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# On the Paperchromatographic Analysis of Amino Acids and Peptides in Tissue Extracts and Enzyme Hydrolyzed Proteins

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During the last year we have been working with the paper chromatographic method of Consden, Gordon and Martin [1] with the aim to apply it to analysis of amino acids and peptide mixtures in blood filtrate, tissue extracts and enzyme digested proteins. Some of our results are given in this communication.

## EXPERIMENTAL AND RESULTS

When not otherwise stated the quick-filtering paper no. 0B supplied by Munktells Pappersfabriksaktiebolag, Grycksbo, was used. Dimensions 48 × 48 cm. This number was the best of the ten Munktell papers available in filter sheets, possibly with the exception of no. 00 in which, however, the fronts of the organic solvents moved very slowly. Two-dimensional parallel runs were also carried out with the Munktell paper no. 0B and the Whatsman paper no. 4 using phenol-cupron in one direction and a mixture of pyridine and amyl alcohol in the other. The resolving capacity of the two papers was about the same. The pair of solvents mostly used was phenol containing 0.1 % of cupron and a mixture of pyridine and amyl alcohol consisting of 35 % by volume of pyridine, 35 % by volume of technical amyl alcohol and 30 % by volume of water, (Edman [2]).

At the time when the experiments were started collidine, isovaleric acid or isobutyric acid were not available. In some of the runs with phenol a 0.15 % solution of ammonia was present in the tray at the bottom of the chamber. A typical result obtained with a two-dimensional run of a casein hydrolysate is given in Fig. 1. Phenol was used in the first direction and pyridine-amyl alcohol in the second. The $R_F$ values are not given since they have not been found to be very consistent when calculated from different chromatograms

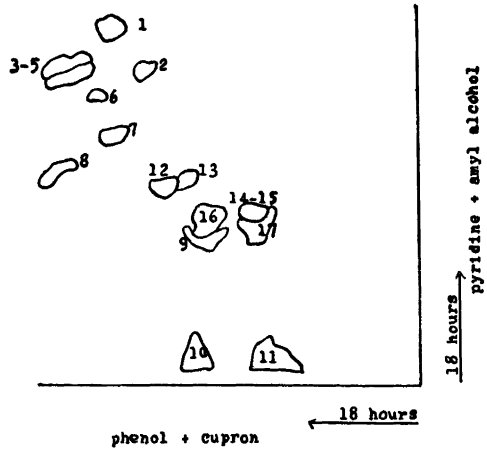


*Figure 1. Phenol-cupron (0.1 %)/pyridine-amyl alcohol diagram, showing the positions of the amino acids from 1.75 mg of a casein hydrolysate fortified with 100 of tryptophane on a two-dimensional chromatogram.*

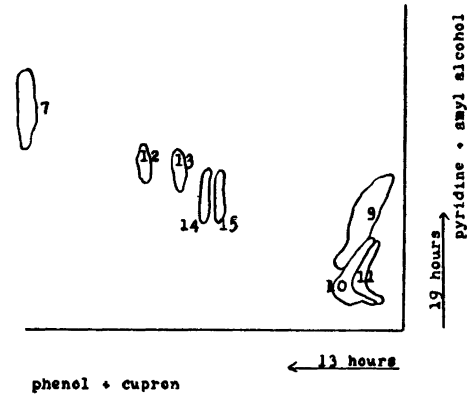


*Figure 2. Pyridine-amyl alcohol/phenol-cupron (0.1 %) diagram showing the positions of different amino acids in amounts from 50—100 γ on a two-dimensional chromatogram.*

*1 = Try 5 = Leu 9 = His 13 = Thr 17 = Asp*
*2 = Tyr 6 = Met 10 = Arg 14 = Gly 18 = (CyS-)$_2$*
*3 = Phe 7 = Val 11 = Lys 15 = Ser 19 = Glu-NH$_2$*
*4 = Ileu 8 = Pro 12 = Ala 16 = Glu*

*Abbreviations according to Brand and Edsall*[25]

(Pratt and Auclair [3]). The resolving capacity of this pair of solvents was comparatively good with some exceptions. The leucines and phenylalanine usually appeared as a single irregular spot. Further on histidine, serine and glycine were sometimes difficult to distinguish from glutamic and aspartic acid. When pyridine-amyl alcohol was run first and phenol afterwards a good separation of all these amino acids was obtained. A typical chromatogram is given in Fig. 2. In this way a better separation of alanine from threonine was also accomplished.

