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Paper Chromatographic Analysis of Amino Acids and other Ninhydrin-Reacting Substances in Deproteinized Human Plasma

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In a recent paper the positions on a two-dimensional chromatogram of 14 ninhydrin-reacting substances from protein-free human plasma were determined [1]. 12 of the spots were shown to correspond to the positions of lysine, arginine, aspartic acid, glycine, serine, glutamine, alanine, threonine, proline, valine, tyrosine and probably α-aminobutyric acid. The positions of some other amino acids and ninhydrin-reacting compounds from protein hydrolysates were also demonstrated. Dent [2] has recently published a similar map. A comparison with his map showed that we obtained other positions for some of the amino acids. The probable explanation to the differences seems to be that we use Swedish filter paper sheets and somewhat different organic solvents. In our chromatograms we still missed some amino acids which from microbiological and chemical analysis are known to be present in the protein-free human plasma [3–5]. An attempt was therefore made to identify the positions of some of the missing amino acids.

EXPERIMENTAL

Solvents. With the exception of lutidine the organic solvents were always purified by distillation. The lutidine was a commercial product of Swedish source. Phenol was used as the first solvent in the two-dimensional runs and a mixture of pyridine and technical amyl alcohol in the second direction. Several substitutes for the pyridine-amyl alcohol mixture were tested. Equal parts of tertiary amyl alcohol and lutidine saturated with water gave the same good resolution or perhaps a little better separation of the spots. Mixtures

of tertiary amyl alcohol and pyridine or amyl alcohol and lutidine saturated with water were less successful. Phenol could be replaced by *o*-cresol in the two-dimensional runs but this solvent had no special advantages. Several two-dimensional runs were made with *o*-cresol and ammonia in the first direction and a mixture of benzyl alcohol and butanol in the second direction. These were carried out in the hope of obtaining a satisfactory resolution of the single spot given by leucine, isoleucine and phenylalanine. Acceptable results were not obtained.

In one-dimensional chromatography a satisfactory separation of the leucine and isoleucine spots were obtained either with the lutidine-tertiary amyl alcohol mixture in runs of 50 hours or with the lutidine-amyl alcohol mixture in runs of 60—80 hours. With the last mentioned solvent mixture a spot corresponding to the position and greyish green colour of phenylalanine could be distinguished from the leucine and isoleucine spots when a 0.1 per cent diethylamine solution was present at the bottom of the glass tray. 5, 10 or 50 per cent solutions of diethylamine gave more diffuse spots.

Paper qualities and dimensions. Of the Munktell filter paper sheets no. 0B and 00 the following three sizes 24 × 24 cm, 28 × 37 cm and 48 × 48 cm were compared with regard to resolving capacities. Number 0B is a quick-filtering paper while the fronts of the organic solvents move more slowly in no. 00 (de Verdier and Ågren [1]). In accordance with previous reports (Dent [6], Pratt and Auclair [7]) it was also found that some amino acids showed a tendency to destruction during the development of the chromatograms. From this point of view the quickfiltering paper no. 0B would generally be preferred. It was also found, when the 48 × 48 cm papers of the two qualities were compared, that it was necessary to work with at least 2 ml of protein-free human plasma on the no. 00 papers to obtain the usual 14 spots while 1 ml was a satisfactory amount on the no. 0B papers. Descending chromatography gave a better resolution of the spots than the ascending procedure. With the 24× 24 cm papers the ascending procedure gave better results than the descending method. 0.5 ml of protein-free plasma was a suitable amount to apply on the papers. Larger volumes of filtrates gave diffuse spots. However, in the pyridine-amyl alcohol direction the resolution of the spots were better when the 48 × 48 cm papers were used.

At last the following procedure was adopted which so far has given the best resolution of the spots. No. 0B papers of the dimension 37 × 28 cm are used. With ascending chromatography the phenol front is allowed to proceed along the 37 cm side of the paper as far as to the upper edge. From the dried paper the yellowish brown material deposited by phenol is cut away. The paper with a dimension of 28 × 28 cm is developed in the second direction

using pyridine-amyl alcohol and descending chromatography. The sheets are dried at room temperature overnight in a cabinet combined with the compressed air system. The dried papers are sprayed with a solution of 0.25 per cent ninhydrin in butanol containing 1 per cent of acetic acid. Drying at room temperature, also used by Dent [2], resulted in larger and more coloured spots as compared with some of the procedures previously used [1].

