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Chromatography in Aqueous Solution with Mineral Precipitates Insoluble in Water

1. APPLICATION TO THE SEPARATION OF AMINO-ACIDS BY ADSORPTION ON Ag_2S

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(Received 8 September 1945)

The application of chromatography in aqueous solution has not yet been fully explored, owing to the novelty of the technique and to the empirical character of the work to which it has given rise. Experimental considerations have so far limited its use to a fairly miscellaneous choice of adsorbents, the composition and chemical properties of most of which are not well known (silicates, activated carbons, synthetic resins, etc.). The only exception is alumina. Silica gel, which also is a chemically well-defined adsorbent, in most cases functions only as a support (Martin & Synge, 1941). The time has now come to free this technique from its present empiricism and to define its sphere of application. We hope that this work will be a contribution in this direction.

At first sight, the utilization of the mineral precipitates commonly available in the laboratory, in chromatography, does not seem to be of particular interest. Alumina appears to constitute a special case; its adsorbent properties with respect to cations, and perhaps to anions, are due to the impurities which it contains. The impurities can accentuate corrosion during activation of the adsorbent by an acid and thus considerably increase the surface. The fact that the use of the mineral precipitates has so far been systematically avoided is equally significant. According to Schwab (Böttger, 1939), they are not suitable, on account of their lack of selectivity and of their too weak adsorbing power.

To assess the interest of the question, however, it suffices to compare the adsorbent properties of the usual adsorbents and of mineral precipitates. The latter, prepared in presence of an excess of one

of the ions of the lattice, show a heterogeneous surface structure entirely similar to that of an 'acid' column of alumina (Fajans & von Beckerath, 1921; Kolthoff, 1934); their adsorbent properties are very well known, thanks to studies in adsorption (Fajans, 1941; Fajans & Erdey-Gruz, 1931; Kolthoff & MacNevin, 1936; Kolthoff & Rosenblum, 1933 *a, b*; Kolthoff, Von Fischer & Rosenblum, 1934), in analytical chemistry (Fajans & Wolff, 1924; Burnstein, 1927; Kolthoff & Lingane, 1936), in colloidal chemistry (Verwey, 1933; Verwey & Kruyt, 1934), to studies utilizing radio-active elements (Hahn, 1926, 1930; Paneth, 1914), and studies on photographic sensitivity (Sheppard, Lambert & Walker, 1939; Trillat & Aubry, 1941; Trillat & Merigoux, 1941). It must be remembered that the determining factor of the adsorption is the insolubility of the adsorption compound which the adsorbed ion forms with an ion of the opposite sign, which is part of the lattice or is itself adsorbed on it (Fajans & von Beckerath, 1921). After the precipitate is washed, the amount of excess ion of the lattice adsorbed is very slight, even in the case of substances which are sparingly soluble; the adsorption phenomena to which they give rise are only observable with solutions of radio-active elements (Kolthoff, 1934, 1936). We shall show, nevertheless, that their adsorptive power is still sufficient to allow of their utilization in chromatography in aqueous solutions.

We have attempted to separate mixtures of natural amino-acids by chromatography on a precipitate of silver sulphide.* Various chromato-

* A preliminary note on this subject has already been published (Hamoir, 1943).

graphic methods discovered in the last few years for separating natural amino-acids have already made possible the isolation of the basic acids (Turba, 1941; Wieland, 1942 *a, b*; Freudenberg, Walch & Molter, 1942), the dicarboxylic acids (Turba & Richter, 1942; Wieland, 1942 *a, b*; Wieland & Wirth, 1943), and the mono-amino monocarboxylic acids (Karrer, Keller & Szönyi, 1943; Schramm & Primosigh, 1943; Turba, Richter & Kuchar, 1943; Wieland, 1942 *c*; Gordon, Martin & Synge, 1943, 1944). But certain separations are still in need of improvement; the methods are not always sufficiently reliable (separation of the dicarboxylic acids), and sometimes their accuracy leaves something to be desired. We have chosen as adsorbent a flocculate of Ag_2S , because of its very great insolubility (which results in the formation of a precipitate with a large surface and implies a high adsorption energy of the ions of the lattice), and because nearly half the natural amino-acids form insoluble silver salts which are relatively well characterized.

