Journal of Chromatography 104 (1975) 63–72

CHROM. 7813

A METHOD FOR THE SEPARATION OF PLASMA AMINO ACIDS BY ION-EXCHANGE CHROMATOGRAPHY COMBINED WITH A SEMI-AUTOMATIC COMPUTATION OF PLASMA AMINO ACID CONCENTRATIONS

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(Received May 22nd, 1974)

SUMMARY

A method is described for combining the separation of plasma amino acids on a single column amino acid analyzer with semi-automatic computation of the plasma amino acid concentrations. The system used for the quantitation of the amino acids consists of an integrator with a teletype printer fitted with a puncher, and a table calculator with a punch reader and a converter. By using this system, the total time required for the calculation of the molar plasma concentration of 30 amino acids from one analytical run is less than 10 min.

INTRODUCTION

The quantitative determination of the amino acids in physiological fluids is often accompanied by time-consuming work on the planimetric calculation of peak areas followed by manual computation of plasma concentrations of the amino acids. In this paper, a method is described for combining a single-column separation of amino acids with semi-automatic computation of the plasma amino acid concentrations by means of an electronic integrator and a digital table calculator.

EXPERIMENTAL

Analytical system

The separation of the plasma amino acids was performed on a Technicon Type NC-II automatic amino acid analyzer using a single chromatographic column and a continuous buffer gradient. The buffer system used and the operating conditions were derived from the method described by Perry *et al.*[1]. A complete separation of the amino acids could not be achieved by following this method exactly, so a few modifications were made, as discussed below.

The resin column, filled with Technicon Chromobeads Type B resin (17 ± 1 μm spherical particles) was regenerated at 70°, first by pumping 0.2 *M* lithium hydroxide solution through it for 30 min, followed by 0.2 *M* lithium citrate buffer

of pH 2.80 for 100 min. After about eight chromatographic runs, the resin was washed on a glass filter as described by Perry *et al.*[1]; this treatment made the peaks narrower.

The pHs of the first and the second lithium buffer were changed from 2.80 to 2.81 and from 3.80 to 3.36, respectively. This was the only modification to the composition and preparation of the lithium buffers. These changes in the pH of the buffers did not result in an altered order of elution of the amino acids proportional to that found by the original method[1], but the absolute elution times were generally longer.

For the adjustment of the pH of the buffers a Model 26 pH meter (Radiometer, Copenhagen, Denmark) fitted with a scale expander was used. Prior to measurement, the pH meter, which had an accuracy of ± 0.002 pH unit, was calibrated by means of a two-buffer adjustment using a phosphate buffer of pH 6.484 (24°) and a phthalate buffer of pH 4.008 (24°).

The amounts of each lithium buffer placed in the nine-chamber Autograd are given in Table I.

TABLE I

21-HOUR GRADIENT FOR PHYSIOLOGICAL FLUIDS

Chamber	*Methanol (ml)*	*0.2 M Li^+ buffer, pH 2.81 (ml)*	*0.2 M Li^+ buffer, pH 3.36 (ml)*	*1.2 M Li^+ buffer, pH 6.10 (ml)*
1	5	70		
2	5	70		
3		75		
4			75	
5			75	
6				75
7				75
8				75
9				75

The rate at which the eluting buffer was pumped through the resin column was 0.5 ml/min. The column was operated first at 34.8° for 8 h and then at 70.5° for the remainder of the run. The change in temperature was initiated automatically as described by Perry *et al.*[1].

Signal detection system

The electrical output signals from the two single-channel colorimeters measuring at 440 and 570 nm are directly proportional to the concentration of the constituent. These signals drive the recorder, which prints out the peaks linearily with optical density (O.D.). An Infotronic Type CRS-210 integrator receives the linear colorimeter signals and performs an integration with respect to time for each peak. During baseline recording between peaks the integrator automatically and continuously corrects for baseline drift.

Special features are present on the integrator in order to handle unusual conditions such as buffer changes or ammonia plateau and switching from the 570-nm channel to the 440-nm channel. These features can be activated by pre-set timers on the integrator or by external commands from the analyzer.

The retention time and the integrated area for each peak are recorded on a Type ASR-33-TAC teletype printer which is directly connected to and controlled by the integrator, The teletype is fitted with a puncher which produces a punched tape on which retention times and peak areas are recorded.

