and Pollard, A. .. *Nature*, 1953, **171**, 41.
8. Polson, A. .. *Science*, 1947, **105**, 603.
9. Stein, H. and Moore, S. .. *J. Biol. Chem.*, 1951, **190**, 103.

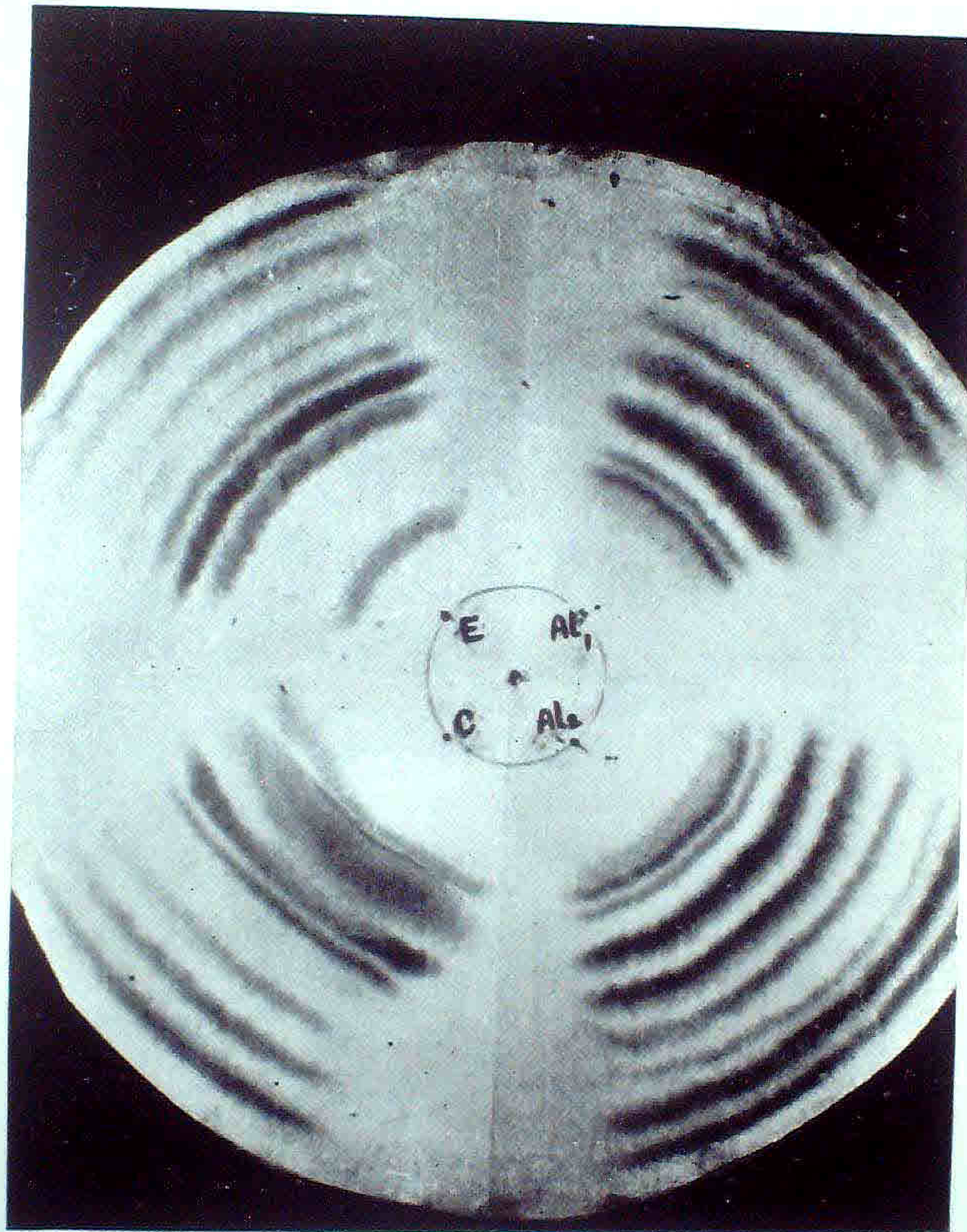

FIG. 1

Comparative study of electrolytic and alcoholic de-salting methods

C—Casein hydrolysed with HCl and neutralised with NaOH.

E—Above hydrolysate after electrolytic desalting.

Al—Above hydrolysate after alcoholic de-salting.

Al_1 and Al_2—represent two different concentrations.