THE EFFECT OF MINERAL NUTRIENT DEFICIENCY ON THE CONTENT OF FREE AMINO ACIDS IN *SETARIA SPHACELATA*

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Abstract

The effects of several single mineral nutrient deficiencies on the free amino acid composition of *S. sphacelata*, grown in water culture, have been examined. Most deficiencies result in an accumulation of the free amino acids. Severe copper deficiency causes a large increase in the alanine content of the leaf. Nitrogen deficiency causes decreases in almost all amino acids.

The data was subjected to an information-statistic classification and multivariate analysis. Zinc deficiency was shown to have the greatest effect, followed by copper.

No single deficiency could be diagnosed by the changes produced in the free amino acid content of the grass.

An amino acid analyser suitable for estimating free amino acids in plant material is described in the Appendix.

I. Introduction

For some years *Setaria sphacelata* has been studied for its value as a productive grass species for the subtropical regions of Australia. Investigations by Birch, Dougall, and Hodgson (1964) showed that *S. sphacelata* differed in several respects from other African grass species, e.g. it was found to have a high ammonia content at certain stages of growth accompanied by a high titratable acidity.

In view of this behaviour and the importance of amino acids in nitrogen metabolism it was decided to investigate the ammonia and free amino acid content of the grass under various nutrient deficiencies. Hodgson (1964) had previously examined the free amino acids at various stages of growth under adequate nutritional status. The effect of mineral nutrient deficiency on the nitrogen compounds of plants and grasses has been investigated by several workers including Richards and Templeman (1936) who showed that the concentration of free amino acids and of amides of barley increased in phosphorus or potassium deficiency. Coleman (1962) reviewed the role of nutrient deficiency in nitrogen metabolism. Further it was hoped that the cause of a red striping of the leaf similar to the symptoms of iron deficiency and which had been observed in field and pot trials on several occasions would be found. The data from

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the present experiment have been examined to determine whether there was a relationship between treatment and amino acid content, and whether the latter could be considered diagnostic or characteristic of the former.

II. Materials and Methods

(a) *Plant Culture*

Seed of *S. sphacelata* cv. Nandi was germinated in HCl-washed sand, and 14 days later was transferred to nutrient solution (Arnon 1938) in 2-litre Pyrex beakers, for all except the experiments on boron deficiency where boron-free glassware was used. There were five seedlings per beaker and three replicates of each treatment. The deficiencies examined were N, P, K, Ca, Mg, Cu, Zn, Mn, Fe, Mo, B, and S. Nutrient change was effected by omitting an element or using a lower concentration in the otherwise complete nutrient solution. Two levels of deficiency were used: (1) minus the element; (2) concentration of element one-tenth of that in the control solution. In the Mo and B experiment only level (1) was used. One replicate of each of Mo, B, and low Mn treatments were later abandoned. The three replicates of the minus Zn treatment were pooled on harvest to obtain an adequate sample size. With three controls, a total of 64 samples was obtained. Iron was introduced twice weekly as ferrous citrate to a concentration of 0·6 p.p.m. Fe. The nutrient solution was aerated with filtered compressed air for 5 min in every half hour. The plants were grown in the glasshouse under natural lighting with a 13-hr day length. Visual signs of deficiency developed on most of the minus treatments and were similar to those noted by C. S. Andrew (personal communication), e.g. in zinc deficiency the main symptoms were yellowing necrotic tips of the leaves with marked interveinal chlorosis and very poor growth. Sulphur deficiency caused slight yellowing of the leaf with red margin and tip wither. Nitrogen deficiency caused yellowing of the leaves and appearance of a pink margin spreading inwards with time. Signs of extreme deficiency were present at 6 weeks of age.

(b) *Preparation of Plant Material Extracts*

The plants were harvested after 6 weeks of treatment. The leaves and stems were removed at 1 in. above the crown. The mass of leaf and stem was noted and 15–20 g (where possible) was extracted with ice-cold 75% ethanol–water within 15 min of harvesting. The sample was macerated for 2 min in a Waring blender then passed through No. 1 Whatman filter paper and the filter-cake washed with ethanol–water (3 : 1 v/v) till the filtrate was colourless. The filtrate was concentrated on a rotary evaporator using a 40°C water-bath. The concentrated solution was removed from the flask and the precipitated chlorophyll washed three times with water, the washings being added to the original concentrate. The volume was adjusted to give the extract a concentration of approximately 5 g of original plant fresh weight per millilitre. This solution was stored at −20°C. Ethanol extracts not concentrated on the day of harvest were also stored at −20°C.

