A NEW SYSTEM OF AUTOMATIC AMINO ACID ANALYSIS

PART I

ANGELO MONDINO
Istituto Ricerche Marxer, Ivrea, Torino (Italy)
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So far evaluation of the peaks in automatic amino acid column chromatography has been a very tedious, time-consuming and a rather imprecise stage in this kind of analysis. Usually manual calculation is made using the formula $H \times W/C$, where H is the net absorbance of the peak, W the width at half net absorbance (in terms of number of dots, when a point recorder is used), and C a constant obtained by standardization[1]. This formula is supposed to be valid only when the symmetry of the peak is perfect, but it is known that even a little overlapping or a slight skewing are causes of deviation from a true Gaussian form and this often occurs.

However, since the color reaction obeys the Lambert-Beer's law, it will be shown in this paper that it is possible in an automatic system to realize the conditions for evaluating the amounts of material in the effluent in terms of peak heights only.

It will easily be appreciated that one of the first conditions required is the necessity to obtain a strict repeatibility in the flow-system, both when analysis of the standards and of the sample are carried out.

One of the main causes of poor repeatibility are the reciprocating micropumps normally used to feed the system. These pumps very easily vary their pumping rates due to the presence of small air bubbles which may develop inside them and remain trapped for some time.

On the other hand, the operation of degassing buffers and reagents is always very critical and it is very difficult to assess whether it has been completely accomplished.

Therefore new syringe-type pumps, whose distinctive features permit the elimination of these troubles, have been devised.

Another very important requirement of the system is obviously the reduction in the time of analysis. In this connection the preparation of the resin and the determination of the size (height and width) of the columns in relation to the nature and grade of the resin itself as well as to the flow-rates in the system, are of paramount importance.

Also the minimum volume of resin still giving the right number of theoretical plates in relation to the quantity of solute applied, as well as to the speed of elution, deserves careful attention; however, this problem will be dealt with in another paper.

The chromatography is a modification of the method of SPACKMAN, STEIN AND MOORE[1]; two columns of the same resin are used. Analysis, including the results of the shorter column takes less than 170 minutes.

Two recorders of the solid-line type providing a linear response in optical

density are employed, so that it is possible to measure the height of the peaks with a ruler and to give the results directly in millimeters.

In this way, the reading of a chromatogram takes no more than two or three minutes, is positively objective, precise and easy.

As far as the sensitivity is concerned, the colorimeters are equipped with a range expander which allows the determination of a few nanomoles. In Fig. 9 an example of a chromatogram of a standard mixture of 25 nanomoles is reported. It should be stressed that this chromatogram was carried out in 170 minutes with a total flow-rate in the colorimeter of 2.2 ml per minute, which means a very high dilution of the color. If the problem of sensitivity were of primary importance, it would be very easy to obtain an increase of sensitivity by using a lower flow-rate both of buffer and reagent; in this case it would be advisable to employ a longer column with a smaller diameter for the same volume of resin.

MATERIAL AND METHODS

Resin and columns

Amberlite IR 120 resin, industrial grade and mesh was purchased. The dried resin was ground in a mill, and after a previous screening through a 200 mesh sieve, was regraded by the method of HAMILTON[2]. The fraction showing a diameter of 25–30 μ by microscopic examination in a Buerker's chamber, was used.

This resin was used to fill the long and the short column, after several washings on a suction funnel first with $6N$ nitric acid, then with $4N$ hydrochloric acid and finally with boiling $4N$ sodium hydroxide[3]. Packing was made according to BENSON AND PATTERSON[4].

The optimal sizes of the columns for protein hydrolyzate analysis were found experimentally to be the following: for the long column, total length 75 cm, diameter 0.9 cm, length of the resin bed 60 cm (measured just after packing); for the short column, total length 35 cm, diameter 0.8 cm, length of the resin bed 20 cm.

On such columns and with this resin, the buffers were pumped at 1.4 ml/min; the resolution was still very good and the pressure did not exceed 20 kg/cm^2.

The columns are jacketed and thermostated by circulating water. The short column is in series with the long one. The water bath can be selectively held at 40° and at 60°.

Pumps

The apparatus uses six syringe-type pumps, as can be seen in Figs. 1 and 2. Fig. 3 illustrates schematically a single pump. The metal cylinder is internally lined with Teflon and has a volume of 250 ml. The stainless steel piston is equipped with special gaskets which can stand pressures up to 80 kg/cm^2, and can be driven selectively by two motors.

One of the motors can move ahead and reverse quickly and is employed to charge the pump rapidly, each pump being permanently connected with its own reservoir and to the system by Teflon tubing.

The second motor is synchronous and drives the piston during analysis. Its motion is transmitted to the piston by means of interchangeable gears which can provide five different speeds. In this way there is no more need for a flowmeter to reset