Computing system

The computing system consists of three components: a Diehl Dilector, which reads the punched tape, a converter, which translates the tape from ASC II code to Diehl code, and a Diehl Algotronic table calculator.

OPERATION

Sample preparation

A 10-ml volume of blood is collected by venal puncture in a glass tube containing 10 mg of EDTA, disodium salt, and immediately centrifuged. A 2.5-ml volume of the EDTA-stabilized plasma is mixed with 100 mg of solid sulphosalicylic acid in a centrifuge tube. After standing for 10 min, the denatured protein is centrifuged for 45 min at about 2100 *g* and 1.00 ml of the clear supernatant is placed on the top of the resin column for analysis.

Programming of the integrator

The Infotronic integrator is started 30 min later than the positive displacement pump in the auto-analyzer system. This delayed start is due to the integrator being programmable only up to 999 min while ammonia and ornithine on the ammonia plateau are eluted later than this. Hence, all elution times recorded by the teletype are reduced by 30 min proportional to the actual elution times on the chromatogram.

Before the integrator is started, it is programmed in order to detect hydroxyproline and proline on the 440-nm channel and to ensure that the amino acids eluted on the ammonia plateau will be correctly integrated (Fig. 1).

Hydroxyproline is eluted about 10 min later than aspartic acid, which has a reduced retention time of about 170 min, *i.e.*, about 200 min on the chromatogram. This means that the integrator is programmed to switch from the 570-nm channel to the 440-nm channel at some time between the retention time of aspartic acid and before the start of the elution of hydroxyproline; the switch-time could be 172 min. The duration on the 440-nm channel is selected to be 19 min, after which the integrator automatically returns to the 570-nm channel.

In order to detect proline, the integrator is programmed in a similar manner. The switch-time is selected to be some time before the elution is started, and the duration should be long enough to ensure that proline has started to be eluted.

Ammonia and the subsequently eluted amino acids are all eluted on the ammonia plateau (Fig. 1). In order to achieve a correct integration of these peaks, it is necessary to program the integrator in such a way that it is able to detect the new baseline on the plateau. Therefore the "buffer switch" is programmed to be activated just before the ammonia plateau begins to occur. The duration should be long enough to ensure that the integrator triggers the new baseline before ammonia starts to be eluted. The second "buffer switch" is programmed to be activated just