(c) *Analysis*

Qualitative examination of all grass extracts was made with two-dimensional paper chromatography using the descending method of Consden, Gordon, and Martin (1944). A phenol–water (4 : 1 w/v) mixture was used as the first solvent on Whatman No. 1 paper followed by the butanol–acetic acid–water (9 : 1 : 2·5 v/v) solvent of Steward *et al.* (1955) in the transverse direction. Where identity of the amino acid was in doubt other solvent mixtures were used, e.g. lutidine–water.

For quantitative analysis a modified Paton–Simmonds automatic amino acid analyser, was used. This incorporated Aminex 150S resin and sodium citrate buffers (Moore, Spackman, and Stein 1958). The sulphur-containing amino acids were not estimated as some degradation occurs in both the extraction and estimation. Proline was not estimated quantitatively. Soluble nitrogen estimations were done on the same samples as the amino acid analyses, using a Kjeldahl digest technique developed in this laboratory and which does not measure nitrate nitrogen. The unidentified non-protein nitrogen fraction is the remainder of the total soluble nitrogen after the identified compounds have been accounted for.

III. Results

(*a*) *Individual Amino Acids*

The data were examined to determine whether or not a relationship between single deficiencies and individual amino acid content was such that it could be used for diagnostic purposes. This was done by arranging the 64 values for each amino acid in order of magnitude and examining the set for major discontinuities of the type in which all replicates of a treatment or possibly groups of treatments were on one side of the discontinuity with the remainder of the values on the other. No such discontinuity was observed. The order set is shown graphically in Figure 1 for two

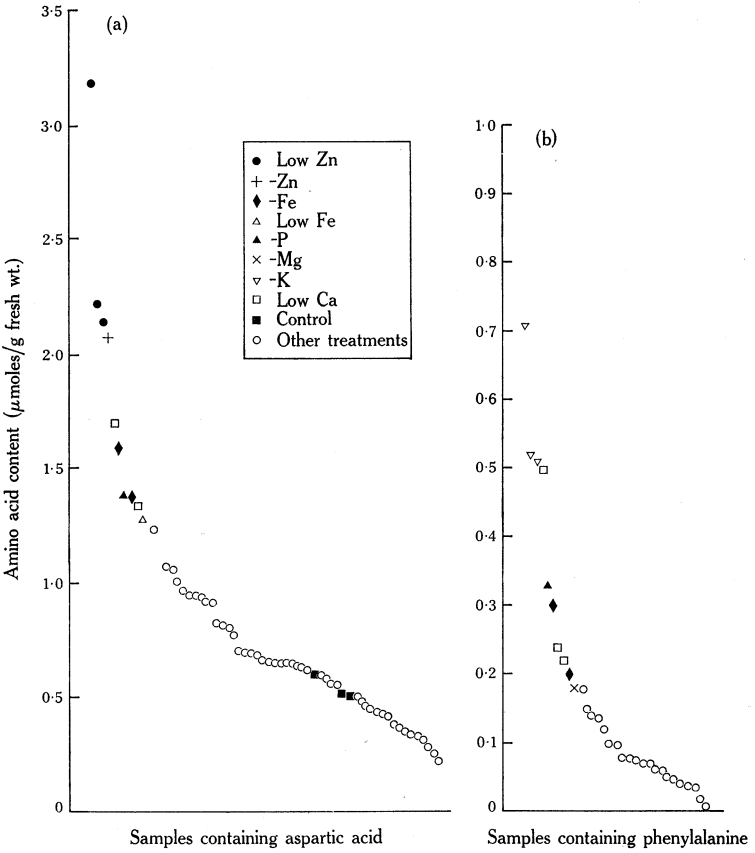

Fig. 1.—Order set showing the concentration in *S. sphacelata* of two amino acids (*a*) aspartic acid, present in all samples, and (*b*) phenylalanine, detected in 31 samples, plotted in descending order of values for all samples with non-zero values. This also shows the treatments producing the highest 10 concentrations for these amino acids.

amino acids: (1) aspartic acid present in all samples; and (2) phenylalanine, which was not detected in 33 samples including all the controls. All the remaining sets showed a similar smooth decline.

The general properties of the low-value end of all the sets were examined. The number of samples in which the amino acid was not detected is summarized in Table 1. For tyrosine, phenylalanine, tryptophan, and arginine the number is very high. However, tryosine and phenylalanine were not detected in all the controls, so that the only possible effect of a deficiency was the abnormal production of these acids; tryptophan and arginine were present in all of the controls, but were so frequently absent elsewhere that they would obviously be suppressed by a wide range of deficiencies. The particular interest lay in any case where the number of complete absences was small or zero, but all replicates of a particular deficiency had low values. As an arbitrary cut-off point the last 10 (zero or non-zero) values were examined for all except the four amino acids mentioned above. Any treatment, all of whose replicates fall within this last 10, has been included in Table 1. Minus N appears four times; but although minus B, minus Mo, and low S also appear, the curves fall so slowly in this region, that the low concentrations are useless diagnostically.