However, a good resolution of the other amino acids was not obtained in this way, possibly with the exception of the three basic amino acids. If, on the other side, ammonia was present in the cabinet when phenol was run in the first direction the $R_F$ values of aspartic and glutamic acid decreased (Consden, Gordon and Martin [1]). This is easily seen by a comparison of Fig. 1 with Fig. 3 which presents such a chromatogram. The $R_F$ values of arginine and lysine were both increased and the spots corresponding to the amino acids usually touched each other when this procedure was used. Later on when

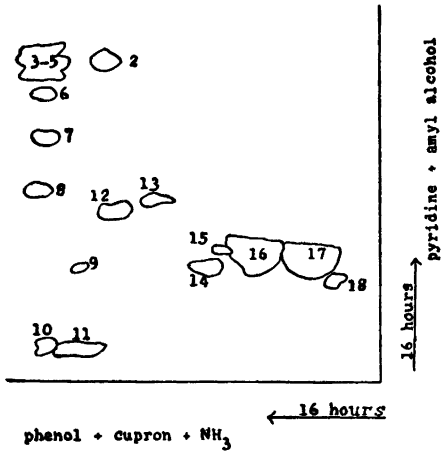


*Figure 3. Phenol-cupron (0.1 %) — ammonia (0.3 %)/pyridine-amyl alcohol diagram showing the positions of amino acids from 0.56 mg of a casein hydrolysate (HCl + HCOOH) on a two-dimensional chromatogram.*

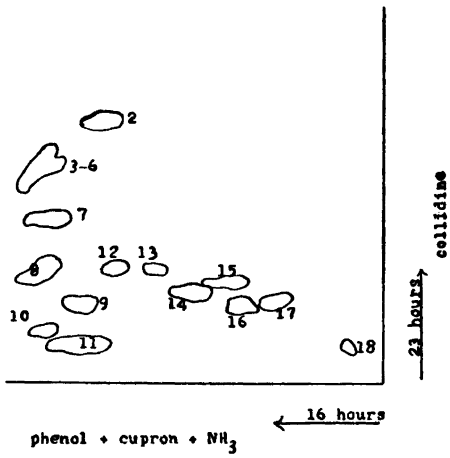


*Figure 4. Phenol-cupron (0.1 %) — ammonia (0.3 %)/collidine diagram showing the positions of the amino acids from 0.56 mg of a casein hydrolysate (HCl + HCOOH) on a two-dimensional chromatogram.*

collidine was available some experiments were carried out in which pyridine-amyl alcohol was exchanged for this solvent in two-dimensional runs with the Munktell paper no. 0B. A typical result is given in Fig. 4.

A comparison with Fig. 3 shows that collidine had some advantages over the pyridine-amyl alcohol mixture. More distinct spots were obtained for the dicarboxylic acids but the spot corresponding to methionine was usually covered by the leucine spot. Several attempts were made to resolve the leucine-isoleucine-phenylalanine group by one or two dimensional chromatograms using butanol with 3 % ammonia and benzylalcohol with 0.1 % cupron (Consden, Gordon and Martin [1]). The best results were obtained in two dimensional runs but clearly separated spots were never seen.

The organic solvents were removed from the papers by drying at 80°. After drying, the papers were sprayed with a solution of 0.1 % ninhydrin in butanol to which in some cases acetic acid was added to a final concentration of 1 % (Consden and Gordon [4]). The papers were then heated at 50° in a constant temperature chamber for 1 to 2 hours. It was observed that the spots usually appeared at quite different times and it occured to us that if these different times for development were characteristic constants for the various amino acids it would be able to use this circumstance in the identifi-

cation of amino acids with similar $R_F$ values. To test this possibility 5 $\mu$l of amino acid solutions were placed on filter papers previously treated with phenol containing 0.1 % cupron and a pyridine-amyl alcohol mixture. The papers were coloured with ninhydrin and placed in the constant temperature chambers. For each of 19 amino acids the time was measured when the colour first appeared. Several series were carried out. However a comparison between the results of the different series showed a considerable spreading of the time values for each amino acid. Thus it seemed impossible to use this method to identify leucine in the presence of isoleucine and phenylalanine, nor would the method increase the possibilities to locate serine in the presence of glycine or alanine in the presence of threonine.