The protein-free filtrates. Detailed comparisons between filtrates obtained by five different deproteinizing methods (*cf.* de Verdier and Ågren [1]) were carried out. The following short comments may be made. Filtrates obtained by dialysis or by precipitation with trichloracetic acid, tungstic acid, ferric hydroxide or ethanol showed the same general pattern of spots on the two-dimensional chromatograms. However, the most clearcut pictures were obtained with the trichloracetic acid, ferric hydroxide and ethanol filtrates and with the dialysates, while the tungstic acid filtrates gave more diffuse spots. Several spots suspected to be caused by peptides were found close to the starting point in the two-dimensional chromatograms of the trichloracetic acid filtrates. Concentrating the investigation at first hand on the free amino acids, ethanol filtrates were used in most of the experiments. It is interesting to note that the ethanol filtrates which always showed lower amino nitrogen values than the other filtrates still gave somewhat larger and more coloured spots. An explanation to this behaviour may possibly be found in the recent find of Martin and Mittelmann [8] that the coloured ninhydrin product consists of several components whose proportions depend upon the conditions under which the colour is developed. The dialysates proved to be of special value for the identification of some amino acids.

The identification of amino acids by the use of colour tests. Pratt and Auclair [7] recently tested the minimum quantities of amino compounds which will give a visible colour with ninhydrin on a two-dimensional chromatogram when viewed by transmitted light. Comparing the listed values with those obtained by chemical and microbiological determinations carried out by Gutman and Alexander [9], Hier and Bergeim [3], Sheffner *et al.*[5] on protein-free plasma some conclusions could be drawn. When 1 ml volumes of protein-free plasma are used it could be assumed that histidine, methionine and tryptophan would not appear on a two-dimensional chromatogram. Weak spots could be expected from arginine and phenylalanine. Accordingly duplicate two-dimensional chromatograms were carried out. — One paper was developed in the usual way with ninhydrin. On the other specific colour reactions for these amino acids were tested.

Histidine. The Pauly diazo reagent was used. The diazotized sulphanilic acid was prepared according to Blatt [10]. Attempts with alcoholic or aceton

solutions of the reagent were unsuccessful. Freshly prepared water solutions (0.1 % of the diazo compound in N $NaCO_3$) had to be used. The amino acid was detectable in amounts of about 10 μg. The bromine reaction carried out according to Woolley and Peterson [11] was less sensitive.

Methionine. The methionine-nitroprusside reaction according to Mc Carthy and Sullivan was carried out in different modification (*cf.* Csonka and Denton [12]) but the sensitivity was to low. The iodoplatinate reaction was used by Consden *et al.* [13] to identify methionine. Recently the reaction was investigated by Winegard and Toennies [14]. According to their figures the reaction is not more susceptible than the ninhydrin reaction. We can corroborate this result.

Tryptophan. The bromine-water reaction [15] was positive only with amounts of 20 μg. The glyoxylic acid reaction according to Shaw and Mc Farlane [16] was tested in the following way. 7 μl of the glyoxylic acid-copper sulphate reagent was applied to the expected position of the amino acid on the paper. The sheet was dried overnight at room temperature and then 7 μ l of 12.5 per cent H_2SO_4 were added. Black spots were obtained after about 4 days. The amino acid was detectable in amounts of about 10 μg. The dimethylaminobenzaldehyde reaction according to Bates [17] was perhaps a little more susceptible and the greenish coloured spots were visible after 48 hours.

Arginine. Sometimes the arginine spot was not clearly separated from the lysine spot. In such cases the presence of arginine had to be verified by use of the Sakaguchi reaction carried out according to Dubnoff [18]. To the expected position of arginine on the cold paper 14 μl of the α-naphtol-urea reagent were added by means of a micro-pipette followed by 7 μl of the NaOBr solution. The amino acid was detectable in amounts of about 5 μg.

Citrulline. 5—10 μg of the amino acid were visible on a two-dimensional chromatogram when the dimethylaminobenzaldehyde reaction (*cf.* Dent [2]) was applied to the paper. The sensitivity of the reaction could be increased by purification of the reagent.

RESULTS

The following routine method is proposed to be used for the detection and identification of a maximum number of amino acids and amino acid derivatives.

