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Identification of the amino acids of cerebrospinal fluid

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IDENTIFICATION OF THE AMINO ACIDS OF CEREBROSPINAL FLUID

By

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I. INTRODUCTION AND METHODS

The greater part of this paper will deal with the development of a paper chromatogrpphic method for analysing cerebrospinal fluid since, although partition chromatography is in wide usage presently, its theoretical background is not such as will allow of predictable behavior on the part of a given biological fluid. A specific analytic problem is thus better attacked at the bench than in the library (3). Hence, the reference that chromatography is an art and not a science.

References to the paper chromatographic separation of free amino acids and other biological substances in tissue, blood, and urine are numerous in the literature but there has been little if any work published on the amino acids of cerebrospinal fluid. A microbiological assay of the fluid has yielded considerable insight as to which amino acids should be expected. (16). This provided the basis for the synthetic spinal fluids which were here utilized for establishing the qualitative and quantitative attack upon the present problem.

The method followed is that of Consden, Gordon and Martin (7) as modified by Williams and Kirby (18). This procedure is the "capillary ascent" method in which the filter paper is stapled into a cylinder and placed upright in the solvent allowing the solvent to climb by capillary action. This calls for a minimum of equipment and yields results comparable with those of the somewhat more complicated descending method (7).

Some of the problems of the project arose from the fact that samples of spinal fluids were only available in 4 to 8 ml. quantities. These samples, according to Solomon, Hier, and Bergeim (16), contained amino acids of the order of 2 micrograms per ml. This necessitated the lyophilization of the fluid in order to bring about the necessary concentration of amino acids. Also biological fluids contain salts, small concentrations of which alter the R_f values of the acidic and basic amino acids (15). Dialysis could not be used since it would remove the amino acids as well as the inorganic salts. Ultimately, an electrolytic "desalting" apparatus was resorted to. Spinal fluid proteins also had to be precipitated by methods other than those which would ultimately add more salt to the solution. The initial precipitation by trichloracetic acid followed by sodium hydroxide neutralization had to be discarded for that reason. A method of alcohol precipitation was resorted to (2).

In chromatographic analysis R_f values may vary with atmospheric conditions, solvents, etc. But, in general the amino acids being separated will have the same pattern of distribution over the paper (9). This means that if enough amino acids are present there is no need to calculate the absolute R_f values at all, but simply to compare the relative positions of the spots with Dent's chart (9). This chart contains the relative positions of some 60 amino acids and related substances. Of course, a given spot is always checked against a standard amino acid spot pattern before a decision is reached concerning its identi-

ty (11).

Standard solutions of amino acids were made up .01 M in distilled water. These were autoclaved in plasma bottles and kept under sterile conditions. An improvement over this would have been the use of Ethyl alcohol as a solvent thereby making unnecessary any particular procedures to assure sterility and bacterial or fungal decomposition of the substances. A further improvement would have been the utilization of a solvent having the same ionic strength as cerebrospinal fluid.

The location of the spots on paper by ultra-violet examination of their fluorescence (14) was found to be inadequate, and a light spraying with a .1% Ninhydrin solution was substituted. This detected some spots which were not obvious by ultra-violet scanning. After the location and identification of the amino acids, a quantitative determination is carried out. This involves cutting out the spot, eluting it, reacting the solution with ninhydrin and comparing the optical density with that of standard solutions on a spectrophotometer. For this quantitative procedure the method of Moore and Stein (13) was followed. The recovery was expressed as per cent by comparing the optical density of the eluted acid with the optical density of a specific amount of standard amino acid developed in the same manner.

II. REAGENTS AND APPARATUS

A. Apparatus

1. Tanks

Two large cylindrical glass tanks of approximately 50" x 20" were used for running the chromatograms.

2. Paper

Filter paper of Whatman grade #1 was used for all chromatograms. These were cut into convenient squares for two-dimensional chromatography.

3. Ultraviolet Lamp

4. Water Bath

For a water bath a shallow pyrex dish 14" x 3" x 6" was supported on two ring stands and heated by two Bunsen burners. This was sufficient to keep a rack of twenty-eight tubes at approximately 100° C for twenty minutes.

5. Drying Oven

An in cubator heated by electric light bulbs equipped with a thermostat was used for drying the chromatograms between runs. The temperature of this oven was kept at 40° C since higher temperatures cause a loss of amino acids when phenol is being removed from the paper.

6. Electric Oven

An electric oven was used to bring out the spots after spraying the dry chromatograms with a dilute (.1%) ninhydrin solution. This oven should be kept at approximately 100° C and 5 to 10 minutes was found to be sufficient length of time for color development of all spots.

7. Spectrophotometer

A Coleman Junior Spectrophotometer Model 6 was used for all readings.

8. Test Tubes and Racks

Pyrex tubes 30 x 150 mm without lips were used throughout. These tubes were calibrated to 10 ml. for constant dilution. For all photometric readings Coleman Cuvettes (Type B) were used. These were not individually calibrated.

9. Atomizer

For ninhydrin spraying an atomizer was constructed out of a 250 ml. Erlenmeyer flask and glass tubing.

10. Lyophilization Apparatus

A standard lyophilizing apparatus equipped with a manifold was used. The manifold permitted lyophilization of up to 4 or 5 cerebrospinal fluids at a time.

11. Pipettes

Regular 0.2 ml. serological pipettes calibrated to 10 microliters were used for the initial qualitative work. For more accuracy in quantitative work , Hgb pipettes calibrated to 20 microliters were used. The narrower tips on these pipettes proved quite satisfactory for spotting the papers. With these it was possible to get spots one half cm. in diameter with reasonable consistency.

12. Hair Dryer

A hair dryer was found useful in drying the spots as they were applied to the paper. This greatly cut down the

amount of time necessary for applying the standards and unknowns to the paper while keeping the spots as small as possible.

B. REAGENTS

1. Amino Acids

Amino acids for standards were obtained from National Biologicals and were used without further purification.

2. Solvents

(a) Phenol - Regular reagent grade Merck phenol was purified by distillation before use. The commercial reagent was found to contain a slight yellow coloration which was undesirable.