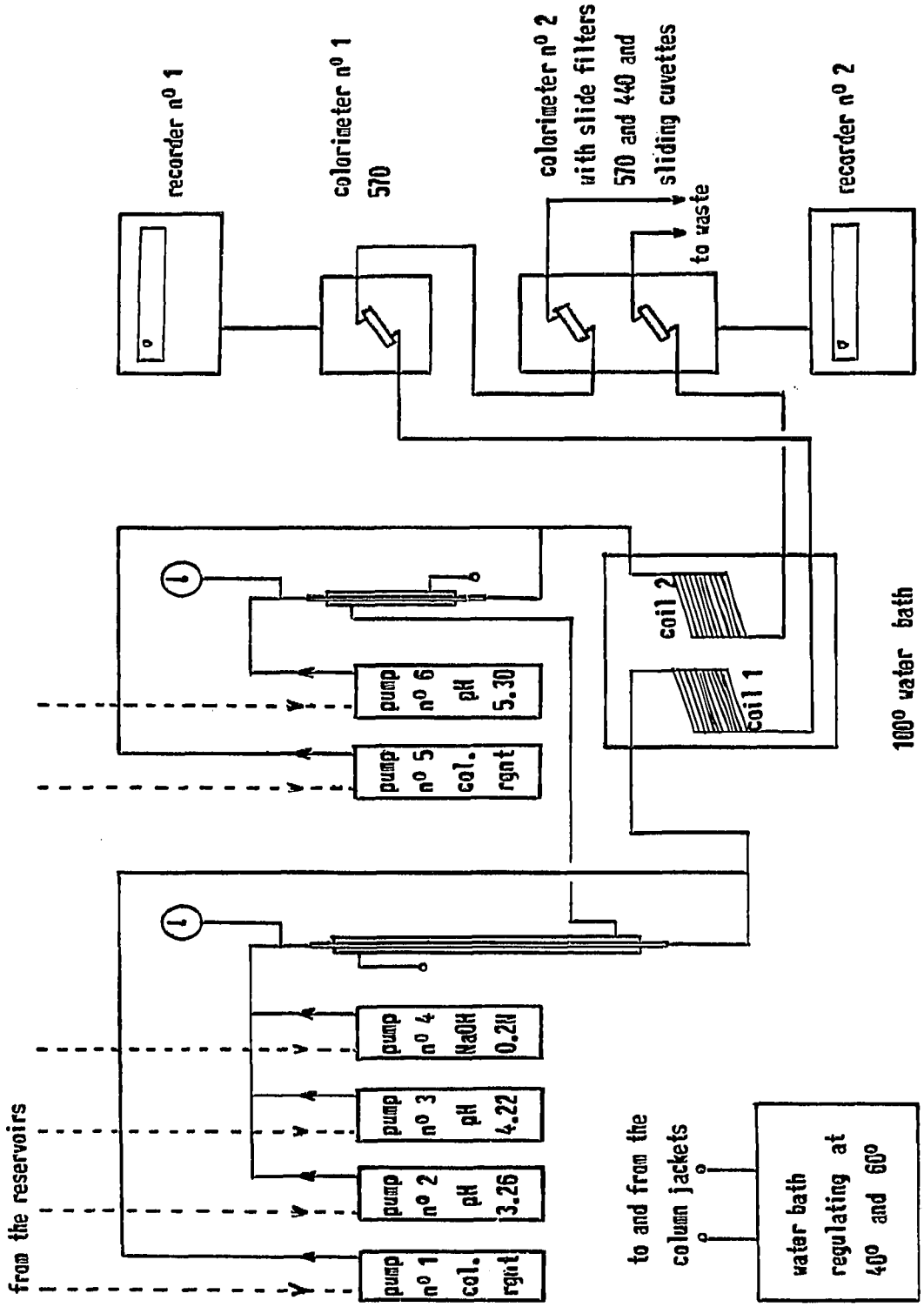

Fig. 1. Flow diagram of the system.

and check the flow-rates when they are changed, inasmuch as the discrete flow-rates are standardly repeatable.

The head of each cylinder is provided with a two-way valve for filling and for operation.

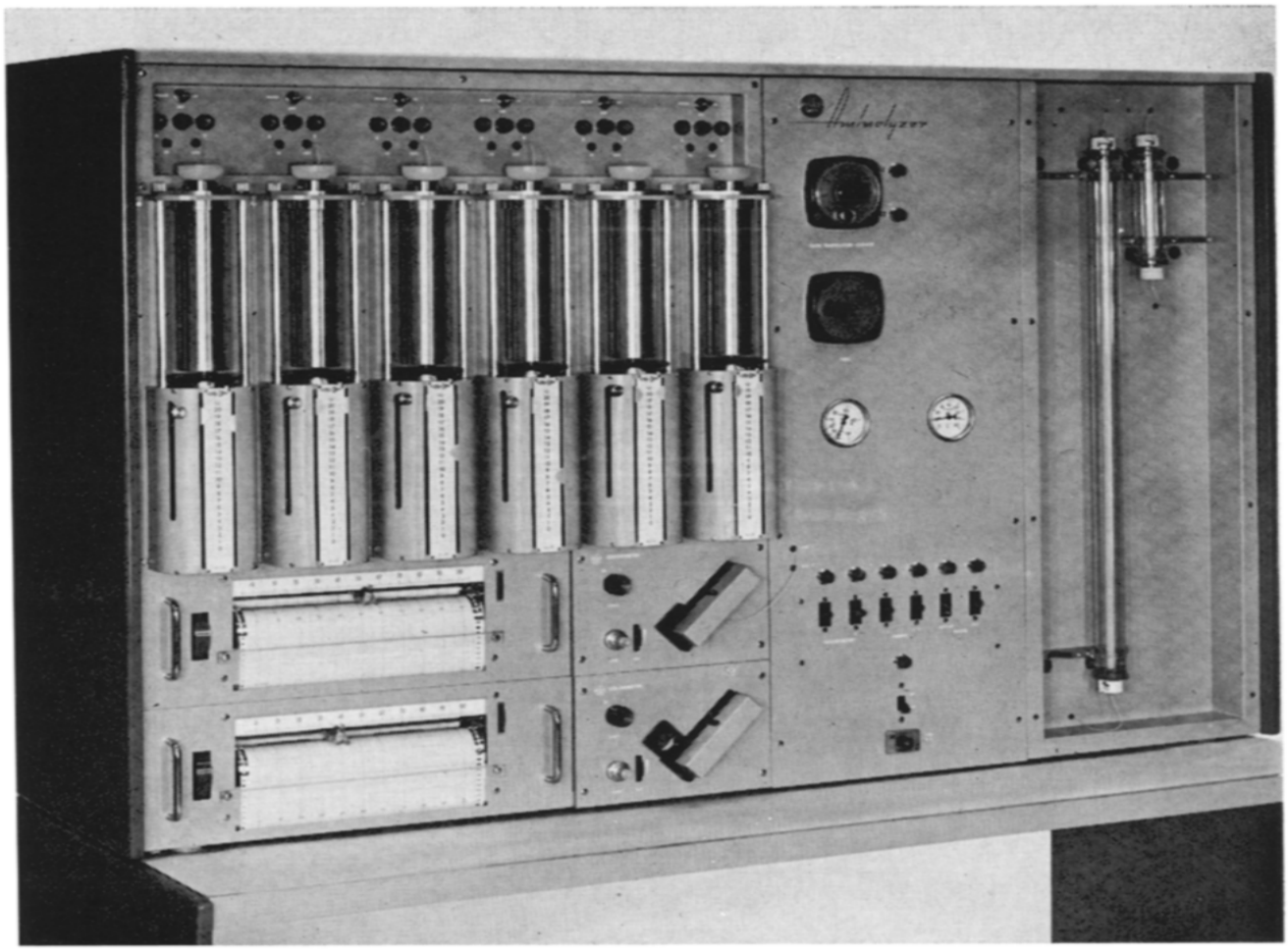

Fig. 2. Photograph of the prototype.