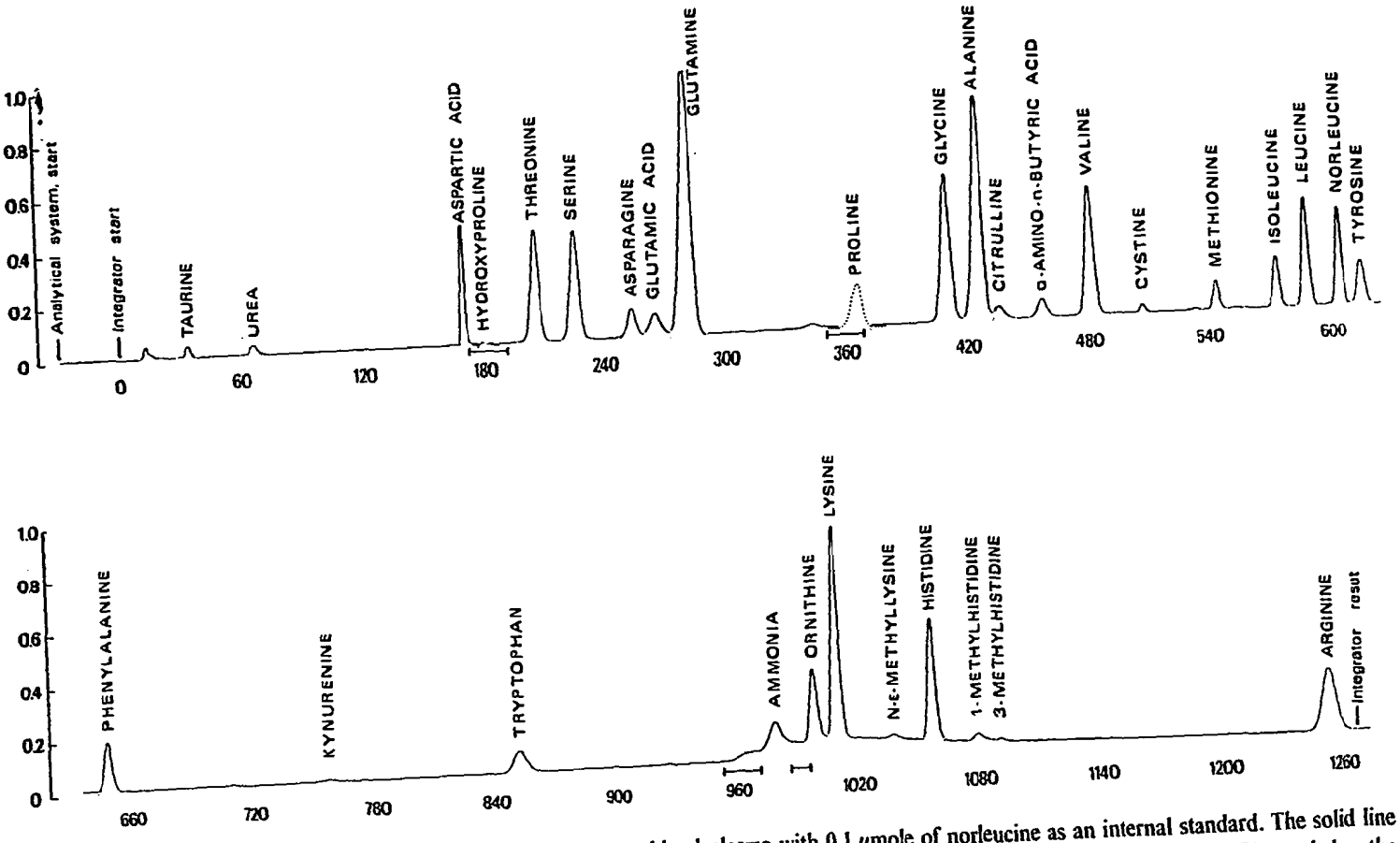


Fig. 1. Trace of a chromatogram of 1.00 ml of deproteinized human blood plasma with 0.1 μmole of norleucine as an internal standard. The solid line indicates optical density at 570 nm and the broken line the optical density at 440 nm. ⊢——⊣, Programming intervals on the integrator. Figures below the traces indicate the reduced elution time in minutes, *i.e.*, the actual elution time less 30 min (see text). Dots below the peaks are produced by the integrator marker pulses, which indicate the start and end of an integration procedure.

after the elution time of ammonia and the duration should be such that it is completed before ornithine begins to be eluted.

A special feature of the integrator is the marker pulses, which indicate when the integration starts and stops and from which baseline the integration takes place. Hence these features indicate a correct or false integration procedure (Fig. 1).

Handling of the punched tape

During an analytical run on a physiological fluid, peaks will be detected that are either unidentified or of no biological interest. The data from these peaks must be omitted from the punched tape before the automatic computation can take place. This is easily achieved by inserting the original paper tape, which is produced during the analysis, in the punch reader on the teletype and starting the puncher. By checking the new transcript on the teletype with the original, it can be seen when the puncher has to be stopped and the original tape moved forwards in order to omit the data from a peak that is of no interest. When this is done, the puncher is re-started and it runs until the appearance of the next set of data that has to be omitted. In this way, the original punched tape is converted into a new punched tape containing the retention times and peak areas of only those peaks which are of interest. The procedure of converting the original punched tape from an analytical run on deproteinized plasma into a new punched tape containing the data for 30 amino acids takes a 2–4 minutes.