1. Munktell papers no. 0B cut to dimensions of 37 × 28 cm are used. Duplicate two-dimensional runs are carried out with phenol and the pyridine amyl alcohol mixture. 1 ml of desalted protein-free plasma (ethanol filtrate) is used. One of the papers is developed with ninhydrin. On the other the presence of

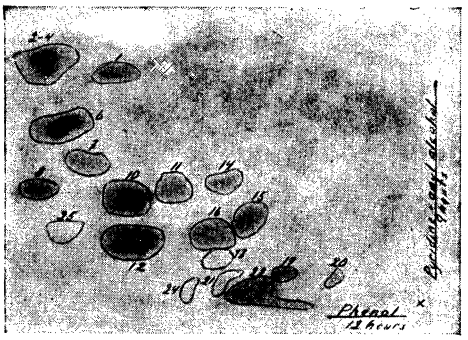

Figure 1. Photograph of a phenol/pyridine-amyl alcohol chromatogram, showing the positions of the amino acids from 1 ml of human plasma filtrate (80 % ethanol precipitation).

1 = Tyrosine	*13 = Citrulline*
2 = Phenylalanine	*14 = Taurine*
3 = Isoleucine	*15 = Serine*
4 = Leucine	*16 = Glycine*
5 = Methionine	*17 = Asparagine*
6 = Valine	*18 = Histidine*
7 = α-Amino-n-butyric acid	*19 = Glutamic acid*
	20 = Aspartic acid
8 = Proline	*21 = Arginine*
9 = Hydroxyproline	*22 = Lysine*
10 = α-Alanine	*23 = Ornithine*
11 = Threonine	*24 = Fast-arginine*
12 = Glutamine	*25 = Peptide*

Figure 2. Photograph of a one-dimensional lutidine-amyl alcohol chromatogram. A = 1 ml of plasma dialysate + 20 μg of tryptophan. B = 1 ml of dialysate + 27 μg of tyrosine. C = 2 × 1 ml of plasma dialysate. D = 1 ml of dialysate + 22 μg of phenylalanine, 6 μg of leucine and 6 μg of isolucine.

26 = Tryptophan	*34 = Ethanolamine-phosphoric acid*
27 = Cystine	*35 = Serine-phosphoric acid*
28 = β-Alanine	
29 = Glucosamine	
30 = Histamine (neutralized)	
31 = Histamine (free base)	
32 = Glutathione	
33 = Peptide	

arginine, histidine and tryptophan may be established by means of the specific colour reactions. A comparison with the paper sprayed with ninhydrin shows the places which should be tested with the different reactions. In this connection we may confirm the statement by Consden *et al.*[19] that when strict duplicates are run simultaneously in the same chamber, differences in R_F values do

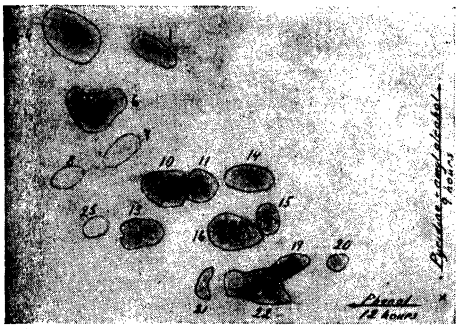

Figure 3. Photograph of a phenol/pyridine-amyl alcohol chromatogram, showing the positions of the amino acids from 1 ml of human plasma dialysate.

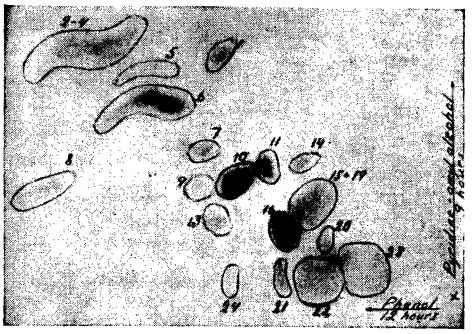

Figure 4. Photograph of a phenol/pyridine-amyl alcohol chromatogram, showing the positions of the amino acids from 1 ml of hydrolyzed plasma filtrate (80 % ethanol precipitation).

not exceed 4 %. It may be mentioned that the arginine and histidine reactions usually are not inhibited by a previous spraying with ninhydrin. In this way nearly all of the plasma amino acids are detected. A typical chromatogram is shown in Fig. 1.