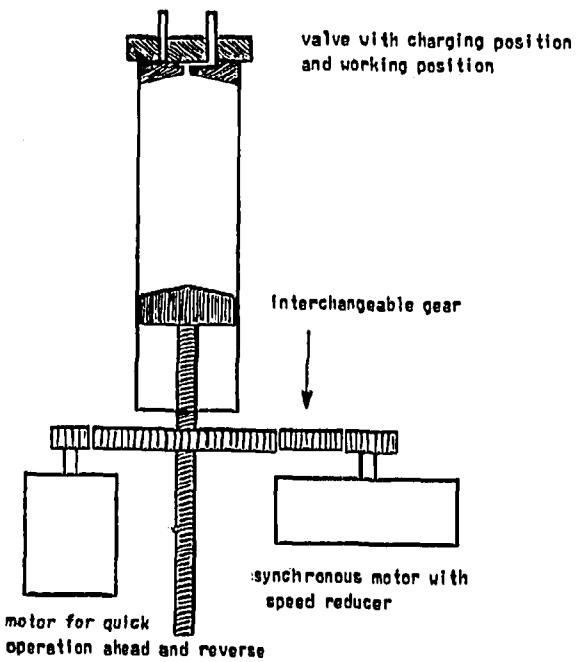

Fig. 3. Schematic section of a pump.

During its run the piston of each pump can operate an adjustable micro-switch which can stop the pump at any desired volume and at the same time can start the successive pump, provided the latter has been previously preset. In this way each pump acts also as a timer.

Colorimeters and recorders

The system employs two colorimeters equipped with interchangeable filters. They can hold flow-cuvettes up to 50 mm in optical path. For the analyses shown in this paper 20 mm cuvettes with a diameter of 2 mm and with a volume of only 0.063 ml were employed. In this way "washing" of the cuvette is very effective.

The cuvettes are cylindrically shaped, made of metal internally lined with Teflon and the quartz windows are plane and parallel.

They are permanently connected, by AWG 22 Teflon tubing, with the flow-system, and, as shown in Fig. 1, the second colorimeter holds two cuvettes on a moving slide; each one of them can be selectively introduced into the light beam.

The light sources are only 2 W and are electronically stabilized.

The photosensitive element is a photomultiplier, whose electronic circuit includes a range expander which permits one to choose a sensitivity of either 0.5, 1 or 2 O.D. units for full scale deflection, and gives a linearized signal which is recorded on two solid-line recorders.

The chart speed usually employed is 3 in. per h.

Reaction coils

There are two coils, one for the long column and a second one for the short column, made of AWG 22 Teflon tubing each 80 m long, immersed in a boiling water bath.

At the total flow-rate of 2.2 ml/minute (1.4 of eluate plus 0.8 of color reagent) it means that the reaction mixture is at 100° for more than 15 minutes.

Buffers and reagents

The buffers for elution of the columns are prepared according to SPACKMAN, STEIN AND MOORE[1] in batches of 50 l. Their pH is adjusted to 3.26, 4.22 and 5.30 respectively, using an expanded scale pH-meter and approximating the third decimal figure. This instrument must be highly reliable. It is advisable to calibrate it with standard buffers of pH near to the working values.

The pH 5.5 buffer for the color reagent is also prepared according to SPACKMAN *et al.*[1]. The color reagent is prepared daily with ninhydrin (Merck) and with hydrindantin prepared in our laboratory by reduction of ninhydrin with ascorbic acid. The methylcellosolve employed is also purified and stabilized in our laboratory. Great care is taken in the preparation of this reagent, which is prepared each time in quantities of 500 ml corresponding to the capacity of the two pumps. A stream of oxygen-free nitrogen is bubbled for at least 15 min both through the buffer and methylcellosolve before use. 10 g of ninhydrin and 0.5 g of hydrindantin are dissolved in 370 ml of methylcellosolve, always under nitrogen, and then 130 ml of pH 5.5 buffer are added.

Before introducing it into the pump, the transmittance is measured at 475 mμ in a spectrophotometer. A good preparation must read about 40 % of transmittance. It has been observed on hundreds of chromatograms that this value can range from

35 % to 45 % without giving appreciable differences in the height of the amino acid peaks. In Fig. 4 the spectrum of this reagent is reported.