(b) Methyl cellosolve - The commercial reagent was distilled before use. This was used for a second dimension solvent on many of the chromatograms.

(c) n-Propanol - This reagent was obtained as the commercial product and it was not found necessary to purify it.

3. Diluents

As a diluent in the Moore and Stein Photometric Method a 1:1 n-propanol-water was used.

4. Citrate Buffer

A citric acid buffer of pH 4.95 was prepared by weighing out 21.01 grams of citric acid, adding 200 ml. of 1 N NaOH and diluting to 500 ml.

5. Ninhydrin Solutions

(a) A 0.2% solution of ninhydrin in water and later a .1% ninhydrin solution in butanol saturated with water were used.

(b) Quantitative Ninhydrin Solution - The Moore and Stein quantitative ninhydrin solution was made up as follows: 0.8 gm of reagent $SnCl_2 \cdot 2H_2O$ in 500 ml. of citrate buffer (pH 5) were added to 20 gm of ninhydrin disolved in 500 ml. of methyl cellosolve.

6. NH_3 was used to get better resolution of the basic amino acids in phenol. Four drops were added to the inside of the tank before each run in phenol.

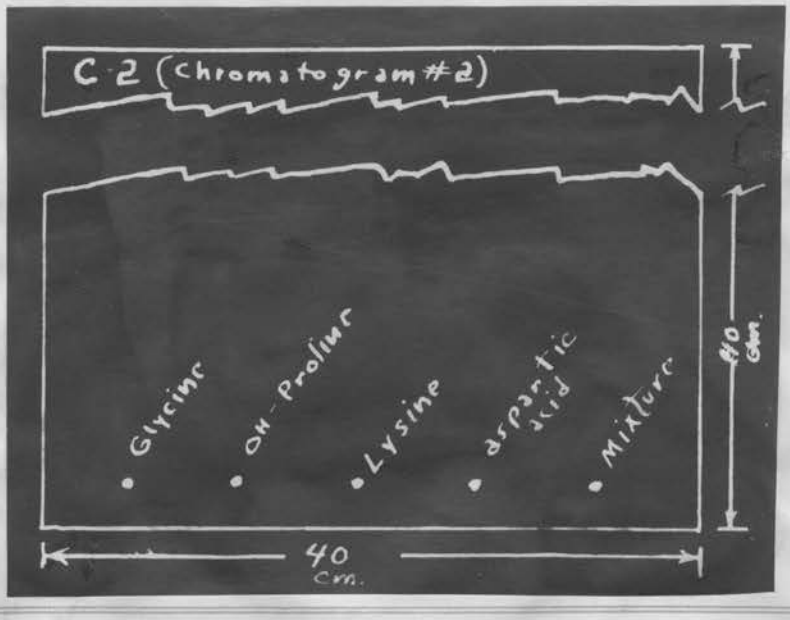
7. HCN was used for getting rid of the "pink beard" in the phenol runs. Sufficient concentration of HCN was maintained during the run by adding KCN to the solvent. Its affect on R_f values, however, caused the abandonment of its use.

III. PROCEDURE

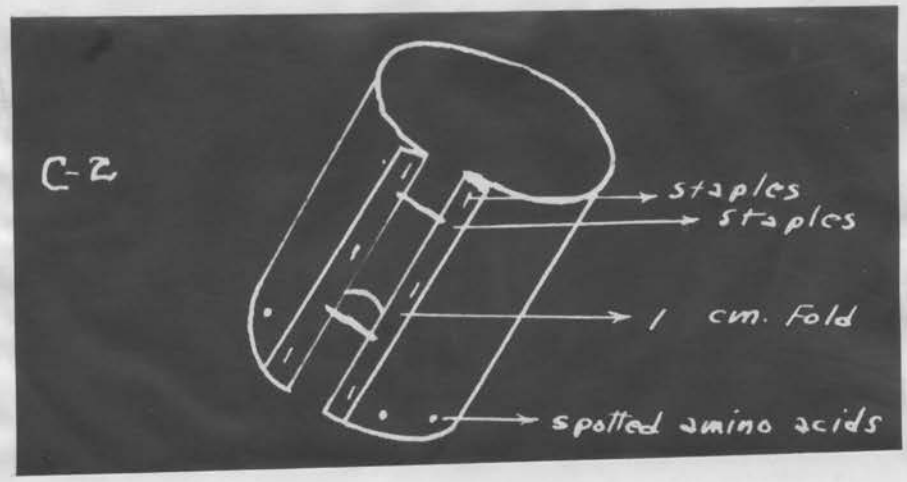
A. Method for Establishing Rf Values

1. A Typical Chromatogram - Preliminary one- dimensional chromatograms were run as follows: By means of sterile syringes and needles the standard amino acids were withdrawn and placed into labeled test tubes. From these tubes the desired amounts were withdrawn with appropriate pipettes and spotted on paper. Exactly 2.8 micrograms of amino nitrogen was usually applied by pipetting out 0.02 ml. of the standard. Some of the early chromatograms were spotted with 0.1 ml. of standard 0.01 M solution (i.e. 14 micrograms of amino acid nitrogen). As pipetting techniques were improved, smaller amounts were applied and found to be adequate.