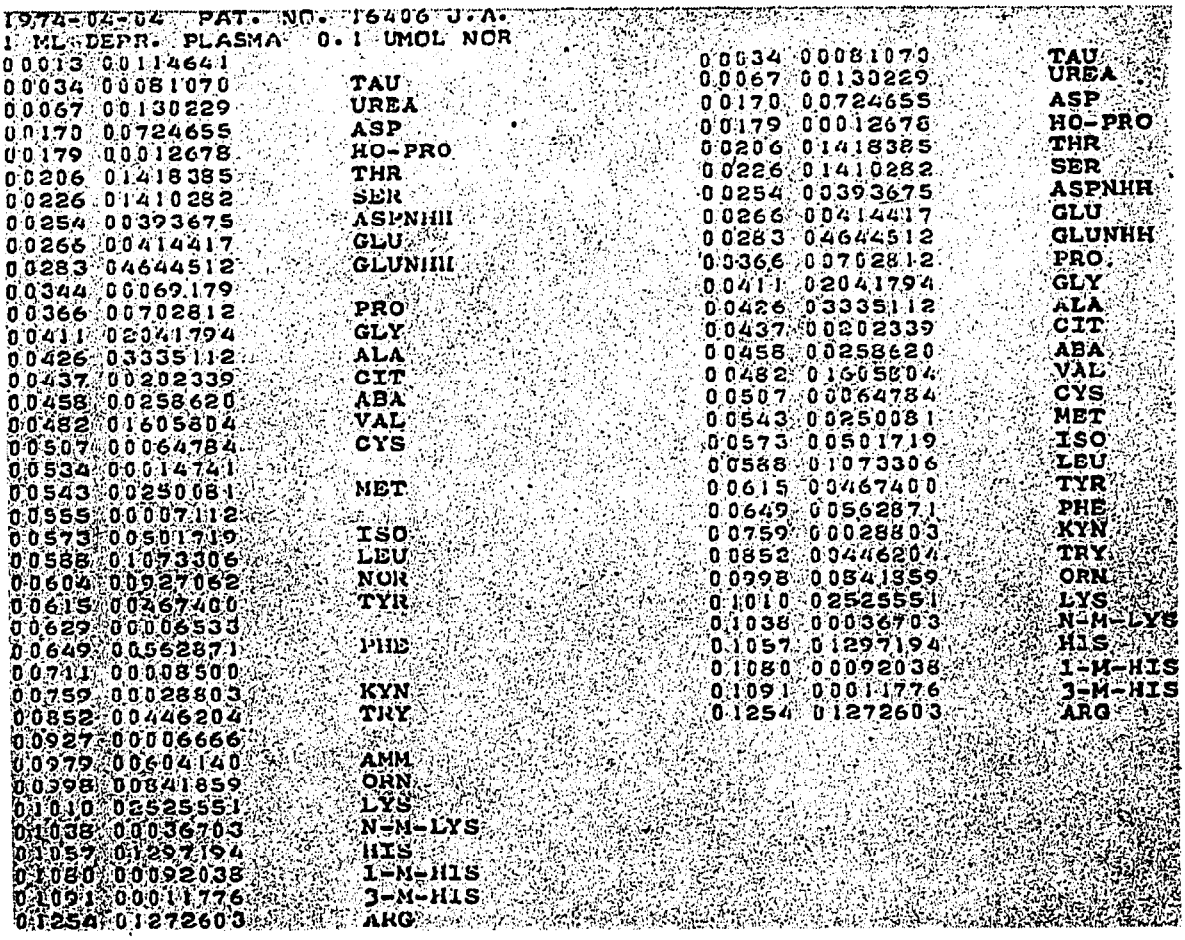
Fig. 2 shows, on the left, the original transcript on the teletype from the chromatogram shown in Fig. 1 and, on the right, the converted transcript produced during the conversion of the original punched tape into the new punched tape. It can be seen that the data from norleucine, which is used as an internal standard, have been omitted in the converted transcript together with the data of those peaks which are either of no biological interest or are unidentified.

Programming of the computer

The Diehl Algotronic calculator can be programmed either automatically by means of a program punch-tape in Diehl code or manually. In the former case, the program strip is inserted in the Diehl Dilector and the "Operate" button is activated. The calculator immediately transcribes the program in clear language, as can be seen in Fig. 3. At the same time, the calculator is programmed for the automatic computation of the plasma amino acid concentrations. In order to execute the program manually, the calculator is programmed from the keyboard by touching the appropriate operating keys in the same sequence as seen in Fig. 3.

The program contains the colour factors for all of the 30 amino acids that are of physiological interest. The value for taurine, which is the first amino acid eluted, is programmed in $\leq$ 30, the value for urea in $\leq$ 29, etc. The colour factor for arginine, which is the latest eluted amino acid, is inserted in $\leq$ 01. The number in $\leq$ P30 replaces the counts for the area data from the integration on the left of the comma. The value in $\leq$ 00 refers to the number of amino acids for which plasma concentrations have to be calculated. This value has to be the same as the number of colour factors in the program. P $\leq$ 00 to P $\leq$ 03 contain the cipher codes that are specific for the calculating program.