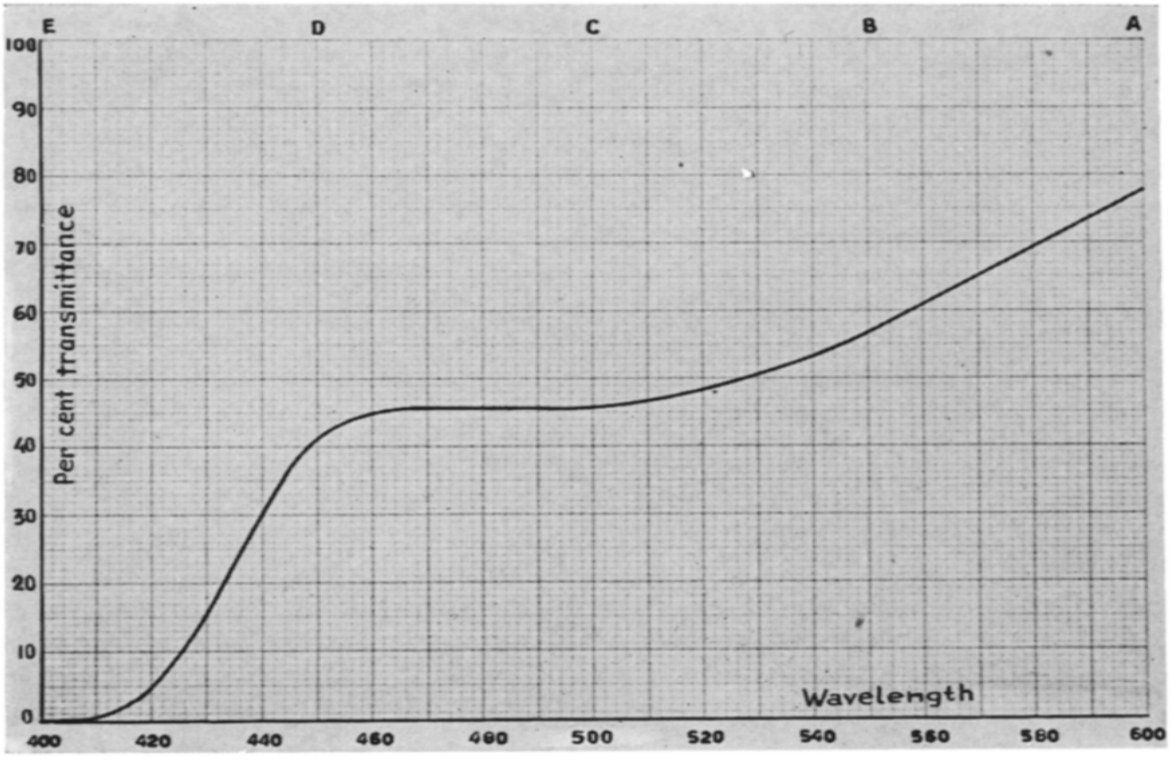

Fig. 4. Spectrum of ninhydrin color reagent.

Operation of the equipment

As can be seen in Fig. 1, in this system there are two channels. One includes the long column, pumps No. 1, No. 2, No. 3, and No. 4, a mixing T, one reaction coil and two cuvettes (one on the first colorimeter and one on the second colorimeter). The second channel includes pump No. 5 and pump No. 6, the short column, a mixing T, one reaction coil and a cuvette on the second colorimeter.

Let us suppose that a standard chromatogram is run at 2 O.D. sensitivity full scale. In this case one will introduce on top of the resin, according to the usual technique, 0.1 ml of the standard solution containing 100 nanomoles of each amino acid, on both columns, which have been previously regenerated.

The pumps are filled as indicated in Table I, which also provides the relative flow-rates.

TABLE I

PUMP FILLING AND SETTING SCHEDULE

Pump No.	*Filling*	*Flow rate (ml/min)*
1	Color reagent	0.8
2	Buffer, pH 3.26	1.4
3	Buffer, pH 4.22	1.4
4	0.2 *N* NaOH	1.4
5	Color reagent	0.8
6	Buffer, pH 5.30	1.4

TABLE II

ROUTINE DAILY SCHEDULE

Time	*Lapsed time (min)*	*Operations*
8:30 a.m.	0	The apparatus is switched on automatically to warm up.
9:00 a.m.	0	Charge the pumps as specified in Table I and start long column analysis. Substitute in the short column the buffer over the resin with 0.2 *N* NaOH (about 6 ml) and then start pumping buffer, pH 5.30, again in order to regenerate the resin.
10:20 a.m.	80	The proline is out, then start the short column analysis.
11:50 a.m.	170	The *first* analysis is complete in both channels. Automatically pump No. 4 starts pumping NaOH through the long column for 15 min (21 ml) and then pump No. 2 will automatically equilibrate the resin again by pumping buffer, pH 3.26, for 25 min (35 ml). Charge the pumps.
12:40 p.m.	0	Start long column analysis. Substitute in the short column the buffer over the resin with 0.2 *N* NaOH (about 6 ml) and then start pumping buffer, pH 5.30, again in order to regenerate the resin.
2:00 p.m.	80	The proline is out, then start the short column analysis.
3:30 p.m.	170	The *second* analysis is complete in both channels. Automatically pump No. 4 starts pumping NaOH through the long column for 15 min (21 ml) and then pump No. 2 will automatically equilibrate the resin again by pumping buffer, pH 3.26, for 25 min (35 ml). Charge the pumps.
4:20 p.m.	0	Start long column analysis. Substitute in the short column the buffer over the resin with 0.2 *N* NaOH (about 6 ml) and then start pumping buffer, pH 5.30, again in order to regenerate the resin.
5:40 p.m.	80	The proline is out, then start the short column analysis.

From now on the apparatus can proceed by itself unattended. It will finish the *third* analysis and regenerate the long column, then automatically it will be switched off.

TABLE III

RESULTS EXPRESSED IN TERMS OF PEAK HEIGHTS IN MILLIMETERS OF STANDARD CHROMATOGRAMS RUN AT 100 AND 50 NMOLE LEVELS

Amino acid	*Chromatogram No.*											
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*
	100 nmole level						*50 nmole level*					
Aspartic acid	118	117	116	119	117	117	58.5	58	58	58.5	59	58.5
Threonine	111	110	109	112	111	110	55.5	55	55.5	56	55	56
Serine	118.5	118	120	119	119	118	60	59.5	59	59	60	59
Glutamic acid	93.5	94	94.5	93	93.5	93	47	46.5	47	46.5	47	47
Proline	19.5	19.5	19	20	19	20	10	10	9.5	10	10	10
Glycine	85.5	85	86	86	84	85	43	43.5	43	42.5	43	42.5
Alanine	79	79.5	80	80.5	79	80	40.5	40	39.5	40	40	39.5
Cystine	59	56	58	57	55	56	28	29.5	29	28.5	29	29
Valine	56	55.5	56	57	55	56	28	27.5	28	28	27.5	27
Methionine	142	142.5	143	142	141	141	71.5	72	71	71.5	71	72
Isoleucine	126.5	127	127	128	125	126	64	63	63.5	63	64	64
Leucine	124	124	125	127	126	125.5	62.5	63	63	62	63	63
Tyrosine	84.5	85	86	86	85	84.5	42.5	42.5	43	43	43	42.5
Phenylalanine	78	77.5	77	78	77	78	38.5	39	38	38	38.5	38.5
Tryptophan	54	53	53.5	54.5	54	53	27	27	26.5	27	27	26.5
Lysine	92	94	93	94	92	93	47	46.5	46	46.5	47	47
Histidine	75	76	77	76	77	76	38.5	38	38.5	38.5	39	39
Ammonia	125	120	130	128	131	124	66	63	70	63.5	67	65.5
Arginine	39	38.5	39	39	38.5	39	19.5	19	19	19.5	19	19.5

Colorimeter No. 1 is set at 570 mμ while colorimeter No. 2 is set at 440 mμ; the stream passes through the first and then through the second before going to waste. The sensitivity of both colorimeters is set at 2 O.D. full scale.