The amino acids were spotted approximately 4 cm. apart and one spot labeled "mixture" received an aliquot of each of the standard amino acids. One of the trial chromatograms had the following appearance before development:

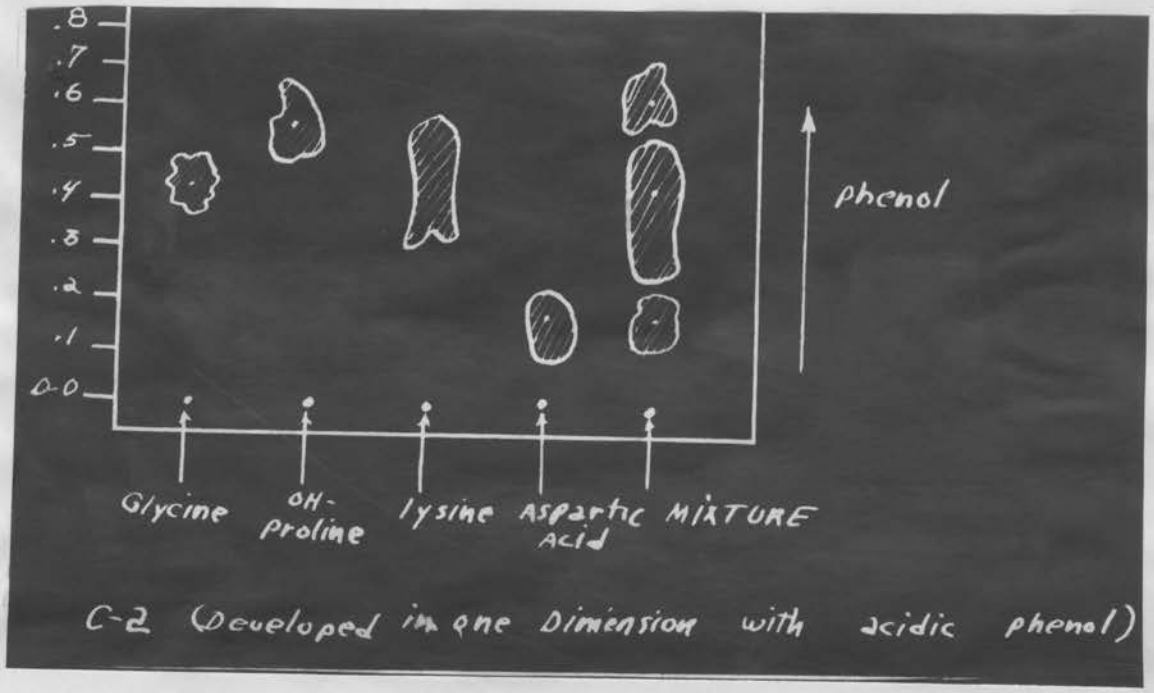


The chromatogram was then formed into a cylinder and stapled as follows:



The cylinder was next placed in a tank at the bottom of which was a petri dish containing a solution of phenol and water (80:20). The solvent was then allowed to climb for a period of 16 hours at which time the solvent front had reached a height of approximately 30 cm. The chromatogram was then dried at 100° C for 35 minutes and examined under ultraviolet light. The fluorescing spots were circled with pencil. It should be noted that attempts to locate spots without heating (i.e. air drying at room temperature) yielded no fluorescing spots. Evidently the heating is a necessary factor in the formation of the fluorescing substances. With increased heating the intensity of the fluorescence seems likewise to increase. The fluorescing substance remains behind upon water-elution; therefore, in doing quantitative work prolonged heating at high temperatures may lead to errors in recovery of amino acids (14).

The distance of this fluorescing spot is measured and the ratio of this distance to the total distance that the solvent front has traveled is the R_f value. (i.e., R_f (rate of flow) = cm. traveled by the amino acid/cm. traveled by solvent) (7).

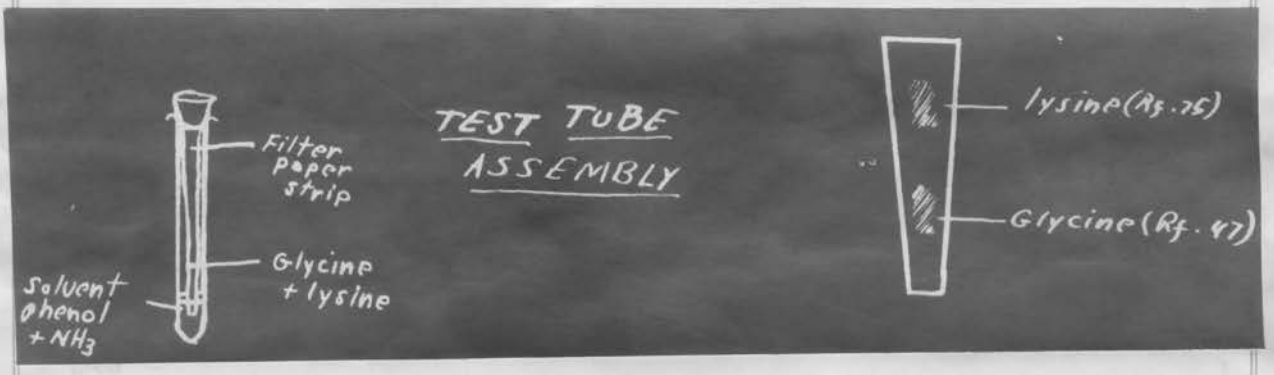


C-2 (Developed in one Dimension with acidic phenol)

Amino Acid	Dent's R_f	our R_f (C_2)	our R_f (C_3)
Glycine	.41	.41	.41
OH-Proline	.63	.63	.67
Lysine	.82	.41	.39
Aspartic acid	.18	.18	—

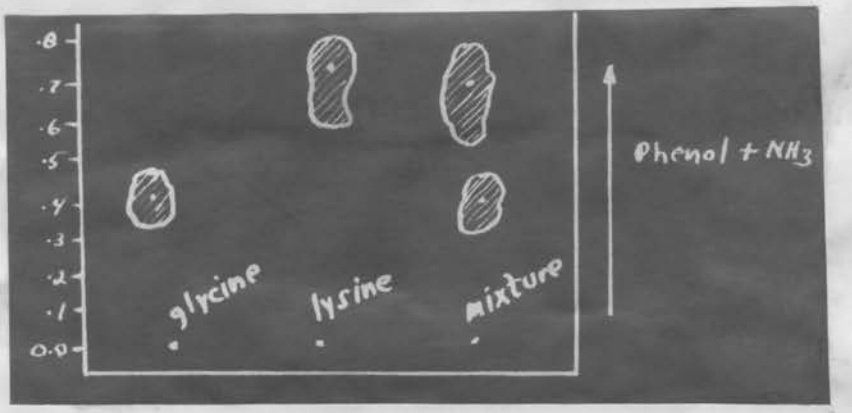
2. Effects of NH_3 on R_f Values

It will be noted from the above figure that the values obtained for lysine do not approximate Dent's values, and this was found to be due to the fact that the amino acid was obtained as the hydrochloride. Since the basic amino acids are slowed under acidic conditions (9), it was decided to test the R_f obtained for lysine under basic conditions. To perform a rapid qualitative test to see if glycine (R_f.41) could be separated from lysine (our R_f.41), the "test tube" method as described by Cassidy was used (6).