2. A one-dimensional chromatogram is run with the lutidine-amyl alcohol mixture. 0.1 per cent diethylamine is placed on the bottom of the tray. In this way the presence of leucine, isoleucine and phenylalanine may be established. Better results have been obtained with dialysates than with ethanol filtrates of human plasma. Filtrate volumes corresponding to 0.5—1 ml of protein-free plasma have given the best pictures. A typical chromatogram is shown in Fig. 2.

3. For the identification of citrulline and β-alanine a two-dimensional chromatogram of a dialysate must be run in the same way as in (1). During the dialysis glutamine is hydrolyzed to glutamic acid. Citrulline and β-alanine which occupy the same position as glutamine on the chromatogram may be detected. Spontaneous hydrolysis of the labile amide nitrogen of glutamine has previously been observed (Archibald [20]). That a proteolysis usually takes place during our dialysing conditions (8 hours at + 2°) was realized when it was found that the non-dialysing proteins of plasma were practically soluble in 80 per cent ethanol at the end of the dialysis. However, the proteolysis is not reflected by a more pronounced increase of the α-amino nitrogen. The hydrolysis of glutamine may also be of enzymatical nature. A volume of the desalted

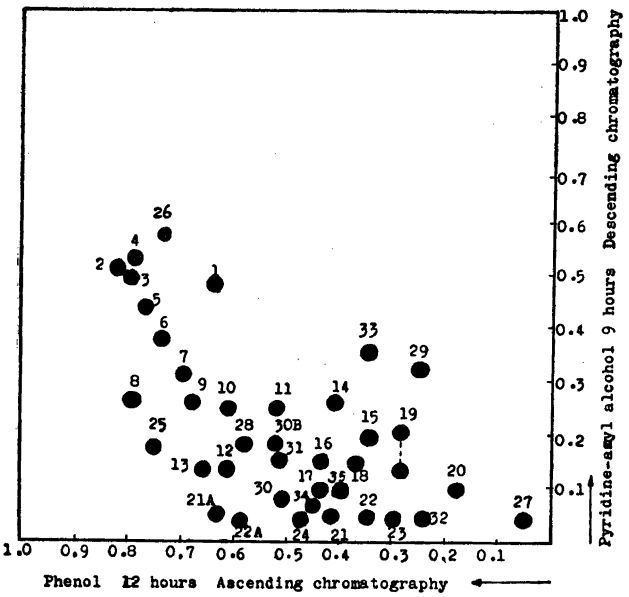

Figure. 5. Phenol/pyridine-amyl alcohol diagram, showing the average positions of the spots given by 23 amino acids and amino acid derivatives together with 2 peptides which have been found in 1 ml of deproteinized plasma. The positions of the spots given by 10 other amino acids and amino acid derivatives which may occur in blood and tissue extracts are also given.

dialysate corresponding to 1 ml of protein-free plasma is used for these chromatograms. The typical pattern is illustrated by Fig. 3.

4. Spots due to peptides disappear from the paper chromatogram after hydrolysis of the material. The hydrolysis is carried out with 2 *N* HCl for 5 hours at 120° or with 6 *N* HCl for 24 hours at atmospheric pressure. The acid is removed by evaporation to dryness followed by desalting. A two-dimensional chromatogram is run as in (1) with hydrolyzed material corresponding to 1—2 ml of protein-free plasma. Chromatograms of our five types of deproteinized plasma have unanimously shown a spot suspected to be caused by a peptide, no. 25 in Figure 1, and more irregularly a second spot no. 33 in Fig. 5. After hydrolysis the two spots disappear and spots corresponding to methionine and hydroxyproline appear on the papers. The typical picture is demonstrated by Fig. 4.

The identity of the different spots was verified by addition of known amino acids to the protein-free, desalted human plasma. Most of these experiments were carried out with ethanol filtrates. Fig. 5 shows the positions of the amino acids which are normally found in such filtrates (*cf.* Figs. 1 and 2). In addition the positions of some other amino acids and amino acid compounds which may occur in tissue extracts are given. The map is supposed to serve as a guide in further experiments.

DISCUSSION

With regard to the ninhydrin colours of the amino acids and their positions on the two-dimensional chromatograms some further remarks may be made. The usual colour of the spots is purple. In the following colour descriptions are given only when a deviation from this rule occurs.