At this point pumps No. 1 and No. 2 are put into operation.

Pump No. 1 is preset for pumping until pump No. 3 stops.

Pump No. 2 is preset to pump 130 ml of buffer, pH 3.26, and then to stop after having started pump No. 3, which will pump 126 ml of buffer, pH 4.22.

At this point pump No. 3 stops, as the chromatogram of the acidic and neutral amino acids is complete (after 170 min), and pump No. 4 will start pumping NaOH to wash the resin, as pump No. 1 has stopped pumping color reagent.

Colorimeter No. 1 is reading and recording throughout all this period.

Just after the proline has been recorded on both recorders, the 440 mμ filter in colorimeter No. 2 is substituted by a 570 mμ filter, and the cuvette of the second channel is slid into the light beam; thus the second colorimeter from now on can read and record on recorder No. 2 the basic amino acids, whose elution from the short column has now begun by switching on pump No. 6. Pump No. 5 starts pumping color reagent for the short column eluate.

Operating in this way the chromatogram of the basic amino acids and the chromatogram of the acidic and neutral amino acids are complete at the same time.

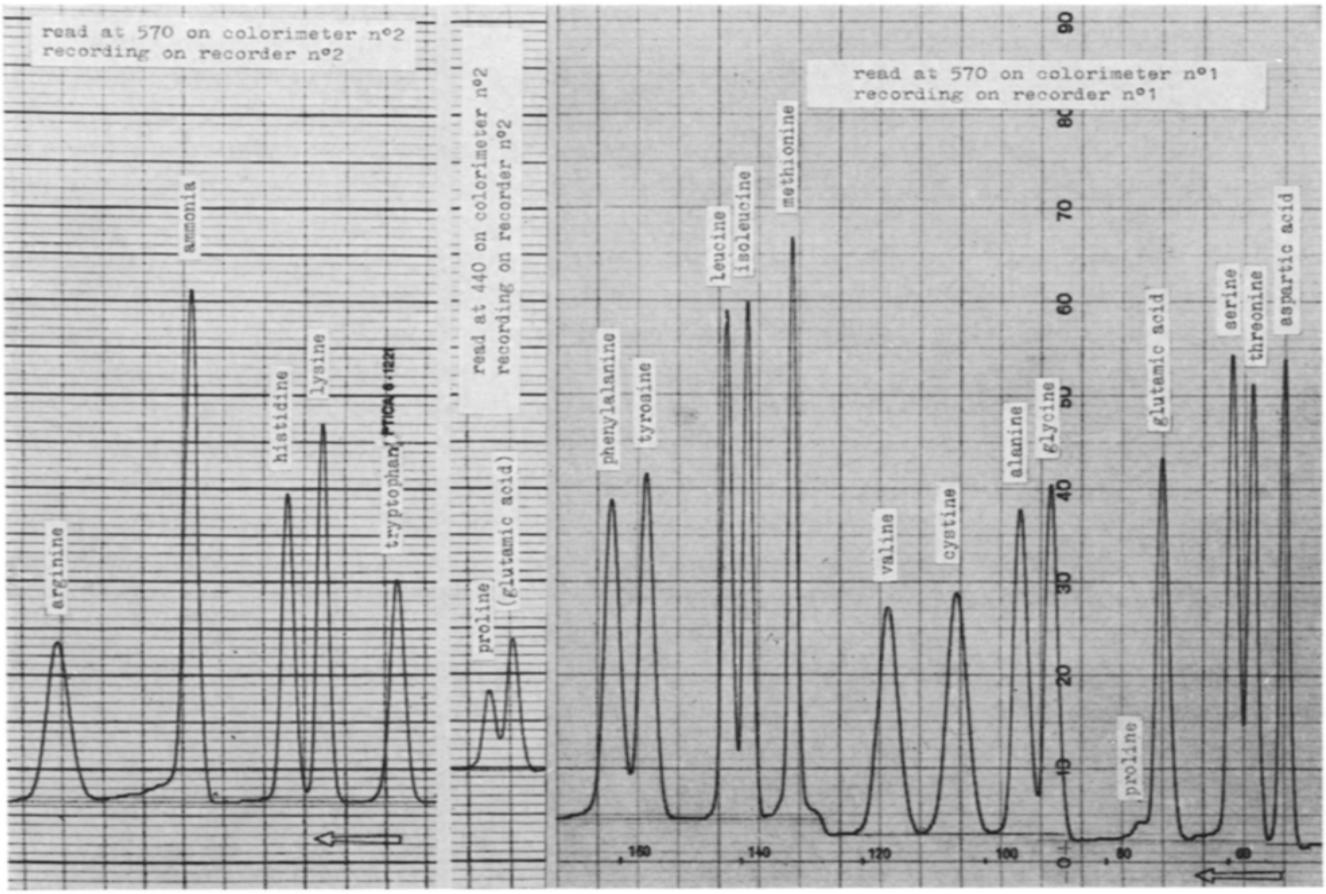

Fig. 5. Recording of chromatogram No. 1 of an amino acid mixture containing 100 nmoles of each amino acid. The beginning of the chromatogram is on the right-hand side. Sensitivity: 2 O.D. full scale. Chart speed: 3 in./h. The basic amino acids have been separated during the run of the acidic and neutral ones, so that the total time of analysis is 170 min.