The above procedure is common for each calculation of the plasma amino acid concentrations using the converted punched tape. A constant that is specific for



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1974-02-04  PAT. NO. 16406 J.A.
1 ML DEPR. PLASMA  0.1 UMOL NOR
0.0013 0.0114641
0.0034 0.0081070    TAU
0.0067 0.0130229    UREA
0.0170 0.0724655    ASP
0.0179 0.0012678    HO-PRO
0.0206 0.0418385    THR
0.0226 0.0410282    SER
0.0254 0.0393675    ASPNHH
0.0266 0.0414417    GLU
0.0283 0.0464512    GLUNHH
0.0344 0.0069179
0.0366 0.0702812    PRO
0.0411 0.2041794    GLY
0.0426 0.3335112    ALA
0.0437 0.0202339    CIT
0.0458 0.0258620    ABA
0.0482 0.1605804    VAL
0.0507 0.0064784    CYS
0.0534 0.0014741
0.0543 0.0250081    MET
0.0555 0.0007112
0.0573 0.0501719    ISO
0.0588 0.1073306    LEU
0.0604 0.0927062    NOR
0.0615 0.0467400    TYR
0.0629 0.0006533
0.0649 0.0562871    PHE
0.0711 0.0008500
0.0759 0.0028803    KYN
0.0852 0.0446204    TRY
0.0927 0.0006666
0.0979 0.0604140    AMM
0.0998 0.0841859    ORN
0.1010 0.2525551    LYS
0.1038 0.0036703    N-M-LYS
0.1057 0.1297194    HIS
0.1080 0.0092038    1-M-HIS
0.1091 0.0011776    3-M-HIS
0.1254 0.1272603    ARG

0.0034 0.0081070    TAU
0.0067 0.0130229    UREA
0.0170 0.0724655    ASP
0.0179 0.0012678    HO-PRO
0.0206 0.0418385    THR
0.0226 0.0410282    SER
0.0254 0.0393675    ASPNHH
0.0266 0.0414417    GLU
0.0283 0.0464512    GLUNHH
0.0366 0.0702812    PRO
0.0411 0.2041794    GLY
0.0426 0.3335112    ALA
0.0437 0.0202339    CIT
0.0458 0.0258620    ABA
0.0482 0.1605804    VAL
0.0507 0.0064784    CYS
0.0543 0.0250081    MET
0.0573 0.0501719    ISO
0.0588 0.1073306    LEU
0.0615 0.0467400    TYR
0.0649 0.0562871    PHE
0.0759 0.0028803    KYN
0.0852 0.0446204    TRY
0.0998 0.0841859    ORN
0.1010 0.2525551    LYS
0.1038 0.0036703    N-M-LYS
0.1057 0.1297194    HIS
0.1080 0.0092038    1-M-HIS
0.1091 0.0011776    3-M-HIS
0.1254 0.1272603    ARG
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Fig. 2. Left: the original teletype transcript from the chromatogram shown in Fig. 1. The left-hand column of figures gives the retention times of all the peaks that are detected during the analytical run and the right-hand column includes the peak area counts in arbitrary units. Right: the converted teletype transcript produced by the conversion of the original tape into the new punched tape that is used for the automatic calculation. The data for norleucine, ammonia and unidentified peaks are omitted.

each calculation is inserted in P $\leq$ 31. This constant is the product of the number of millilitres of deproteinized plasma used for analysis with the number of counts representing the area of norleucine in this specific run, diveded by the amount of norleucine in micromoles.

The calculator is now ready to be started. The converted punched tape is inserted in the Diehl Dilector and the converter, which translates the tape from ASC II code to Diehl code, is activated. After pushing the "Operate" button, the table calculator starts to read out the retention times and the plasma concentrations (μmole/ml) for each of the 30 amino acids. Part of the read-out is shown in Fig. 4.