Similar experiments were also carried out with the following series of peptides which were synthezised in this laboratory (*cf.* Ågren [5]):

Glycyl-DL-leucine
Glycyl-DL-alanine
Glycyl-L-tyrosine
Glycyl-glycine
Alanyl-glycine
Glycyl-DL-alanyl-glycine
Glycyl-glycyl-glycine

In accordance with the results recently published by Consden, Gordon and Martin [6] we found that peptides in which glycine carried the free amino group on heating with ninhydrin gave first a yellow colour, then, grey and finally purple. The colour came slower for the two tripeptides than for the dipeptides.

Several attempts have been made to apply quantitative methods in the paper chromatographic procedure (*cf.* Consden [7]). In this laboratory it has been found advantageously to proceed in the following manner. One-dimensional runs were extracted in the apparatus described by Consden, Gordon and Martin [6] and on the small volume thus obtained, about 0.25 ml, the ninhydrin reaction was carried out as described by Moore and Stein [8]. The molar extinction coefficients for the different amino acids were determined and used for this purpose.

## The preparation of blood filtrates

A paper chromatographic investigation of the free amino acids in blood plasma has so far only been carried out by Dent [9]. He prepared the blood filtrates by treating plasma or serum with ten times its volume of 95 % ethanol. Values for the amino nitrogen in the filtrates were not given, and the figures of the chromatograms only displayed 4—5 amino acids. Accordingly, determinations of the amino nitrogen in filtrates prepared by this method were carried out and compared with those obtained on filtrates prepared by

other methods. Cattle serum was used in these experiments. Zinc hydroxide precipitation according to Somogyi [10], tungstic acid precipitation according to Hier and Bergeim [11], ferric hydroxide precipitation according to Somogyi [12], dialysis and trichloracetic acid precipitation were carried out. The clear filtrates both from zinc hydroxide, ferric hydroxide and trichloracetic acid precipitations contained small amounts of protein which coagulated upon concentration. The largest amounts were found in the filtrates from zinc hydroxide precipitation. Since inorganic salts are known to be a disturbing factor in paper chromatographic analysis (Consden and Gordon [4]) the large amounts of sodium sulphate present in the filtrates after zinc hydroxide and ferric hydroxide precipitation must be removed if such an analysis should be carried out. The solubility of sodium sulphate in higher concentrations of ethanol is very low, the following figures being obtained at 2° when calculated per 100 ml of solvent: 27.5 mg in 70 %, 5.2 mg in 80 % and 3.6 mg in 90 % ethanol. The filtrates from zinc hydroxide and ferric hydroxide precipitation were accordingly partially desalted by addition of 95 % ethanol to a final concentration of 80 %.

In the trichloracetic acid precipitation 10—50 % solutions of the reagent were added to a final concentration of 5—10 % followed by rapid heating to boiling and subsequent cooling to room temperature. The surplus of trichloracetic acid was first removed by boiling under reflux (Eichloff and Lossen [13]) for 3 hours when all of the reagent had formed chloroform. During the boiling a brown colour appeared when the final concentration of reagent was 10 % mainly depending on destruction of tryptophane. When precipitation was carried out by addition of a 10 % solution of the reagent to a final concentration of 5 % the filtrate remained colourless during the reflux. Subsequent amino nitrogen determination showed, however, that the peptides present in these filtrates must have been hydrolyzed during the reflux (*cf.* Table 1). Accordingly the reagent was removed by ether extractions. Ninhydrin controls showed that amino acids were not removed in this procedure.

Dialysis was carried out in cellophane tubes, Ø = 5 mm, at 2° for 8 hours during constant stirring against distilled water which was exchanged three times. The different filtrates were analyzed by Van Slyke determinations. The values obtained on three different samples of serum are given in Table 1. Tungstic acid filtrates and ferric hydroxide filtrates contained about the same amounts of $\alpha$-amino nitrogen, while the zinc filtrates gave lower values. Somogyi [14] investigating non-protein nitrogen in the same kind of filtrates also found the lowest values in the zinc filtrates while the nitrogen values of iron filtrates lay between those of zinc and tungstic acid filtrates. Agreeing $\alpha$-amino nitrogen values were obtained in the filtrate series from 10 and 5 %