This demonstrated the fact that NH_3 in phenol did have a profound effect on the R_f values and that on chromatograms run in a basic environment, lysine could be distinguished from glycine with phenol as the solvent. Future chromatograms were run by placing 4 drops of concentrated NH_3 down the inside of the tank immediately before sealing in the phenol runs.

A more elaborate chromatogram run in NH_3 gave the following separation:

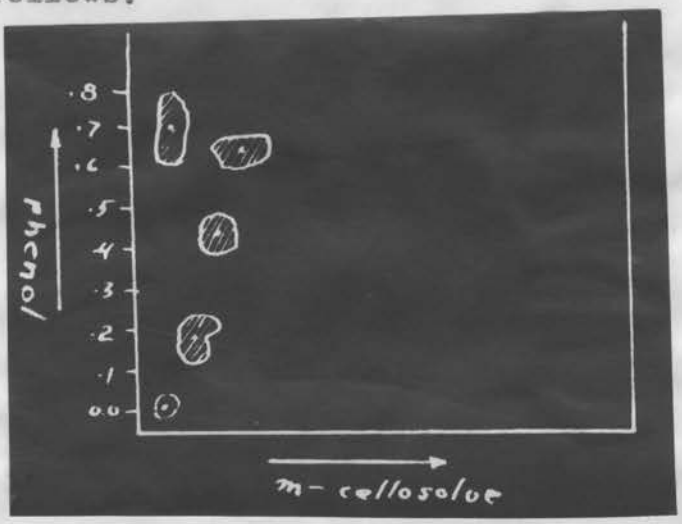


All future chromatograms in phenol were run under these conditions.

3. Two Dimensional Chromatograms

The R_f values obtained from one-dimensional chromatograms were used for the location and identification of amino acids on the two dimensional papers. After permitting a spreading of the amino acids according to their Rf's along the phenol edge, the chromatogram was dried and made into a new cylinder with the partially spread amino acids parallel to the surface of the second solvent.

The results indicating a complete separation in this case are as follows:



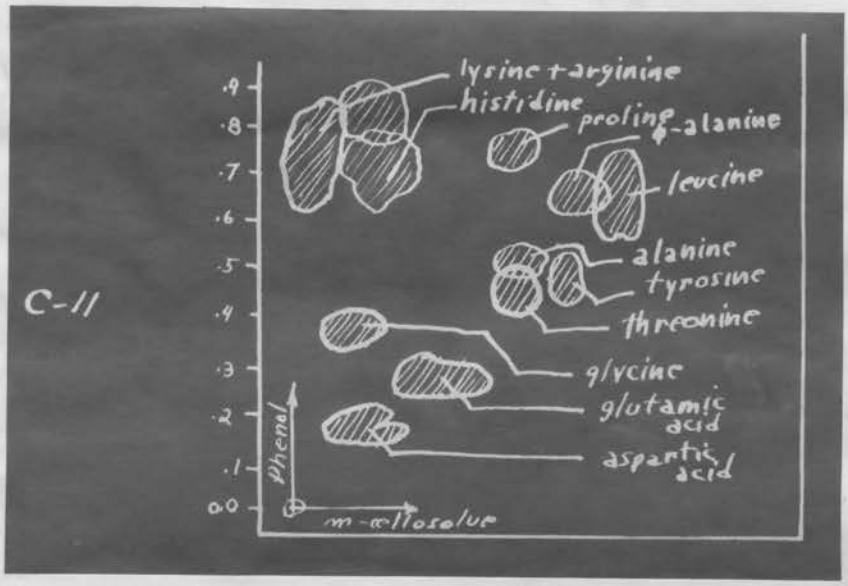
All further separations were accomplished according to the above procedure as described with the necessary variations such as length of running time, solvents, etc.

B. Synthetic Spinal Fluids

On the basis of the microbiological investigation (16) of the CSF, chromatograms were made up containing those amino acids which would be encountered plus a few others of known biological occurrence. The synthetic fluid contained the following amino acids:

1) alanine
2) arginine
3) aspartic acid
4) glutamic acid
5) histidine
6) leucine
7) lysine
8) phenylalanine
9) proline
10) threonine
11) tyrosine
12) glycine

The paper containing the synthetic spinal fluid was run in phenol and then methyl cellosolve (1) giving a pattern which was readily reproducible.



Many of the amino acids gave spots of characteristic shape and color. (i.e. Proline always gave a round spot which was a bright yellow.) On these chromatograms containing the synthetic spinal fluid complete separations were not obtained, but for actual quantitative work these chromatograms would be run for longer periods of time. If separation were not then complete the combined spot of two or more amino acids would be eluted and re-spotted on a new paper. This then would be run in a third solvent to effect the separation.

C. Quantitative Recovery of Amino Acids

One-dimensional chromatograms were run for determining the degree of quantitative recovery possible. 2.8 Micrograms

of amino acid N (i.e. 0.02 ml. of the 0.01 M standard stock solutions of the amino acids) were spotted on the paper for each acid. Standard tubes for each acid were set up to contain 2.8, 5.6, and 8.4 micrograms of amino acid N. (i.e. 0.02, 0.04, and 0.06 ml. of the standard 0.01 M stock solutions). The standard tube optical densities were used to plot standard curves for each of the amino acids run. The chromatograms were developed with propanol-water, 80:20, as the solvent. They were dried in air and then sprayed with a 0.1% ninhydrin solution in butanol saturated with water. The papers were then placed in the electric oven for 5 minutes to allow color development of the spots. The spots were circled, cut out from the paper and set into test tubes for elution. A representative spot was cut out from the paper to serve as a blank.