The automatic programming of the calculator by means of the program

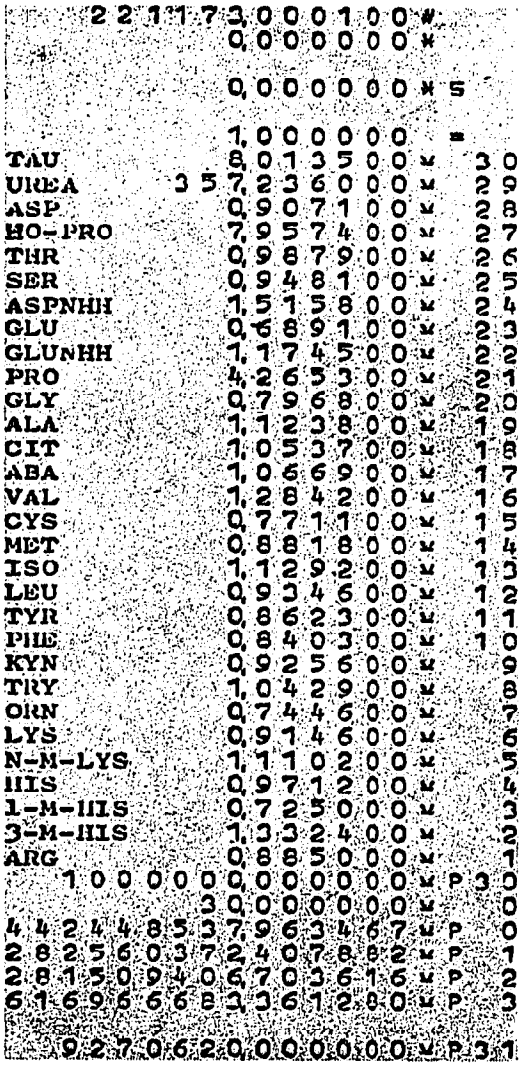

Fig. 3. Program transcript from the calculator produced during the automatic programming of the calculator by means of a program punched tape .The last value in P ≍ 31 is inserted manually. For details, see text.

punched tape takes 1 min and the automatic calculation of the plasma concentrations of 30 amino acids by means of the converted punched tape takes 4 min.

RESULTS

A synthetic standard containing the same amino acids as found in human plasma is made up in such a way that the concentration of each amino acid is about the same as that found in the plasma. Eight analytical runs were carried out on the synthetic standard and the mean colour factor ± the standard error of the mean

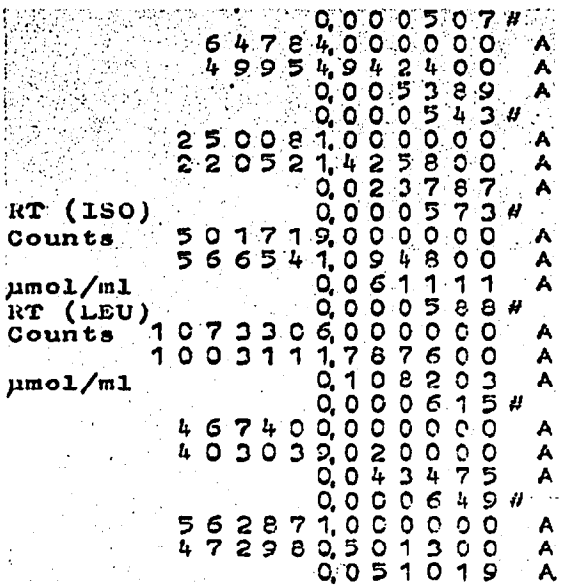

Fig. 4. Part of the calculator read-out produced during the automatic calculation of the plasma amino acid concentrations by means of the converted data punch-tape. The read-out contains the retention times and amino acid concentrations (µmole/ml) for each of the amino acids.

(S.E.M.) is calculated for each amino acid. Most of the amino acids are determined quantitatively with such an accuracy that the S.E.M. on the colour factor is less than 1% of the colour factor value. However, when determining 0.05 µmole of taurine, the S.E.M. is 2%, for 2.50 µmole of urea 3.5%, for 0.02 µmole of hydroxyproline 1.2% and, in determining 0.10 µmole of ammonia the S.E.M. on the colour factor is 3% of the mean colour factor.

Except for ammonia, the rather high S.E.M. values (exceeding 1%) are due to the small peaks. The complications that arise during the determination of ammonia are due to the time of elution of ammonia, which takes place during the increase of the ammonia plateau.

In order to determine the upper limit for linear integration in relation to the optical density, a series of norleucine standards of different concentrations in 0.1 *N* hydrochloric acid was prepared. Each standard was pumped through the analytical system for 120 sec and the peak area was automatically integrated and recorded on the teletype. Between the peaks, 0.1 *N* hydrochloric acid served as a blank solution. Three experiments were carried out on each norleucine standard. The results are shown in Table II.