METHODS

Preparation of the adsorbent and of the adsorption columns. The sulphide precipitate is prepared by passing an excess of H_2S into 2 l. of a solution of silver nitrate or sulphate (equivalent to 12.5 g. of sulphide), and then leaving for several hours in the presence of the saturated solution of H_2S . The excess H_2S is then completely removed, and the precipitate carefully pulverized in a mortar. Thereafter, CO_2 -free water is used. The precipitate is left for about 3 hr. in the presence of a dilute solution of 1–2% silver nitrate, then washed till the reaction with NaCl is perfectly negative. This is the activation process.

For preparing the adsorption columns, tubes with a sintered glass bottom (Allihn, Schott & Gen., Jena) are used, permeability 2, 3 or 4, into which the precipitate is packed by suction (Fig. 1). The porous plate should be very clean; after a certain time it should be washed with a concentrated solution of KCN , even if the permeability does not seem to be lessened. The columns obtained are completely homogeneous in spite of the size of the grains, and whatever the height (generally 2–3 cm., sometimes 10 or more). The method of operation is simple, rapid and easy.

Methods used for quantitative determination. The adsorption experiments have been followed by means of certain very sensitive colour reactions performed on the filtrate after concentration *in vacuo*, and by Van Slyke's micro-method for determining amino-nitrogen (Van Slyke, 1929). We have not been able to reproduce the precision of 0.5 μg .

obtained by this author; we have observed slight variations in the blanks and in determinations of 1.5–3 μg . As the quantities to be determined in the course of our separation tests were only about 50 μg ., the errors amount to a few per cent, but the method is sufficiently accurate to establish the quantitative character of the technique.

Attention should be drawn to certain causes of error: (a) the presence of moulds affects the determination adversely, their reducing power causing an additional liberation of nitrogen; (b) phenolic derivatives, such as thymol form, under Van Slyke's conditions, a *p*-nitroso derivative, then an unstable diazo derivative; (c) an aqueous solution of acetic acid concentrated under reduced pressure at 50° in the presence of traces of a heavy metal (Mn) decomposes, forming compounds which liberate traces of nitrogen or other inert gas in the Van Slyke amino-N determination.

RESULTS

Properties of the adsorbent

A flocculate of silver sulphide adsorbs irreversibly considerable amounts of silver ions which in turn adsorb those anions forming sparingly soluble silver salts. In practice, a column of 12.5 g. of sulphide does not retain histidine, but after activation, it adsorbs quantities of the order of 1 mg. Any sparingly soluble precipitate activated by adsorption of one of the ions of the lattice can, therefore, judging from the behaviour of silver sulphide, be used for chromatography in aqueous solution. The ratio of the quantity adsorbed to the weight of adsorbent for Ag_2S (1/10,000) places these substances among the adsorbents which are relatively only slightly active; for the usual adsorbents, the ratio is 1/1000. On the other hand, the adsorbing power both of different preparations, and of the same preparation at different times, is very constant; no change in the quantity of histidine fixed is observed by modifying the temperature of preparation (by working at 0°), the speed of precipitation (by accelerating the passage of H_2S or by mixing quickly a solution of sodium sulphide and silver nitrate), and the conditions of precipitation (by using or not using mechanical agitation). Further, the same adsorption column can be used for more than a month.

In an attempt to increase the adsorbing power of the sulphide, several factors have been studied.

(a) *Ageing of the precipitate.* If the precipitate is washed and activated immediately after it is prepared, the adsorbing power is approximately doubled, but does not show the same selectivity. It becomes impossible to redissolve certain amino-acids which are not precipitated by silver in aqueous solution, e.g. leucine. As far as we are aware, this is the first time that the phenomenon of the ageing of silver sulphide has been pointed out.

(b) *Optimum time of activation.* 12.5 g. of sulphide, activated for a few minutes only, retain

Fig. 1. Diagram of adsorption column.

0.8–0.9 mg. of histidine; after 3 hr. 1.38 mg., and after 16 hr. 1.43 mg. Thus, the activation is practically complete after 3 hr. This rather long time may be due to diffusion of traces of H_2S occluded, or to a slow displacement of the silver ions adsorbed towards sites which are less accessible.

(c) *Modification of the state of aggregation.* No change of adsorbing power is obtained by grinding the previously dried precipitate (temp. $\leq 60^\circ$) in an agate mortar till it passes through a sieve of 20μ . Much more extensive pulverization is obtained by flattening between two polished glass plates, without previous desiccation, yet the activity is only doubled and the same diminution of selectivity is observed as in the case of the unaged precipitate.