To each test tube containing the filter paper 3 ml. of distilled water were added. The tubes were then immersed in a bath of boiling water for 1½ hours. After completing the elution 2 ml. of the Moore-Stein ninhydrin reagent were added in citrate buffer (pH 5) to each of the unknown and standard tubes. All the tubes were then immersed in a bath of vigorously boiling water for 20 minutes to allow full color development. The tubes were then removed, cooled slightly, and brought up to a volume of 10 ml. with a sufficient amount of n-propanol-water (1:1) diluent. The tubes were then read on the Coleman Jr. Spectrophotometer and their optical densities recorded. In the instances where the readings were off scale, proper dilutions

were made.

Per cent recovery was then determined by comparing the optical densities of the eluted amino acids with the optical densities of the corresponding standard tubes. (i.e. The tube containing 2.8 micrograms of amino acid N)

Optical Densities (uncorrected for blanks)

Tube	.02 ml.	.04 ml.	.06 ml.	eluted acid
Blank	.095	.095	.164	.30
alanine	.65	1.28	1.84	.53
arginine	.78	1.28	1.88	.56
glycine	.63	1.22	1.76	.44
histidine	.65	1.20	1.60	.45
lysine	.71	1.44	1.82	.53
tyrosine	.60	1.44	1.64	.54

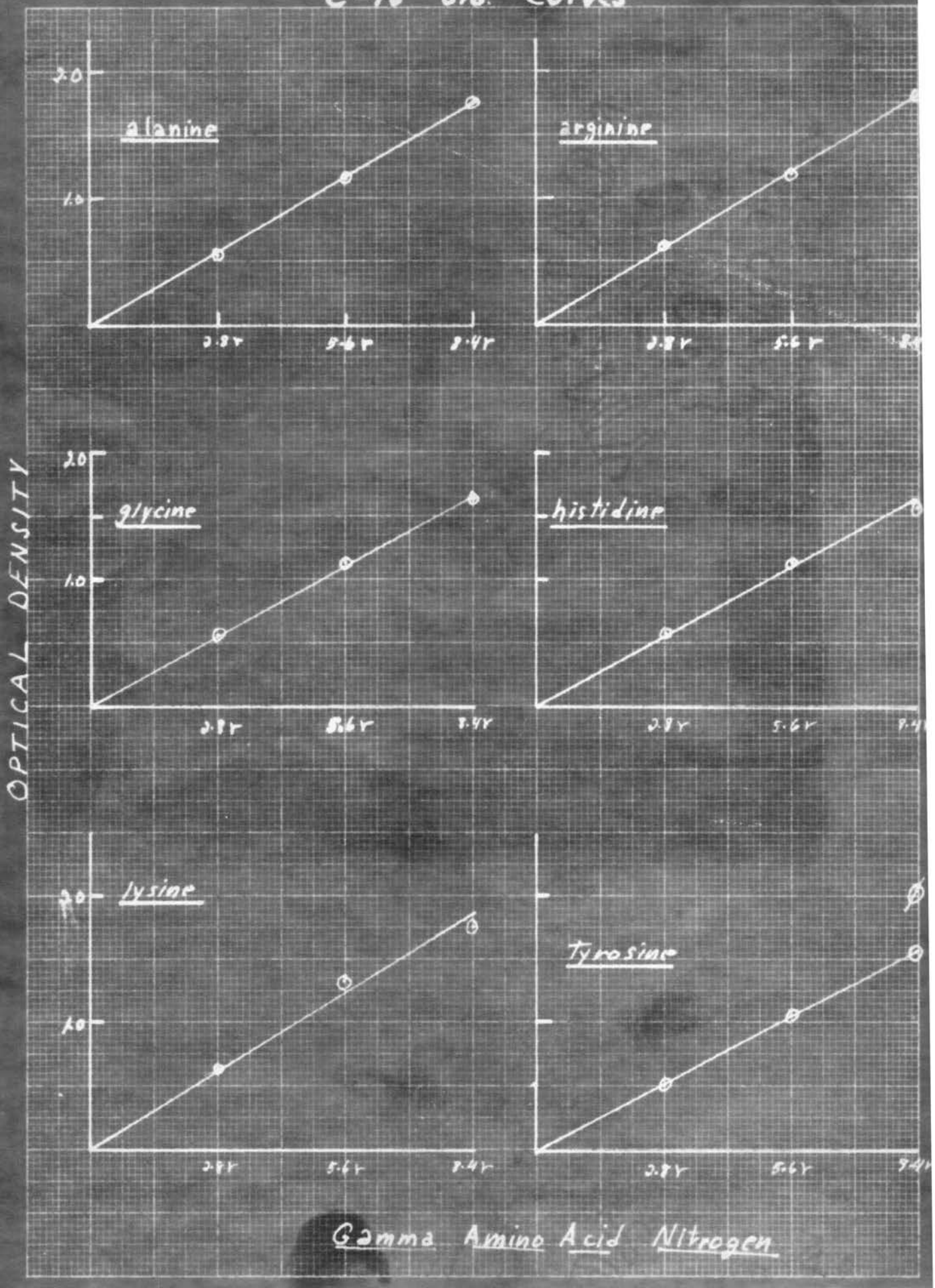
Optical Densities (Corrected)

Blank	0	0	0	0
alanine	.55	1.18	1.74	.28
arginine	.68	1.18	1.78	.26
glycine	.58	1.12	1.66	.14
histidine	.55	1.10	1.50	.15
lysine	.61	1.34	1.72	.23
tyrosine	.60	1.04	1.54	.21

Tube	% Recovery
(2) alanine	42.0
(3) arginine	39.0
(4) glycine	26.5
(5) histidine	27.0
(6) lysine	38.0
(7) tyrosine	54.0

Within the ranges used the optical densities of the amino acids gave straight lines which passed through the origin.

C-16 Std. Curves



Gamma Amino Acid Nitrogen

D. Discussion of Results

In the earlier quantitative experiments serological pipettes were used for spotting the amino acids. Highly inaccurate and unpredictable results were obrained due to the inherent inaccuracy in this type of pipette and in the method of applying the spots. Improved results were later obrained when Hgb pipettes calibrated to 0.02 ml. were used. Reproducibility as far as the standards were concerned was consistently obtained.