*Table 1. Comparison of deproteinizing agents. α-amino nitrogen of various filtrates of cattle serum. Values are in mg α-amino nitrogen per 100 ml of serum and each value is an average of two or more analysis.*

| Sample | Serum no. | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| $Zn(OH)_2$-filtrate | 3.95 | 4.10 | 4.0 |
| $Fe(OH)_3$-filtrate | 4.75 | 4.80 | 4.75 |
| Tungstic acid filtrate | 4.75 | 4.85 | 4.75 |
| Filtrate from trichloracetic acid precipitation. Final reagent concentration 10 %. Reagent removed by boiling | 9.50 | 9.80 | 9.60 |
| Filtrate from trichloracetic acid precipitation. Final reagent concentration 10 %. Reagent removed by 4 ether extractions | 5.25 | 5.40 | 5.30 |
| Same as preceding but with final reagent concentration 5 %. Reagent removed by ether extraction | 5.30 | 5.45 | 5.30 |
| Dialyzable α-amino nitrogen | 4.95 | 5.05 | 5.0 |
| Filtrate from 80 % ethanol precipitation | 3.80 | 3.90 | 3.80 |

trichloracetic acid precipitations where the surplus of reagent was removed by ether extractions. A comparison with the results of the filtrate series from which the reagent had been removed by boiling showes that these filtrates contained bound α-amino nitrogen. This explanation may also be valid for the dialysates which also contained more α-amino nitrogen than the ferric hydroxide and tungstic acid filtrates (*cf.* Christensen and Lynch[15]). Finally, the α-amino nitrogen in the ethanol filtrates lay mainly on the same level as in the zinc hydroxide filtrates. One possible explanation to the few amino acids found by Dent[9] in his chromatograms of the free amino acids in blood plasma could thus be the precipitation method and another an incomplete resolution in the runs. In this investigation the interest was mainly focused on the latter possibility. Precipitation with 80 % ethanol was used.

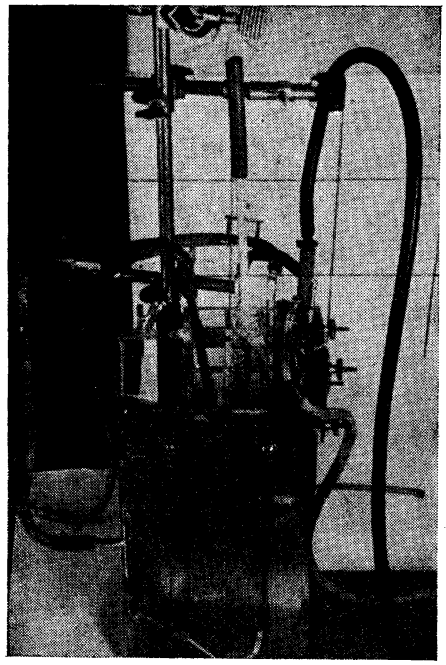

*Figure 5. The desalting apparatus.*

## Results obtained on blood filtrates

When the first two-dimensional paper chromatograms were run with ethanol filtrates very unclear pictures were obtained. Extraction of the dried alcohol filtrates with dry ethyl ether removed some interfering substances but the results still were far from satisfactory. However, when the ether treated extracts were desalted more appropriate pictures were obtained. The apparatus used by us was a modification of the desalter described by Consden, Gordon and Martin [6] (*cf.* Fig. 5). Platina was prefered as anode and it was found favourable to applicate a cellophane bag around the anode in addition to the membrane which separated the middle compartment from the anode compartment *. A comparison was made between descending and ascending paper chromatography (Williams and Kirby [16]). As far as our experience goes we prefere to use the ascending procedure in this case. A typical chromatogram is shown in Fig. 6. This was carried out on a 48 × 48 cm paper with an ethanol filtrate from normal human plasma. Similar runs performed on 24 × 24 cm papers did not give the same good resolution of the amino acids when ascending chromatography was used. In some cases filtrates from 70 %

* A similar apparatus was used by H. T. Macpherson in Chibnall's laboratory.