(d) *Modification of the state of the surface.* It has been possible to double the adsorbing power without influencing the selectivity, merely by activating the Ag_2S with 0.1M- HNO_3 for several hours, and then washing with 0.1M-acetic acid. Negative groupings S_nO_m form on the surface, giving rise to new active centres after displacement by the acetate ions. The corrosive action of the nitric acid increases the heterogeneity of the surface. The question of augmenting the adsorbent power of mineral precipitates still has very numerous experimental possibilities and requires further study.

Behaviour of the different natural amino-acids

The adsorption of individual amino-acids has been studied, and where it occurs, the quantity adsorbed by 12.5 g. of sulphide and the stability of the adsorption compound in a more or less acid

A few mg. of amino-acid are filtered through the column, and the excess eliminated by washing with 200 ml. of boiled distilled water (three portions, 0–100, 100–150, 150–200 ml.). Then, according to circumstances, a more or less acid solution is filtered through (0.001M-acetate buffer of pH 5, 0.1M or M-acetic acid) and the speed of elution determined by tests on successive portions of the filtrate.

In general, an excellent parallel has been observed between the adsorbability and the corresponding insolubility of the silver salt. Methionine is the only exception; although its silver salt is very soluble it is very strongly adsorbed to the extent of 0.5–1 mg. The stability differences of the adsorption compounds as a function of pH allow us to classify the natural amino-acids in four groups:

Group 4. This comprises the amino-acids not adsorbed at pH 6–7. They are glycine, alanine, serine, valine, threonine, leucine, proline, hydroxyproline, lysine and arginine. The results obtained with leucine proper (amino-*iso*-caproic acid) are probably equally valid in the case of *isoleucine* and *norleucine*. 100 ml. water are sufficient to wash these acids into the filtrate, except in the case of leucine and of lysine, for which in practice 150 ml. are necessary for complete elimination.

Group 3. This consists of the acids adsorbed at pH 6, with easy elution; they are, in order of decreasing adsorbability, glutamic and aspartic acids, tryptophan, histidine, phenylalanine and tyrosine. The amounts of phenylalanine and tyrosine adsorbed are distinctly less than those of the other amino-acids (0.4 and 0.3 mg. respectively). Different

Fig. 2. Rate of elution of different amino-acids adsorbed on 12.5 g. Ag_2S by 0.001 M-acetate at pH 5, (a) from a column packed with the help of a pestle, (b) packed by suction.

medium have been ascertained. Threonine and hydroxyproline are the only acids not studied. It is supposed that their behaviour is identical with that of the corresponding non-hydroxylated amino-acids because of their chemical relationship, and because the silver salts of these two acids have not been described and therefore very probably are soluble.

factors can influence these adsorption phenomena: (a) The pH of the medium. Fig. 2, showing the elution speeds of these acids with a solution of 0.001M-acetate buffer at pH 5, indicates that it should be possible to separate phenylalanine and tyrosine from the other amino-acids in this way. The difference in behaviour of histidine in the two

elution curves is due to the greater homogeneity of the column packed by suction. The reversal of the elution speeds shown in (b) is probably due to the buffering power of the histidine. (b) The presence of salts in solution. Any salt containing an anion whose silver salt is not very soluble is capable of modifying the adsorption equilibrium of the acids, to act, that is, as an eluting agent. The same column saturated with tyrosine, histidine and tryptophan was washed with 0.001M solutions of Na acetate and Na_2SO_4 . The elution of the tyrosine is complete after washing with 150 ml. of sodium sulphate, whilst 75 % of tryptophan and 25 % of histidine pass into the filtrate. The elution of the tyrosine is also complete after washing with 200 ml. of 0.001M-sodium acetate. The reversal of the order of stability observed for histidine and tryptophan is due to the fact that the silver salt of the imidazole group is much more stable to SO_4 ions than to H ions. (c) The nature of the displaced ion. The stability of the adsorption compound of tyrosine is so slight that the acid passes into solution in the first 100 ml. of washing water, even when the amount involved is only 0.1 mg. To make the retention quantitative, it is enough to treat the column with 0.01M solutions of soda and then of nitric acid, so that the NO_3 ions are substituted for the acetate ions retained electrostatically by the silver ions adsorbed (the value of 0.3 mg. given above relates to a 'nitric' column, all the others to 'acetic' columns). This change does not modify the behaviour of the column with respect to the acids of group 4.