As far as the eluted spots were concerned, once the use of the Hgb pipettes was instituted, recovery always remained less than 100%. This was attributable to a number of factors greatest of which seems to be the manner of elution. Various methods of elution were tried, the optimal recovery being obtained by the procedure outlined above.

Another factor involved in the low recovery of amino acids was the incomplete delivery of the amino acids from the Hgb pipettes on to the paper. This would tend to give a small but constant loss. Another source of error might lie in the preliminary drying of the chromatograms after development (5, 12). Also after development of the spot, the ninhydrin complex has been shown to be non-elutable (13). High blanks have also tended to contribute to the error of the technique in that when the blanks constiture a large percentage of the combination of blank plus eluted amino acid, a normal variation in blank reading will have a tremendous effect on per cent recovery of the

amino acids.

The color development procedure seems to be satisfactory since standards all agree rather well. All per cent recoveries are now approximately in the same low range. In an attempt to improve these returns from the paper a series of preliminary experiments was run to find just where the majority of the loss was occurring.

E. Elution Experiments

Before actual investigation of the point of loss was undertaken multiple determinations were run in order to see if the loss was a constant one. With this established two chromatograms, each having duplicate spots for each acid, were then run simultaneously and carried through in identical manner with the exception of the location of spots. Both chromatograms were sprayed with 0.1% ninhydrin solution as usual, but the spots of chromatogram A were allowed to develop in air while chromatogram B was dried in an oven at 100° C for five minutes for spot development. The results were as follows:

Tube	% Recovery	
	A	B
alanine	42	53
glutamine	43	45
leucine	42	47
threonine	44	46
tyrosine*	140	138
glycine	21	49

*No attempt is made to explain the unusual behavior of tyrosine, and the above evaluation is made without any consideration of tyrosine.

It will be noted that although there is a slight increase in percentage recovery from the heated paper, the change is negligible as compared to the big loss. Consequently, further experiments were carried on.

Squares of filter paper 4 x 4 cm. were treated in the following manner:

(a) 0.02 ml. of standard leucine solution was applied to one set of squares.

(b) 0.02 ml. of standard leucine solution was applied to another set and the squares were heated for 5 minutes.

(c) 0.02 ml. of standard leucine solution was applied to a third set and these were sprayed with 0.1% ninhydrin and heated for 5 minutes.

(d) 0.02 ml. of standard leucine solution was applied to a fourth set which were sprayed with the ninhydrin solution. These squares were allowed to dry in air until the spots were moderately visable.

All the above papers were run in quadruplicate. Elution and color development was carried through in the usual manner. Results for the quadruplicate runs were averaged and the results were as follows:

Tube	Optical Density
Blank	.15
Standard	.28
A	.75
B	.78
C	.75
D	.75

These results indicate that the manner in which the paper is treated with respect to spotting, spraying and heating makes comparatively little difference in quantitative return. The loss must occur someplace in the development of the chromatograms with their respective solvents. No conclusive experiments have been completed as yet. However, the problem can be attacked systematically making use of the above outline. In this case the solvents would become the variables.

F. Suggestions for Future Work

With respect to the quantitative recovery of amino acids from chromatograms there are several recent improvements which have not been utilized as yet (4).

After the spotted amino acids are dry, those acids available as hydrochlorides can be effectively neutralized by holding the chromatogram over a bath of 4 N NH_4OH for four minutes (4). The chromatograms can then be hydrated by steam treatment for 10-15 minutes. The hydration brings about more uniform chromatograms with more compact spots. Following the run in phenol, a strip 2 or 3 cm. below the solvent front should

be cut off. This removes a large portion of the phenol decomposition products which interfere with the running of the second solvent.

Further improvement of results may be obtained by a complete removal of the phenol solvent by washing the paper with ether. The effects of phenol have been mentioned elsewhere. Also, traces of NH_3 and other nitrogenous substances may be removed by spraying the paper with 1% KOH in methanol.

IV. CEREBROSPINAL FLUID

A complete qualitative separation of the amino acids in cerebrospinal fluid has not yet been accomplished although chromatograms now show the desired degree of consistency. Salt interference, which inhibited a good qualitative separation, has been overcome by use of a "desalting" apparatus which will be described.

A. Preparation of CSF for Chromatography

The CSF has been treated in the following manner:

1. All cellular debris is spun down for 15 minutes in a refrigerator centrifuge.
2. To the supernatant is added 5 volumes of EtOH for protein precipitation.
3. The solution is then centrifuged at 2000 r.p.m. to bring down the protein.
4. The supernatant is brought down to dryness on a steam bath.
5. The residue is taken up in 10 ml. of distilled water.
6. This solution is then desalted by means of the "desalting" apparatus. (See desalting apparatus)
7. The "desalted" solution is then brought down to dryness on a steam bath. This final residue is then desolved in approximately 0.25 ml. of distilled water and spotted on a chromatogram.