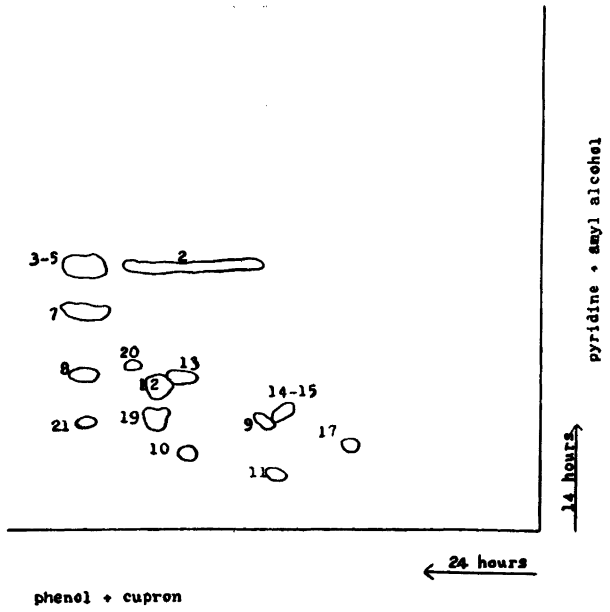


*Figure 6. Phenol-cupron (0.1 %)/pyridine-amyl alcohol diagram, showing the positions of the amino acids from 1 ml of human plasma filtrate (80 % ethanol precipitation) on a two dimensional ascending chromatogram.*
*20 = α-amino-butyric acid? 21 = unidentified spot.*

ethanol precipitation were also investigated. The same number of ninhydrin spots were obtained as with the filtrates from 80 % ethanol precipitation. However, with filtrates from the same amount of plasma, 1 ml, the ninhydrin colour of the spots were stronger when the lower ethanol concentration was used. In conclusion one reason to the discrepancy between our results and those of Dent's is that we removed some interfering lipids and inorganic salts.

## Results with liver extracts

In connection with investigations of the growth effects of certain liver extracts and enzyme hydrolyzed proteins on lactic acid bacteria (Ågren [17]) paper chromatographic analysis were also performed. As described in that paper a highly purified liver extract (Heptomin) containing the antipernicious anemia liver factor could be divided in three microbiologically active fractions by electrodialysis. The anode fraction was subjected to the one-dimensional technic using a butanol acetic acid mixture (Partridge [18]). Four ninhydrin positive spots were observed. Using the method described by Dent [9] for

isolation of similar substances three of them seemed to increase the growth of *Lactobacillus casei* cultivated on the medium of Henderson and Snell [19]. The microbiological assay technic previously described (Ågren [17]) was used. Data from the assays will be published elsewhere.

The middle fraction run in a mixture of pyridine-amyl alcohol was resolved in at least three ninhydrin positive and four ninhydrin negative but fluorescing spots (Phillips [20]). The material corresponding to the positions of the spots was again isolated and tested for growth effect on *Lactobacillus lactis*. Two of the ninhydrin positive and one of the ninhydrin negativ but fluorescing spots stimulated the growth of this microorganism. The result is of interest since this microorganism was used in the standardization of vitamin $B_{12}$ (Shorb [21]) and also depending on the presence in the middle fraction of the antipernicious anemia liver factor (Ågren [17]). The three substances giving the ninhydrin reaction after hydrolysis were again subjected to one-dimensional runs in the mixture of pyridine-amyl alcohol. All of them seemed to contain valine, leucines or phenylalanine, glycine or serine and perhaps aspartic acid.

A two-dimensional run with the cathode fraction of electrodialyzed Heptomin showed the presence of five ninhydrin positive spots. After hydrolysis of the cathode fraction the following amino acids were found in a two dimensional run with phenol and the pyridine-amyl alcohol mixture: leucine, isoleucine, phenylalanine, methionine, valine, threonine, alanine, histidine, arginine and lysine. The cathode fraction also contained a complex mixture of basic peptides. This fraction was the only one of the three fractions separated by electrodialysis which gave a biuret positive spot on paper (Killander [22]). Microbiological assays were not carried out on this fraction.

The Wilson liver fraction L was also electrodialyzed in three fractions which all stimulated the growth of *Lactobacillus casei* (Table 2). This is of interest with regard to the present discussion of the constitution of strepogenin (Wolley [23]). So far only the cathode fraction has been analyzed by paper chromatography. In two-dimensional runs with phenol and the pyridine-amyl alcohol mixture 5 ninhydrin spots were obtained. The substances corresponding to the position of the spots on the paper were isolated and each of them hydrolyzed and run again in one-dimensional chromatography with pyridine-amyl alcohol. The results indicated that the cathode fraction contained five polypeptides of different amino acid composition. Leucine or isoleucine was found in all of them. The two slowest running peptides contained comparatively large amounts of lysine and the peptides with the highest $R_F$ values showed the largest content of leucines. In this preliminary survey microbiological assays of the different substances were not carried out.