β-Alanine. A spot corresponding to this amino acid has so far not been observed with the amounts of deproteinized plasma used in our two-dimensional runs. If present in sufficient amounts it should have been visible as a spot on the chromatograms of the plasma dialysates and the hydrolysates of deproteinized plasma. When 5 μg of β-alanine was added to a plasma dialysate a small blue coloured spot appeared on the chromatograms close to the citrulline spot but clearly separated from this spot. On the other hand β-alanine can not be observed on the chromatograms of the other types of deproteinized plasma where the glutamine spot overlaps both the β-alanine and the citrulline spots.

α-Amino-n-butyric acid. The position of this amino acid was verifed by addition of 5 μg of synthetic material to the protein-free human plasma. According to Pratt and Auclair [7] the compound should be detectable in amounts of 0.2 μg. The figure given by Dent [2], 4 μg, is more in agreement with our experience. There is no microbiological or chemical method available for the determination of the small amount of free amino acid present in human plasma. However, since the spot is barely visible when 1 ml of protein-free human plasma is used only a few μg of the amino acid should be present in this volume. α-amino-*n*-butyric acid has not been found in acid hydrolysates of proteins. According to Fromageot and Clausen [21] it should be formed *in vivo* by decomposition of methionine.

Arginine. The two basic amino acids arginine and lysine travel faster when ammonia is present during the phenol runs. The position of their spots are then changed and they will occupy a place on the chromatograms below the glutamine spot while the histidine spot is overlapped by the glutamine spot (*cf.* spots no. 21 A and 22 A in Fig. 5). The arginine and lysine spots are not better separated by this procedure and the Pauly diazo reaction presents some difficulties with the large amounts of glutamine present in blood plasma. On similar chromatograms of dialysates histidine will occupy nearly the same position as citrulline. Accordingly we prefer not to use ammonia in the phenol runs. Arginine is detectable in amounts of 10 μg. On a two-dimensional chromatogram of an arginine solution two spots appear, one in the expected position but a weaker spot is also found close to the citrulline spot. However, the spot does not coincide with the citrulline spot when both amino acids are

run together. Previous investigators [7] have already stated that arginine is decomposed by the solvents used in chromatography. The ninhydrin-positive decomposition product found by us is so far of unknown nature.

Asparagine gives an orange-brown coloured spot and is easily detectable on the two-dimensional chromatograms when amounts of about 5 μg are added to 1 ml of an ethanol filtrate of human plasma. The spot corresponding to this small amount of amide moves close to the glycine spot (*cf.* Fig. 5) but is not overlapped by this large spot caused by the high glycine content of human plasma (*cf.* Dent [2], Gutman and Alexander [9]). A small spot giving the same colour and occupying the same position as the asparagine spot is also observed on the chromatograms of the filtrates from 1 ml of ethanol precipitated human plasma without any addition of the amide. This spot is not observed after hydrolysis of the ethanol filtrates. Accordingly it seems highly probable that free asparagine is present in small amounts in normal human plasma. The asparagine spot may sometimes be overlapped by a blue purple spot formed by the decomposition of arginine.

Aspartic acid gives a small blue coloured spot (*cf.* no. 20, Figs. 1, 3, and 4). According to previous investigators [4] the amounts of aspartic acid in human plasma should be neglible. Our experience is that a small aspartic acid spot is a rather constant phenomenon on the two-dimensional chromatograms of human plasma.

Citrulline. The citrulline spot is not detectable on the chromatograms of protein-free filtrates obtained by the precipitation methods. These filtrates contain large amounts of glutamine which occupy about the same place as citrulline on the chromatograms. However, on two-dimensional chromatograms from 1 ml of human plasma dialysates a spot is observed which by addition experiments and colour reagent (Dent [2]) has been verified to be citrulline. When two-dimensional chromatograms of this amino acid were carried out two spots were observed, one in the expected position, the other occupying the same place as the norvaline spot. Nitrogen determinations of the citrulline preparation (Hoffman-La Roche) gave the theoretical yield. A partial decomposition of this amino acid during the development of the chromatogram must occur. The amino acid is detectable in amounts of 5 μg.