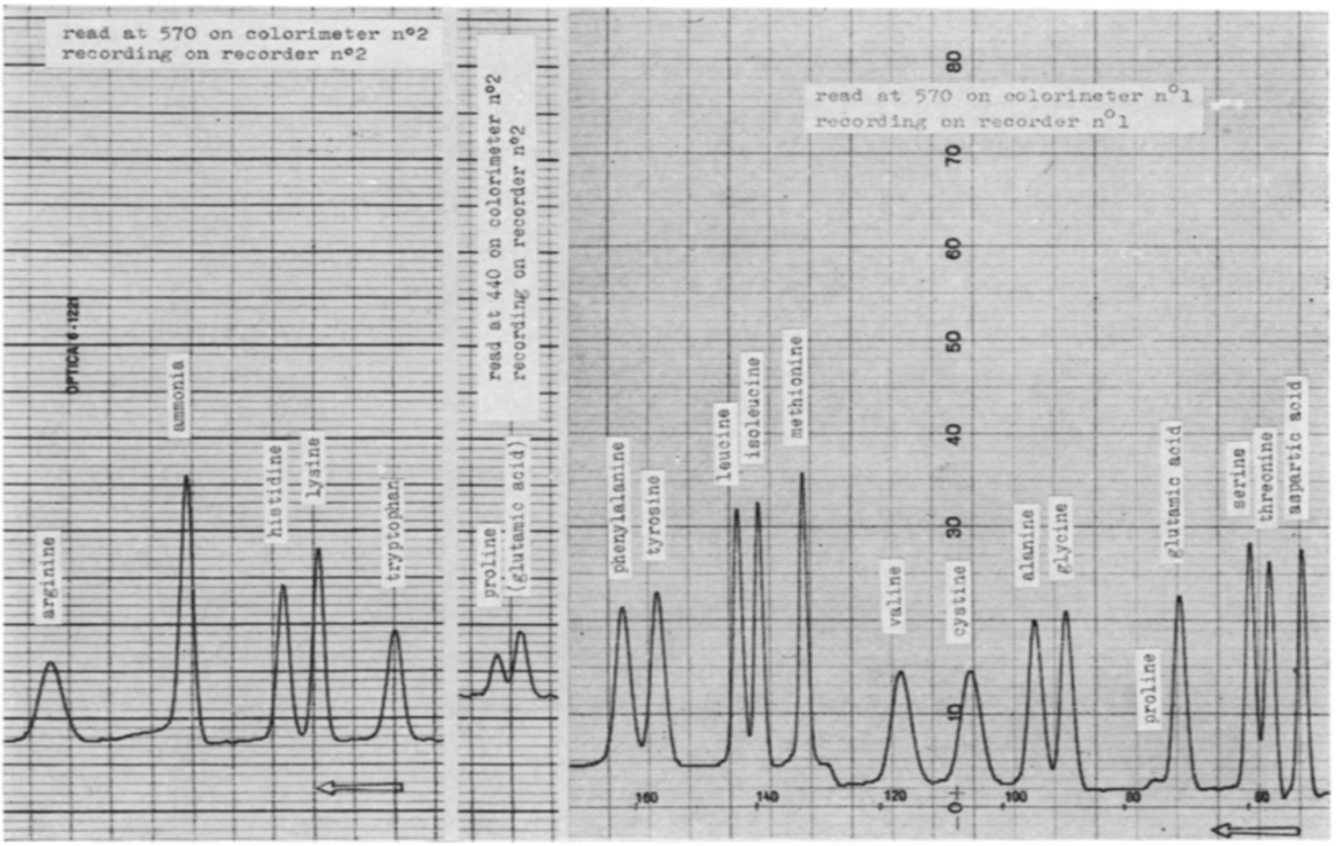

Fig. 6. Recording of chromatogram No. 7 of an amino acid mixture containing 50 nmoles of each amino acid. The beginning of the chromatogram is on the right-hand side. Sensitivity: 2 O.D. full scale. Chart speed: 3 in./h. The basic amino acids have been separated during the run of the acidic and neutral ones, so that the total time of analysis is 170 min.

As far as the temperature change from 40° to 60° is concerned, it takes place at the 35th minute and it is operated by an adjustable micro-switch, which has been preset on pump No. 1. When this pump stops pumping, the temperature is brought again automatically to 40°.

A routine daily schedule for three complete analyses including the regeneration of both columns is summarized in Table II.

RESULTS AND DISCUSSION

To determine the accuracy and the precision of the system, six analyses were performed at 100 nanomole level and six at 50 nanomole level with a sensitivity of 2 O.D. full scale.

The results of these analyses are shown in Table III and are expressed in terms of the peak heights *H* measured with a ruler in millimeters above the base-line.

The results of the chromatograms No. 1 and No. 7 shown in Figs. 5 and 6, are drawn on squared paper as illustrated in Figs. 7 and 8 in order to show the strict obedience of this system to Beer's law.

Only ammonia slightly deviates from linearity. As can be seen in Table III the reproducibility for this substance is not very good. As a matter of fact the color yield for ammonia is strictly dependent upon the concentration of hydrindantin in the color