*Table 2. Growth of Lactobacillus casei after supplementing the basal medium of Henderson and Snell with electrodialyzed fractions from Wilson liver fraction L. Photoelectric readings and titrations after 24 hours of incubation.*

| | Anode fraction | | | | Middle fraction | | | | Cathode fraction | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| pH at the end of electrodialysis | 2.0 | | | | 5.0 | | | | 10. | | | |
| Total nitrogen added per tube in mg | 0 | 1.0 | 0.1 | 0.01 | 0 | 0.5 | 0.05 | 0.005 | 0 | 0.8 | 0.08 | 0.008 |
| Photoelectric readings * | 4 | 69 | 25 | 20 | 4 | 160 | 105 | 20 | 3 | 105 | 65 | 25 |
| Titration values ** | 0 | 3.4 | 0.6 | 0.1 | 0 | 4.9 | 2.0 | 0.2 | 0 | 3.5 | 1.4 | 0.4 |

## Result with enzyme hydrolyzed casein

In the recent paper of Ågren [17] a commercial tryptic digest of casein (Aminosol) was used as a source of strepogenin for the microbiological work. This preparation was now resolved in three fractions by electrodialysis and all fractions stimulated the growth of *Lactobacillus casei*. The assay technic was the same as described in that paper. The results are given in Table 3. The anode fraction was run with one-dimensional chromatography using butanol, pyridine-amyl alcohol, collidine, phenol with addition of ammonia, hydrochloric acid or acetic acid, butanol with addition of acetic acid and finally a mixture of *iso*butyric acid and *iso*valeric acid. Only the three last mentioned organic solvents gave a resolution of the anode fraction in two spots, also when two-dimensional runs were performed. A comparison with simultaneously run amino acids showed that one of the spots coincided with aspartic acid, the other with glutamic acid.

Microbiological analysis with *Lactobacillus casei* showed that growth stimulating effect was present only in the spot laying on the same level as aspartic acid. The material of this »aspartic acid spot» was hydrolyzed before and after deamination according to Consden *et al.*[6] and analyzed with one- and two-dimensional technic using phenol in the first case and phenol and a pyridine-

* Given as scale readings on the Klett-Summerson photoelectric colorimeter. Mean values of three tubes corrected for the blank titrations.

** Given as ml of 0.05 *N* NaOH to titrate 6 ml of final solution. Mean values from three tubes corrected for the blank titrations.

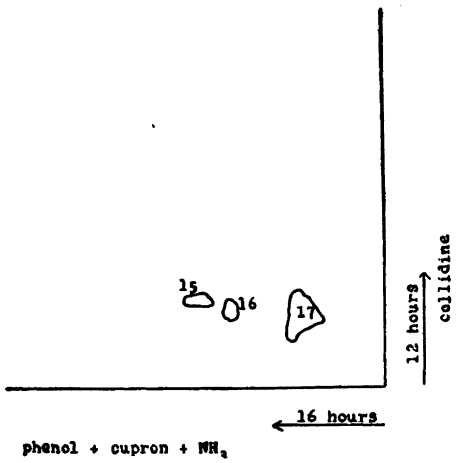


*Figure 7. Phenol-cupron (0.1 %)-ammonia (0.3 %)/collidine diagram, showing the positions of the amino acids from hydrolyzed »aspartic acid spot» on a two-dimensional chromatogram.*

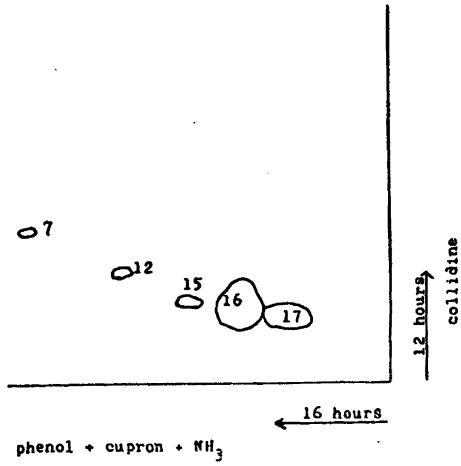


*Figure 8. Phenol-cupron (0.1 %)-ammonia (0.3 %)/collidine diagram, showing the position of the amino acids from hydrolyzed »glutamic acid spot» on a two-dimensional chromatogram.*