Cystine and cysteine. Spots denoting the presence of these two compounds are usually not seen on two-dimensional chromatograms when 1 ml samples of ethanol filtrates are analyzed. According to Alexander [4] cystine plus methionine should comprise about 5 per cent of the free α-amino nitrogen in human plasma. The methionine content of 1 ml of protein-free plasma is estimated to about 4 μg (Sheffner *et al.*[5]). Accordingly an amount of about 15 μg of cystine plus cysteine could be present in the same volume. A comparison between

one-dimensional chromatograms of 10—15 μg samples of cystine or cysteine with either phenol or the pyridine-amyl alcohol mixture as organic solvents showed for both compounds very faint, reddish purple coloured streaks in the phenol runs and weak but more sharply defined purple coloured spots in the pyridine-amyl alcohol runs. A decomposition of the amino acids obviously took place in the phenol solvent. After treating the amino acid solutions with hydrogen peroxide larger spots of cysteic acid were obtained but still a decomposition of cysteic acid took place in the phenol runs. With regard to cysteine it may be further pointed out that this amino acid easily forms a thiazolidine compound during the conditions prevailing in the protein-free plasma (Ågren [22]). The conclusion was drawn that the amounts of cysteine or cystine present in 1 ml of protein-free plasma, wether treated with hydrogen peroxide or not, would not give clearly visible spots on the two-dimensional chromatograms.

Ethanolamine is detectable in amounts of 10 μg and gives a bluish purple spot. It has never been observed in our experiments with protein human plasma. When added to the plasma filtrates it occupies the position shown in Fig. 5.

Ethanolamine-phosphoric acid. A sample synthesised in this laboratory was used. The compound gives a purple coloured spot and is detectable in amounts of about 20 μg. It has never been observed on our paper chromatograms of plasma filtrate. The position of the spot is given in Fig. 5.

Glucoseamine is detectable in amounts of about 10 μg when added to deproteinized human plasma. It gives a purple-brown colour. It has not been observed on the chromatograms with the amounts of protein-free plasma used in the present investigation. A spot close to the position of glucoseamine (no. 33, Fig. 5) was at first thought to be this compound. However, the spot did not move when ammonia was present during the phenol run (*cf.* Dent [2]). Moreover, after hydrolysis the spot disappeared. The most probable explanation is that the spot is related to a peptide present in the filtrates from ethanol precipitated human plasma.

Glutamic acid is mainly present as the amide in the protein-free human plasma. The large amounts of the amide, 60—120 μg, found in 1 ml of the plasma (Hamilton [20]) give an easily recognized spot on the papers. On the chromatograms of dialysates or hydrolysates the glutamine spot has disappeared and a spot corresponding to the position of added glutamic acid appears. In his recent paper Dent [2] has described several irregularities with regard to the position of the glutamic acid spot. We can corroborate his findings. The amino acid is detectable in amounts of about 2 μg.

Glutathione of blood is present in the red blood corpuscles [24, 25] and accordingly can not be extected to appear on the chromatograms of deproteinized plasma. When added to such samples it will occupy the position shown in Fig. 5.

Glycine gives a reddish purple colour. It is one of the main amino acids in plasma.

Histamine gives a bluish purple spot which after some time changes to dull greenish. It is detectable in amounts of 5—10 μg. So far it has not been observed on the chromatograms of protein-free plasma. As demonstrated in Fig. 5 the free base and the hydrochloride do not occupy the same position on the paper when they are added to an ethanol filtrate of plasma.

Histidine. According to Hier and Bergeim [3] 1 ml of deproteinized human plasma contains 14 μg of histidine. Previous investigators [2, 7] have stated that about 20 μg of this amino acid is the smallest amount which will give a spot on a two-dimensional chromatogram. It is therefore not surprising to find that the histidine spot usually is missing on our papers. When a further 10 μg of histidine is added to 1 ml of ethanol precipitated plasma a greyish blue spot will appear as shown in Fig. 5. Hence the presence of histidine in 1 ml samples of deproteinized plasma is better detected by means of the Pauly diazo reaction as described previously in this communication.

Hydroxyproline gives a brownish yellow spot and is detectable in amounts of 5 μg. The spot touches that of alanine but the two spots are easily distinguished by differences in colours. Hydroxyproline can not be detected on a two-dimensional chromatogram of 1 ml of protein-free human plasma without further proceedings. Following hydrolysis it appears at the same time as two ninhydrine positive substances disappears (spots no. 25 and no. 33 *cf.* Fig. 5). The most probable explanation is that hydroxyproline occurs in peptide bound form in deproteinized plasma.