The two weakest norleucine standards produce peaks within the limits of the recorder paper, which represents 1.0 O.D. unit. The optical density measured on these peaks is used when calculating the theoretical optical density for those peaks which exceed 1.0 O.D. unit. The last column shows the mean transcript from the integrator in counts ± standard deviation (S.D.). Fig. 5 shows the graph produced from the data in Table II. It can be seen from Fig. 5 that the integration is linear with optical density up to about 2.0 O.D. units.

TABLE II

DETERMINATION OF THE UPPER LIMIT FOR LINEAR INTEGRATION

Norleucine standards in different concentrations were soaked through the sample tube for 120 sec.

Norleucine concentration (μmole/ml)	*O.D.*		*Integrator counts ± S.D. (n = 3)*
	Measured	*Calculated*	
0.05	0.263	—	425907 ± 9719
0.10	0.533	—	876311 ± 13055
0.25	—	1.325	2047959 ± 27746
0.35	—	1.855	2880236 ± 20065
0.50	—	2.650	3559773 ± 15749
0.75	—	3.975	4188222 ± 22888
1.00	—	5.300	4604875 ± 9467

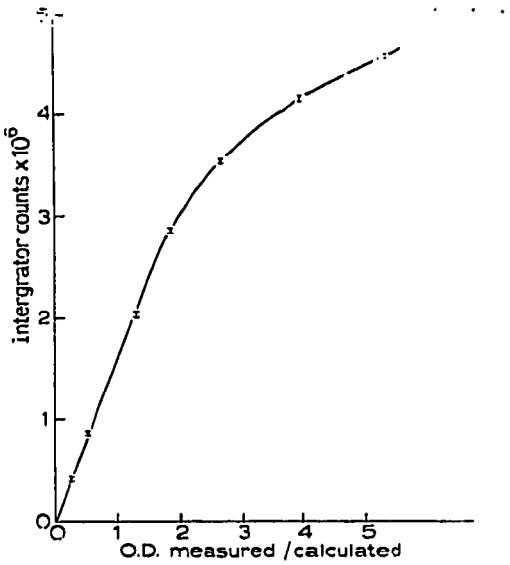

Fig. 5. Graphical illustration of the data from Table II showing a linear integration performance up to about 2.0 O.D. units. Bars represent the mean ± standard deviation of three determinations.

DISCUSSION

The special way of connecting the integrator to the analytical system has many advantages. As the integrator is not connected to the recorder but to the colorimeters, any mechanical or electrical noise from the recorder will not disturb the integration procedure.

Glutamine is often present in plasma in such amounts that its peak height during a routine analytical run will exceed the maximum of 1.0 O.D. unit on the linear recorder. When integrating manually on the recorder paper, this problem can be overcome by installing a second 570-nm colorimeter with a shorter flow cell. If

the first 570-nm colorimeter shows a sample off-scale, the second colorimeter will give a readable peak.

During the direct connection of the integrator to the colorimeters, the 570-nm colorimeter with the short flow cell can be omitted because a correct integration procedure depends essentially on the colorimeters, and they give a linear reading even when the optical density is above 1.0. This has been demonstrated by pumping norleucine in different concentrations through the analytical system and comparing the integrator transcript with the calculated optical densities for the peaks.

The integrator used is fitted with several features that make the instrument convenient for the integration of any type of peak. Among these features is the peak sensor gain, which determines the peak sensor sensitivity; this function is selectable in five positions. The tracking rate, measured in optical density units per minute, is also selectable and this function determines the maximum rate at which the automatic zeroing can change. Other important functions are the minimum peak area, which determines the minimum area counts that will be reported, and the threshold level, which determines the threshold level measured in optical density units above which integration cannot be terminated; this function is important in the case when baseline drift will occur during the recording of a peak.

The table calculator together with the punch reader and the converter are low-cost computer accessories. It has proved to be a very time-saving system for the automatic calculation of the plasma concentrations of amino acids. As the calculating system is not directly connected to the teletype output from the integrator, the system will be occupied for only about 5 min for each analytical run. This means that the table calculator can be used for other purposes, *e.g.*, the calculation of data from a liquid scintillation system where the data are presented on a punched tape in Diehl code or ASC II code.

REFERENCE

1 T. L. Perry, D. Stedman and S. Hansen, *J. Chromatogr.*, 38 (1968) 460.