*Table 3. Growth of Lactobacillus casei after addition of fractions from electrodialyzed Aminosol to the basal medium of Henderson and Snell. The amounts added are expressed as mg of total nitrogen. Photoelectric readings and titrations after 24 hours of incubation.*

| | Anode fraction | | | | Middle fraction | | | | Cathode fraction | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| pH at the end of electrodialysis | 3.0 | | | | 4.8 | | | | 10.0 | | | |
| Total nitrogen added per tube | 0 | 0.35 | 0.035 | 0.0035 | 0 | 0.52 | 0.052 | 0.0052 | 0 | 0.20 | 0.020 | 0.0020 |
| Photoelectric readings * | 2 | 99 | 22 | 10 | 3 | 73 | 15 | 10 | 2 | 62 | 20 | 9 |
| Titration values ** | 0 | 1.84 | 0.72 | 0.20 | 0 | 1.30 | 0.44 | 0.20 | 0 | 1.30 | 0.58 | 0.10 |

amyl alcohol mixture in the second. The results of several of these analysis (*cf.* Fig. 7) indicated that the eluted material consisted of free aspartic acid

* Given as scale readings on the Klett Summerson photoelectric colorimeter. Mean values of three tubes corrected for the blank titrations.

** Given as ml of 0.05 *N* NaOH to titrate 6 ml of final solution. Mean values from three tubes corrected for the blank titrations.

and a seryl-glutamic acid peptide, probably containing several glutamic acid molecules per serine molecule (*cf.* Wolley [23]). The material from the »glutamic acid spot» was analyzed in the same way and seemed to be a mixture of glutamic acid and an asparagyl-peptide (Fig. 8) probably containing serine, alanine and valine. The results of the microbiological assays are given in Table 4. The technic is the same as previously described (Ågren [17]). The amino nitrogen in the anode fraction amounted to 57 % of the total nitrogen. 5 mg of substance was used in the chromatographic resolution and it was calculated that the amount of growth stimulating seryl-glutamic acid peptide added in the assays amounted to about 0.1 mg per tube, which gave a maximal growth effect.

*Table 4. Growth of Lactobacillus casei on the medium of Henderson and Snell supplemented with substances isolated from the anode fraction of electrodialyzed Aminosol. Incubation time 24 hours.*

| | »Glutamic acid spot» | | »Aspartic acid spot» | |
|---|---|---|---|---|
| Amount of substance added per tube | 0 mg | 4 mg | 0 mg | 1 mg |
| Ml of acid produced * | 1.3 | 1.5 | 1.4 | 8.6 |
| Photoelectric readings ** | 84 | 94 | 83 | 300 |

The cathode fraction of Aminosol was investigated with one-dimensional technic using the pyridine-amyl alcohol mixture. So far only peptides containing histidine and valine have been identified and may be responsible for the growth effect of this fraction. This result is of interest with regard to the present discussion of the chemical constitution of strepogenin (*cf.* Wolley [24]).

## SUMMARY

1. Several details in the paper chromatographic procedure have been investigated. In two dimensional runs with phenol in the first direction we have compared the resolving capacity of collidine with that of a pyridine amyl

* Given as ml of 0.05 *N* NaOH to titrate 6 ml of final solution. The values are the mean values from three tubes and have been corrected for the blank titrations.

** Given as scale readings on the Klett-Summerson photoelectric colorimeter. The reading 0 corresponds to 100 per cent transmission. The values are the mean values from three tubes.

alcohol mixture in the second direction without finding any special advantage when using collidine.

2. Different methods for the preparation of blood serum and plasma filtrates containing the free amino acids have been compared with regard to the yield of $\alpha$-amino nitrogen and fitness for use in paper chromatography. It was found necessary to remove lipids and inorganic salts which interfered with analysis. Ascending chromatography was prefered when working with desalted blood plasma filtrates.

3. A highly purified preparation containing the antipernicious anemia liver factor was investigated by means of electrodialysis followed by a combined paper chromatographic and microbiological analysis. In this way it could be demonstrated that the preparation was very far from pure. Each of the three fractions from electrodialysis was found to contain a mixture of peptides which in some cases showed a growth stimulating effect on *Lactobacillus casei* and *Lactobacillus lactis*.

4. A similar analysis of an enzyme hydrolyzed casein preparation was also carried out. The anode fraction from the electrodialysis was resolved in at least one biologically inactive peptide and one growth stimulating peptide which probably was a seryl-glutamic acid peptide. The cathode fraction, which also stimulated the growth of *Lactobacillus casei*, contained a mixture of peptides.

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