Lysine gives a greyish purple spot. It is larger than any of the two closely situated arginine and ornithine spots and often shows streaking to the right. It sometimes partially overlaps the ornithine spot. The two spots are best separated on the chromatograms of the hydrolyzed and desalted ethanol filtrates. (Fig. 4.)

Methionine. According to Sheffner *et al.*[5] 1 ml of deproteinized human plasma contains about 4 μg of the amino acid. This amount obviously is not large enough to give a spot in our two-dimensional chromatograms. Dent [2] also states that the amino acid is detectable in amounts of 10 μg. When 4 μg were added to 1 ml of protein-free plasma a methionine spot appeared on the papers. A methionine spot was also observed when 1 ml of deproteinized and

hydrolyzed plasma was run (*cf.* Fig. 4). It seems possible that a certain amount of the amino acid is bound in peptide form.

Ornithine. In agreement with previous investigators we have estimated the detectable amount of this amino acid to be about 5—10 μg. The ornithine spot is most clearly separated from the lysine spot on chromatograms of deproteinized plasma which has been hydrolyzed (*cf.* Fig. 4). The increase of the ornithine spot on these papers makes it probable that in addition to the preformed free amino acid some amount is formed during the hydrolysis either from arginine or possibly from the peptides present in the plasma filtrate.

Phenylalanine. According to Hier and Bergeim [3] the phenylalanine concentration amounts to about 14 μg per ml of deproteinized normal human plasma. This quantity of amino acid is barely sufficient to give a small greenish blue spot clearly separated from the leucine and isoleucine spots on the one-dimensional chromatograms.

Proline is detectable in amounts of about 5 μg. It gives a yellow spot. According to Hier and Bergeim (quoted by Alexander [4]) the concentration of proline in plasma should be neglible. Our impression is that the size of the proline spot on the chromatograms indicates a concentration higher than 5 μg per ml of protein-free plasma.

Serine-phosphoric acid. A sample synthezised in this laboratory was used. The compound gives a purple colour and is detectable in amounts of 5—10 μg. The position of the spot is given in Fig. 5. It has not yet been found in deproteinized plasma.

Tryptophan. The concentration of free tryptophan is estimated to 11 μg per ml of human plasma. This amount of amino acid will not give a ninhydrin spot on our two-dimensional chromatograms. The colorimetric tryptophan reaction of Bates [17] has been used by us and seems to be somewhat more sensitive than the ninhydrin reaction. However, the results with 1 ml of deproteinized plasma have been irregular. In experiments with 2 ml samples of deproteinized human plasma the ninhydrin spot appears in the expected position.

Unidentified substances. Protein-free human plasma contains at least two peptides (*cf.* spots no. 25 and 33, Fig. 5). The amino acid composition of these substances has so far not been investigated in any detail. However, when the deproteinized samples are hydrolyzed and subsequently analyzed with two-dimensional chromatography hydroxyproline and methionine spots will appear on the papers. The spots given by lysine and especially by ornithine are enlarged (*cf.* Figs. 1 and 4). It is probable that these four amino acids are integral parts of the peptides. In his recent paper Dent [2] describes a spot given by a substance tentativelly named fast-arginine. The suggestion is

made that the compound should be the guanidine analogue of lysine. A spot in a similar position appears on our chromatograms. Since the Sakaguchi reaction when applied to this spot is negative the proposed structure seems less probable.

SUMMARY

1. By combining one- and two-dimensional paper chromatographic procedures in the analysis of the amino acid content in 1 ml samples of deproteinized human plasma the presence of the following 21 compounds have been established: α-alanine, α-amino-*n*-butyric acid, arginine, asparagine, aspartic acid, citrulline, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, ornithine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine.

2. Apart from the free amino acids the following 4 ninhydrin positive substances have been found: two peptides probably containing hydroxyproline, lysine, methionine, ornithine and possibly other amino acids, thirdly taurine and finally the unidentified spot described as »fast-arginine» by Dent. This substance seems not to be a guanidine compound.

3. A map is shown which gives the positions of these 25 substances and 10 other biologically occurring and ninhydrin-positive substances.

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