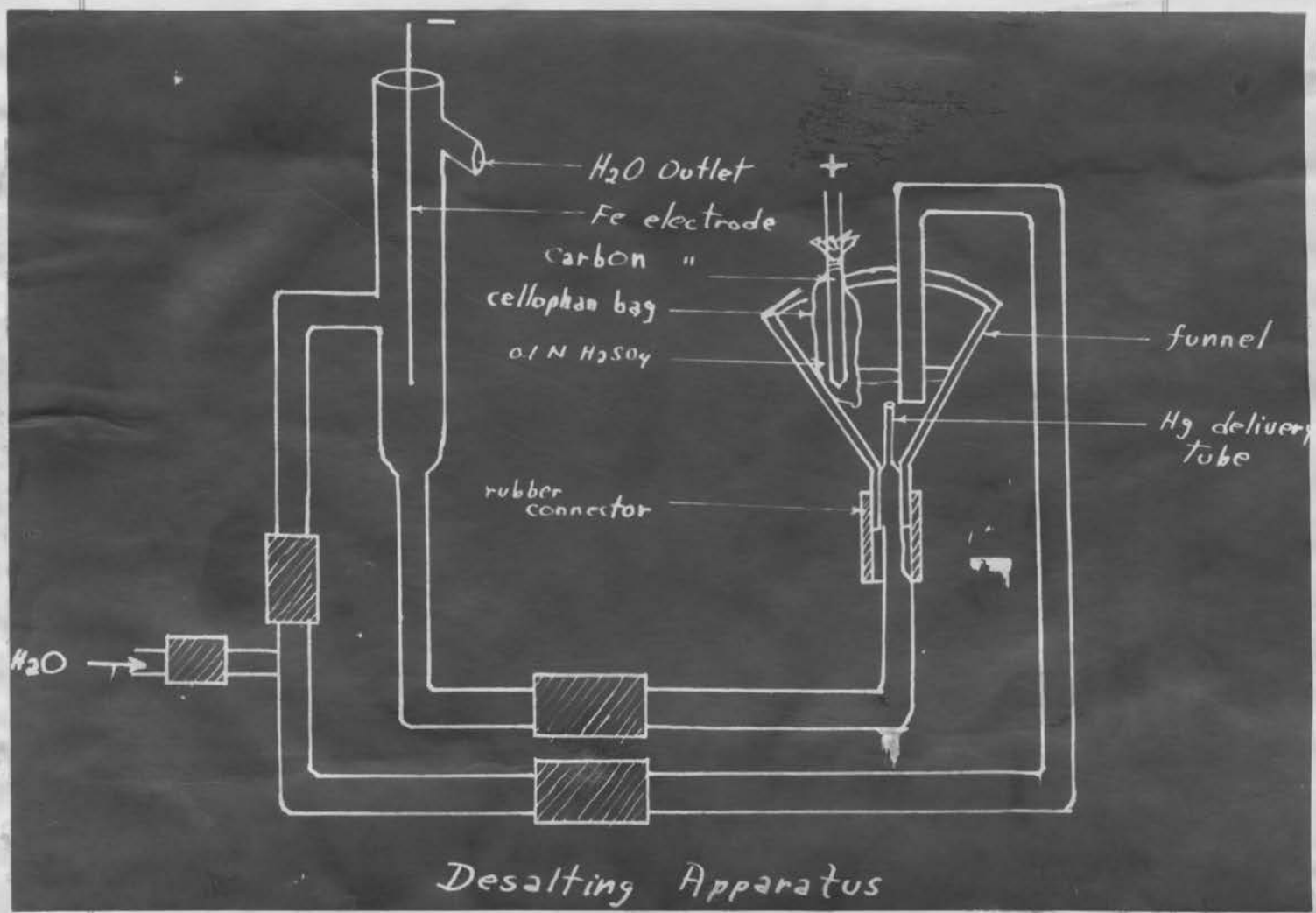
B. Salt Interference

Spinal fluids cleared of proteins in the classical manner have yielded fairly consistent results which, however, did not allow any qualitative identification of amino acids. Since the ions of the inorganic salts of biological fluids move characteristically during the development of the chromatogram (17), the application of the chromatographic method on untreated biological fluids for amino acids present in only trace amounts is inadequate (10). The inorganic ions would come to occupy large areas of the paper finally "shouldering off" the amino acids causing them to appear as distorted streaks surrounding parts of the salt areas. For example, glutamine, glutamic acid and aspartic acids are noticeably affected in this way.

There is now a method for prior removal of most of the salts (8) which can be readily adapted to from one to ten ml. of solution. Dent states that after "desalting" in this manner, over 3 ml. of cerebrospinal fluid can be analysed satisfactorily on one chromatogram even though the original fluid gave distortions when only 0.1 ml. was used (10). This provides a method, therefore, for the detection of amino acids in concentrations as low as 1 microgram per ml.

C. Desalting Apparatus (8)

The apparatus consists essentially of a funnel through which mercury is circulated by a water lift pump. The solution to be desalted floats on the mercury into which dips the graphite anode which is enclosed in a cellophane bag containing 0.1 N H_2SO_4. This membrane permits the passage of anions of strong electrolytes to the anode while hydrogen ions pass in the opposite direction.