Group 2. Methionine, which constitutes this group, differs from the acids of the previous group by the distinctly greater stability of its adsorption compound at acid pH. The elution of glutamic acid is complete after washing with 50 ml. of 0.1M-acetic acid. Table 1, on the other hand, shows the result of filtration of 0.4 mg. of methionine and washing with 0.1M-acetic acid.

Table 1. *Speed of elution of methionine adsorbed on Ag_2S by 0.1M-acetic acid*

Cut (ml.)	0-40	40-70	70-100	100-150	150-200
Methionine content (% of the amount put on)	0	0	24	25	21

This acid is recovered quantitatively by washing the column with 150 ml. of m-acetic acid. We are concerned here with a different type of adsorption due to the presence in the molecule of a very polarizable atom. It is the first example of a general method for isolating the sulphur-containing and halogenated organic compounds by adsorption in aqueous solution on insoluble sulphides. The carrying down of methionine by a precipitate of Cu_2O (Lucas & Beveridge, 1940; Bailey, Chibnall, Rees

& Williams, 1943) may have the same origin. It is suggested that our results should be compared with those of these authors.

Group 1. There remain cystine and cysteine, which are adsorbed in the presence of m-acetic acid and, so far, have not been recovered by elution. In this case the type of adsorption appears different; the amounts adsorbed are about 3 mg., one part being irreversibly fixed, while the other is slowly eliminated by washing with solutions of 0.001 or 0.01M-acetic acid, for example. If the amount of cystine or cysteine put on is only 1 mg., no amino-N or SH grouping (nitroprusside reaction) appears in solution on washing with N-acetic acid, N- H_2SO_4 or 0.04M-ammonia, in spite of the solubility of their silver salts under these conditions. It does not seem probable that we are here concerned with the type of adsorption shown by methionine. Probably the following soluble complex $(\text{C}_3\text{H}_5\text{NSO}_3\text{Ag})\text{H}$, which is very stable, forms on the surface. The structural formula suggested for this complex is

The precipitate of silver cysteinate may be the silver salt of this acid. This would explain not only our adsorption experiments, but also the behaviour of cystine and cysteine with silver salts in aqueous solution (Vickery & Leavenworth, 1930).

Quantitative separation of mixtures of natural amino-acids

A mixture containing 10 mg. each of ten natural amino-acids, i.e. the acids of group 3, excluding phenylalanine, and the following five acids of group 4: alanine, serine, valine, leucine and arginine, were dissolved in water, the pH adjusted to 6, and the solution diluted to 200 ml. 57.7 % of the amino-N should pass into the filtrate.

If an attempt is made to separate the mixture on an 'acetic' column, tyrosine passes into solution; the filtrate obtained by introducing into the column only the five amino-acids of Group 3 gives a positive diazo reaction, and in the case of the mixture of the ten acids, about 65 % of the amino-N passes into the filtrate. The tyrosine is held in so labile a way that even a considerable diminution of the amount of amino-N fixed (down to 42 % of the amount of histidine adsorbed by the column) does not appreciably improve the retention. A 'nitric' column must be used. The separation is then quantitative, except, obviously, when the limit of saturation of the column is exceeded. Thus the mixture of the five acids of Group 3, corresponding to 0.156 mg. of

amino-N were put on 25 g. of sulphide previously activated by 0.1M-HNO₃; the cuts 0-150, 150-250 and 250-350 ml. gave completely negative diazo reactions and the elution was practically quantitative after washing with 200 ml. of 0.1M-acetic acid. Then, on the same column, the mixture of the ten amino-acids corresponding to (a) 0.27 mg., and (b) to (e) 0.294 mg. of absorbed amino-N were run through. The following results were obtained (Table 2).

Table 2. Quantitative separation of a mixture of ten natural amino-acids by adsorption on Ag₂S

(The quantitative separation corresponds to a passage of 57.7% of amino-N into the filtrate.)