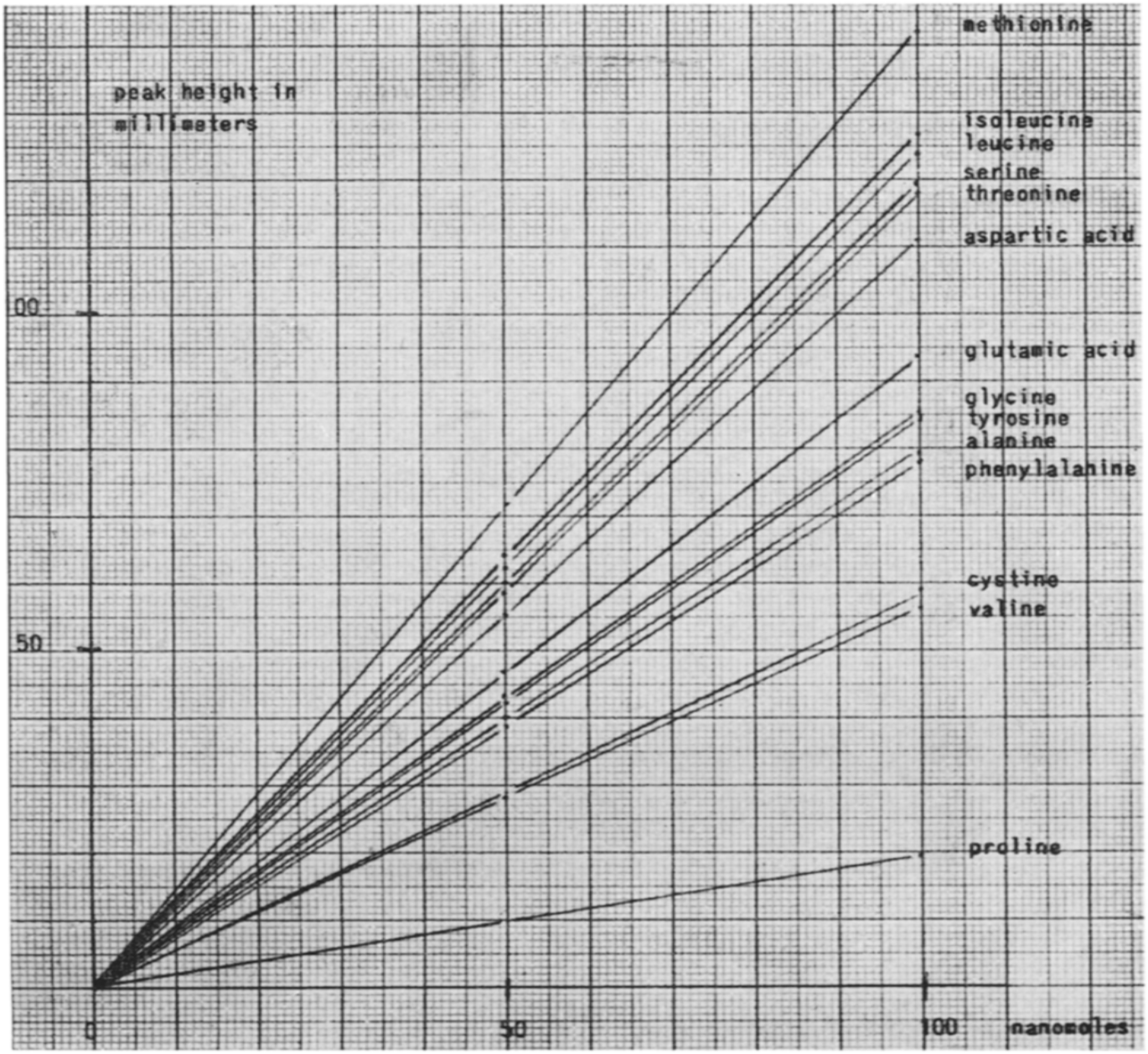

Fig. 7. Plotted results of chromatograms No. 1 and 7 (acidic and neutral amino acids) measured in terms of peak heights in mm, showing a strict linearity for all the amino acids.

reagent. Therefore if it were required to assay ammonia accurately, the color reagent should read a constant value of transmittance as indicated above. Otherwise it must be corrected for its hydrindantin content.

In Fig. 9 a chromatogram performed at 25 nanomole level with a sensitivity of 0.5 O.D. full scale can be seen. The base-line noise is still so slight that a sample of less than 5 nanomoles of each amino acid can be evaluated quite well. The rise of the base-line due to the break-through of pH 4.22 buffer after valine, has become more evident, actually four times higher than it was at the 2 O.D. sensitivity level.

The asymmetry of the peak of cystine, which is very evident at this sensitivity level, is very probably due to mesocystine present in the standard. In fact, all the

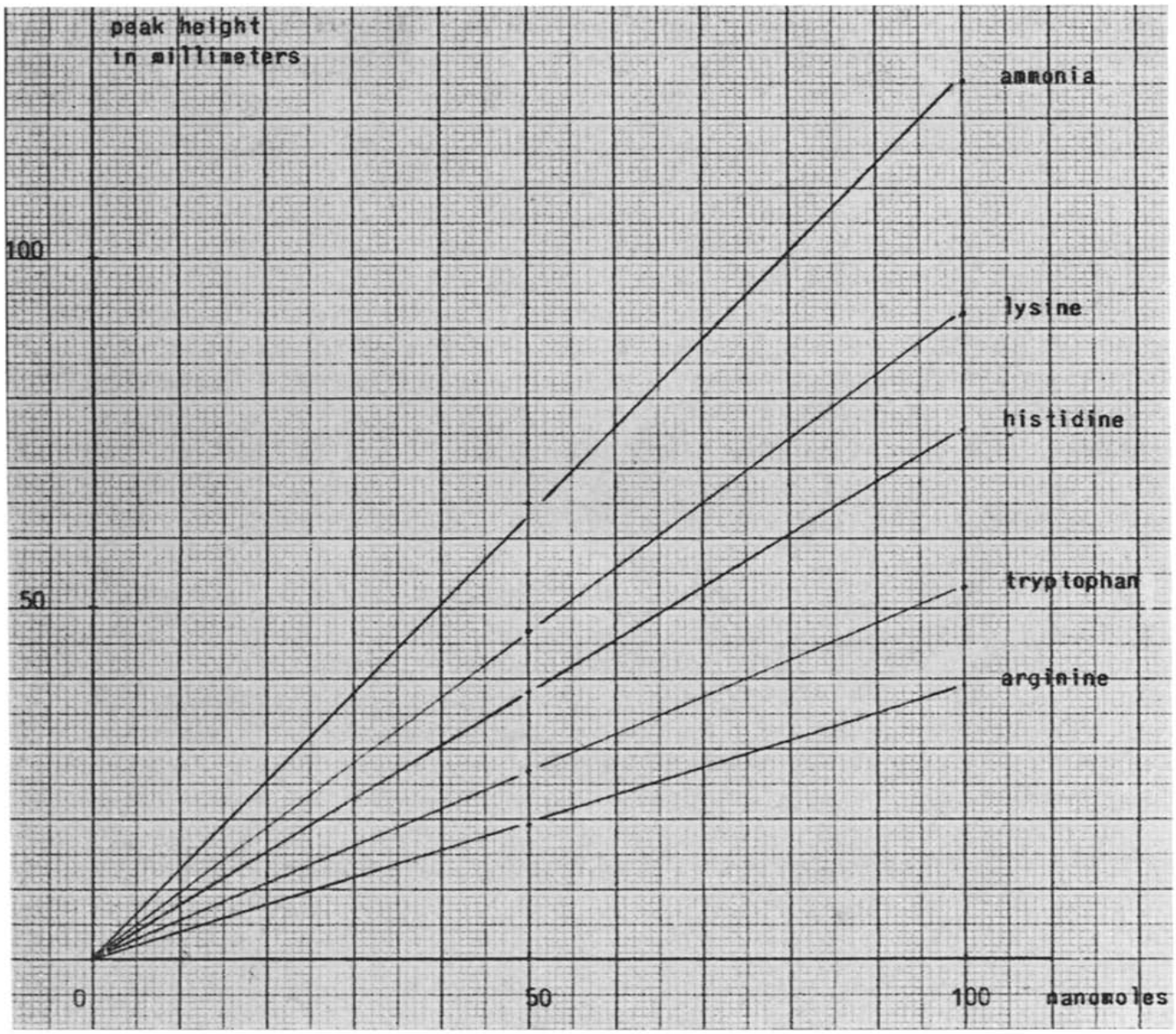

Fig. 8. Plotted results of chromatograms No. 1 and 7 (basic amino acids) measured in terms of peak heights in mm, showing a strict linearity for all the amino acids except ammonia.

chromatograms shown in this paper were performed with standard solutions of 95 % purity grade amino acids manufactured by B.D.H. Company, London.