Desalting Apparatus

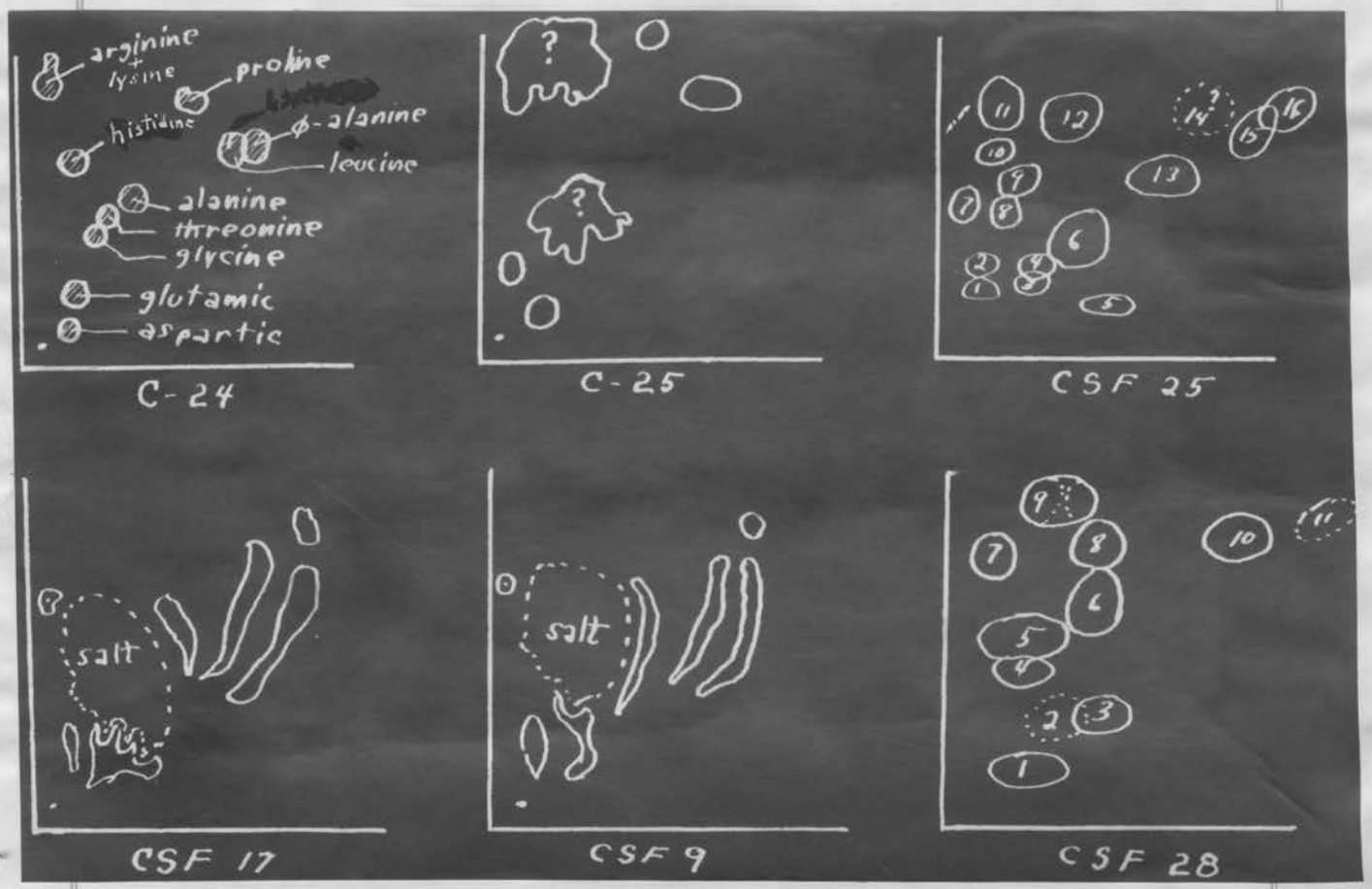
The amino acids, including the dicarboxylic acids, acquire a net positive charge within the membrane and are thus less likely than uncharged molecules to reach the anode compartment. The mercury cathode discharges hydrogen ions and other inorganic cations, hydrogen being liberated and the metals dissolve in the mercury. These metals are washed out of the mercury by the pump water. As the concentration of the solution falls, the resistance of the apparatus rises and the current falls. This small steady value can be used to determine the endpoint of the desalting process.

To test the apparatus for amino acid loss either by its passing into the anode compartment or by being carried away in droplet form by the mercury, standard solutions of amino acids were made up in physiological saline and desalted. The color that was developed by the amino acids in the desalted fluid was then compared photometrically with the standards which were not desalted. No loss was detected.

Tube	Optical Density
"desalted"	.625
standard	.615

D. Experimental Results

Desalted chromatograms had improved separations as will be demonstrated by the following study:



Chromatogram #24 was a synthetic cerebrospinal fluid containing amino acids made up in distilled water. Chromatogram #25 has the same amino acid complement as chromatogram #24, but illustrates the effects of salts. This was accomplished by applying a physiological saline solution to the spot of amino acids. The result is the same as if the biological

fluid itself had been spotted. It is obvious that no identification of amino acids is possible. The chromatograms of cerebrospinal fluid (CSF 17 & 19) are typical of biological fluids showing the characteristic effects of salts. CSF 25 & 28 are separations of amino acids after the biological fluid has been desalted.

The amino acids identified on the basis of these improved separations are as follows:

CSF 25

1. (?)
2. (?)
3. glutamic acid
4. glycine
5. cystine (*)
6. threonine
7. lysine
8. glutamine (*)
9. histidine
10. γ-NH_2-butyric acid
11. arginine
12. OH-proline
13. alanine
14. (?)
15. valine
16. phenyl-alanine

CSF 28

1. aspartic acid
2. (?)
3. cystine (*)
4. glutamic acid
5. glycine
6. threonine
7. glutamine (*)
8. α-alanine
9. arginine, OH-proline*
10. β-alanine, valine (*)
11. leucine (*)

*The identity of these amino acids is not positive.

V. EVALUATION OF PROCEDURE AND RESULTS

The final evaluation of the results in the preceding experimental procedures can be summarized as follows:

A. Separation and identification of amino acids in cerebrospinal fluid can be adequately achieved using phenol-water (80:20) with ammonia and proponal-water (80:20) as the developing solvents. Within limits of experimental procedure it was found that all chromatograms so treated tended to reproduce themselves fully.

B. On the basis of such reproduction, identification of amino acids was made. The validity of such a procedure has already been established by many others (9). Recognition is given here to the fact that the most positive identification lies in the elution of the spots and running them in a third solvent in order to confirm the R_f values in the solvent against standard solutions of known amino acids. Due to the lack of time this more rigorous method of identification was by-passed in favor of the single two-dimensional chromatogram.

C. The adequacy of the desalting apparatus was firmly established. Solutions were desalted to the point wherein no precipitate was observed upon the addition of a 1% solution of silver nitrate. It was also shown that the desalting process caused no loss of amino acids.

D. Quantitatively all that can be stated at the present time is that reproducible but low results have been obtained. The effects of spotting, heating, drying and elution were studied individually and shown not to cause any significant loss in recovery attempts. Other possible factors have been mentioned.

E. The amino acids of cerebrospinal fluid which were identified may be summarized as follows:

glutamic acid
glycine
cystine
threomine
lysine
glutamine
histidine
γ-NH_2-butyric acid
arginine
OH-proline
α-alanine
valine
β-alanine

This is in agreement with the work of Solomon, Hier, and Bergeim with regard to the kinds of amino acids which are present in cerebrospinal fluid. In addition there were found some amino acids which these workers did not include in their original report. (i.e. OH-proline, aspartic acid)

F. A rough visual estimation of the quantity of the amino acids based on the color intensity of the colored spots tends to correlate with the results of Solomon, Hier and Bergeim.

Thus it was found that alanine and glutamine were present in the greatest concentrations while glycine and arginine were present in moderate amounts. The remaining acids were present in lesser amounts.

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