Exp.	Fraction	Filtrate amino-N		Eluate amino-N	
		mg.	% of amino-N put on	mg.	% of amino-N put on
(a)	0-200	0.1540	57.1	0.114	42.2
	200-250	0.0030	1.0	0.004	1.7
	250-300	0.0000	0.0	0.003	1.2
	300-500	0.0000	0.0	—	—
			58.1		45.1
	Total			103.2%	
(b)	0-200	0.1515	56.2	0.111	41.1
(c)	0-200	0.1585	58.7	—	—
(d)	0-200	0.1760	60.0	0.1190	40.5
	200-250	0.0010	0.5	—	—
			60.5		40.5
	Total			101.0%	
(e)	0-200	0.1800	61.0	0.1050	36.0
	Total			97.0%	

The amount put on in Exps. (d) and (e) is higher than the saturation limit; diminishing the amount used again permitted quantitative retention to occur (negative diazo reaction). In practice, the quantity used will be kept below the saturation limit of the column, determined preferably with the acid the least strongly adsorbed; alternatively, the quantitative character of the separation obtained will be controlled by a colour reaction or a second experiment on a new column. It seems unnecessary to give further similar examples of analysis. We confine ourselves only to drawing attention to the fact that the modification of the adsorption by the influence of pH, of salts and of the nature of the displaced ion provides methods of separation with manifold possibilities applicable to any mineral precipitate.

DISCUSSION

Protein hydrolysates can be quantitatively separated by chromatography on precipitated silver sulphide. If the weak adsorbing power of the precipitate makes this technique less convenient in actual fact than other chromatographic methods,

on the other hand it allows separations to be made which are very different from those so far obtained: quantitative isolation of methionine almost directly from a protein hydrolysate, and separation, in the same sample, of amino-acids differing as much as those in Group 3. Because of its originality it can be used for the analysis of proteins and for contributing to the solution of microbiological problems.

But the results just described are of interest from the more general point of view of chromatography in aqueous solution. They show that this technique can be applied to silver sulphide and that this precipitate behaves in an exactly similar way to that of an 'acid' column of alumina. There is the same irreversibility of the adsorption (histidine, etc.), the same influence of the displaced ion and the existence of the same elution phenomena. But silver sulphide differs from the other adsorbents, in that it forms well-defined adsorption compounds whose solubility properties are well known. This permits of predictable separations, and the *a priori* determination of adsorption and elution conditions of the different constituents of a mixture. (The exceptions themselves are easily explained, as we have seen for S-containing amino-acids.) As the adsorbent properties of the sulphide depend only on its insolubility (which determines the formation of a precipitate which has a large surface and implies a high adsorption energy of the ions of the lattice), it can be foreseen that the other mineral precipitates, and particularly those which are least soluble, will behave in the same way. This is completely confirmed by similar laboratory investigations with a precipitate of barium sulphate (Tarte, 1945). This generalization puts at the disposal of investigators a series of new adsorbents which have very numerous possibilities (formation by adsorption of insoluble salts, of complexes, and of addition compounds stable in aqueous solution). Chromatography in aqueous solution should be profoundly modified by giving it a more deductive character. It shows indeed that this technique, which so far is so arbitrary and empirical, should be systematized.

Further, our investigations may permit a better understanding of the phenomena of the adsorption of organic compounds on the surface of mineral precipitates in aqueous solution. In particular they can throw light on the amino-acids that are selectively lost when mineral precipitates are discarded during a protein analysis, and suggest methods for controlling or preventing these losses.

SUMMARY

1. The use of sparingly soluble mineral precipitates, commonly available in the laboratory, as adsorbents for chromatography in aqueous solution, is suggested.

2. A flocculate of silver sulphide, left for several hours in contact with a dilute solution of silver nitrate, acquires considerable and highly selective adsorbent properties.

3. A new quantitative method for separating natural amino-acids is based on these properties. Different types of adsorption are observed which can be regarded as examples of more general phenomena which occur on the surface of mineral precipitates.

4. These investigations indicate new preparatory

and analytical possibilities in the sphere of micro-biochemistry and suggest methods for eliminating the adsorption of organic compounds at the surface of mineral precipitates, a phenomenon which so often invalidates the determinations of these compounds.

Thanks are due to Prof. Dubuisson for the very valuable help he has given in the course of this work, and to Miss M. Horsman (Wool Industries Research Association, Leeds) for her kindness in translating the text.

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An Artificial Standard for Use in the Estimation of Haemoglobin

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(Received 17 August 1945)

Colorimetric procedures for determining haemoglobin have the disadvantage that they must normally be standardized by oxygen capacity or iron determinations on blood, and this process must be repeated at intervals to guard against deterioration of the standard. To avoid the necessity for these

measurements, artificial standard solutions have been proposed for some of the commoner methods of estimating haemoglobin. They have not been widely adopted because they are suitable only for use in visual colorimeters under definite conditions of illumination. The absorption spectra of the