The results are reported in Table IV, where an extraordinary coincidence with the results obtained at the 100 nanomole level with a sensitivity of 2 O.D. full scale, can be noticed.

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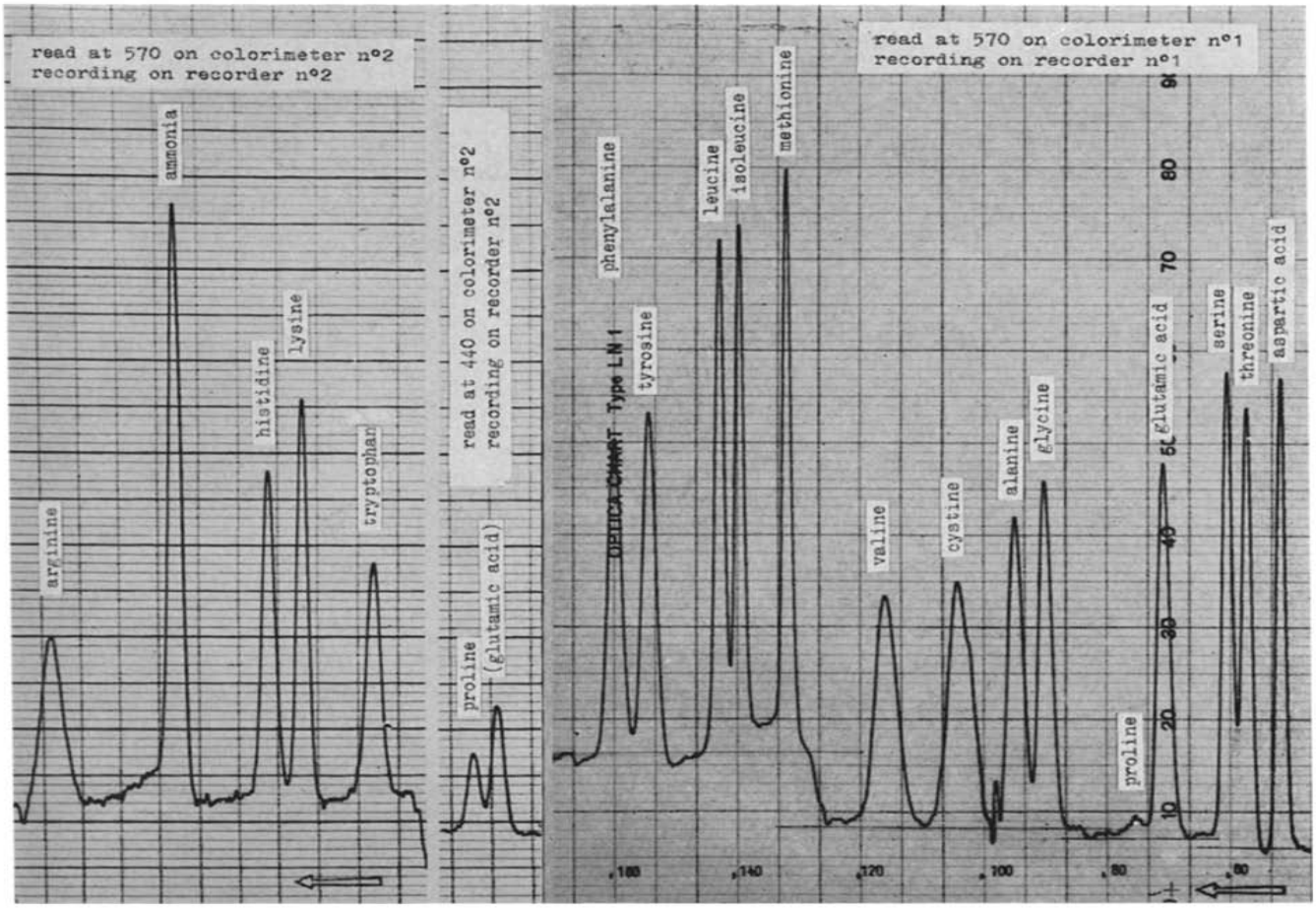

Fig. 9. Recording of the chromatogram of an amino acid mixture containing 25 nmoles of each amino acid. The beginning of the chromatogram is on the right-hand side. Sensitivity: 0.5 O.D. full scale. Chart speed: 3 in./h. The basic amino acids have been separated during the run of the acidic and neutral ones, so that the total time of analysis is 170 min.

TABLE IV

RESULTS EXPRESSED IN TERMS OF PEAK HEIGHTS IN MILLIMETERS OF THE CHROMATOGRAM SHOWN IN FIG. 9

Amino acid	*Peak height (mm)*	*Amino acid*	*Peak height (mm)*
Aspartic acid	117	Isoleucine	130
Threonine	110	Leucine	126
Serine	118	Tyrosine	85
Glutamic acid	93	Phenylalanine	78
Proline	20.5	Tryptophan	56
Glycine	86	Lysine	96
Alanine	78	Histidine	78
Cystine	59	Ammonia	146
Valine	57	Arginine	39
Methionine	144		

SUMMARY

A new two-channel automatic apparatus for amino acid analysis based on ion exchange chromatography, is described. New syringe-type pumps and recording colorimeters, linear with respect to optical density, have been used, so that the peaks of the chromatogram can be evaluated simply by measuring their height. The total run, including elution of arginine, takes less than 170 minutes. A degree of sensitivity as great as a few tenths of a nanomole can be reached very easily. Three chromatograms can be performed in an 8-hour day.

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