TABLE 1

EFFECT OF NUTRIENT DEFICIENCY ON THE PRESENCE OF SOME NITROGEN COMPOUNDS IN *S. SPHACELATA*

64 samples from 12 single-element nutrient-deficiency experiments at two levels of deficiency were analysed for the compounds listed. The table shows (1) the number of samples in which the nitrogen-containing compounds were not detected, and (2) the deficiencies in which all replicates were included in the lowest 10 values for that particular compound

Compound	No. of absences	Deficiency	Compound	No. of absences	Deficiency
Aspartic acid	—		Tyrosine	23	
Threonine	3		Phenylalanine	30	
Serine + asparagine			γ-Aminobutyric acid	2	
+ glutamine	—	−N	Ammonia	—	−N
Glutamic acid	—		Lysine	—	
Glycine	—		Histidine	—	
Alanine	—	Low S	Tryptophan	16	
Valine	—	Low S	Arginine	30	
Isoleucine	8	−N, −Mo	Unidentified non-protein		
Leucine	9	−B, −Mo	nitrogen	—	−N

The high-value end of each set was then examined. The treatments corresponding to the first 10 values have been included in Figure 1; and as examples the consistent behaviour (despite the large intra-treatment variation) of low Zn for aspartic acid, and minus K and low Ca for phenylalanine may be seen. Those treatments for which such high level concentrations occurred for all replicates among the first 10 values have been summarized in Table 2. The most striking feature of this table is the strong "blocking" effect shown: deficiencies in P, Cu, and Zn produced excess quantities of the more acid amino acids and including the unidentified residue, which may therefore belong in this region; K, Ca, and Mg produce increases in the basic amino acids and ammonia. S and Mn similarly affect the more basic fractions. The complete isolation of the three blocks suggests that they are reflections of three quite distinct phenomena.

The quantitative aspects of these relationships were investigated. Unfortunately, consideration of mean values of the nitrogen compounds was complicated by the poor agreement between replicates of certain treatments, and for more detailed examination it seemed desirable to exclude from discussion those treatments whose replicates failed to behave consistently. Two screening procedures were used. First, those treatments which had shown the consistent effects summarized in Tables 1 and 2 were retained. Secondly, the entire (64 × 19) matrix was subjected to a conventional information-statistic classification (the program used was the Canberra taxonometric program MULTBET) and a level sought at which, for as many treatments as possible, all replicates had fused. Most of the fusions were complete by the time the population had been fused into five groups. Treatments not consistent in Tables 1 and 2, and

TABLE 2

EFFECT OF NUTRIENT DEFICIENCY ON THE RELATIVE LEVEL OF SOME NITROGEN COMPOUNDS IN *S. SPHACELATA*

+ marks the deficiencies having all replicates in the highest 10 values for the compounds estimated

Amino acid	Deficiency							
	P	Cu	Zn	K	Ca	Mg	S	Mn
Unidentified non-protein nitrogen	+		+					
Aspartic acid			+					
Threonine		+	+					
Serine+amides	+		+					
Glutamic acid			+					
Glycine		+	+					
Alanine		+	+					
Valine								
Isoleucine				+	+	+		
Leucine								
Tyrosine				+	+			
Phenylalanine								
γ-Aminobutyric acid				+				
Ammonia				+	+			
Lysine							+	
Histidine							+	+
Tryptophan								
Arginine								

not fused at the five-group level, were discarded; these were low Cu, minus S, low Mg, low Mn, minus Fe, low Fe. Since minus Zn was based on a single bulked sample, and since it almost everywhere behaved precisely as low Zn, it too was discarded. Finally, one of the four controls also behaved anomalously and was discarded. The resulting set of replicated controls and 15 treatments will be referred to when necessary as the "restricted set" of data.

Table 3 shows, for this restricted set, the control means and the deviation of the treatment means from the control means. This table, too, shows some striking features. Considering first the treatments, the columns for minus N and low N are predominantly negative; the only treatment approaching these in its tendency to cause a reduction of amino acid fractions was minus B. Of the amino acids, the rows for tryptophan and arginine are almost everywhere negative. With these exceptions, however, the table is overwhelmingly non-negative; in other words, all deficiencies except N, and possibly B, tend to result in the accumulation of amino acid fractions, presumably as a result of the impairment of protein synthesis. As expected, the main increases were in the "serine and amides" fraction, and in ammonia as these

TABLE 3

EFFECT OF VARIOUS SINGLE NUTRIENT DEFICIENCIES ON THE NITROGEN COMPOUND ANALYSIS AND YIELD OF S. SPHACELATA

Results are expressed in μmoles/gram fresh weight of plant material for all values except yield which is grams fresh weight

Amino acid	Control	Deviation from control mean for following treatments:														
		−N	Low N	−P	Low P	−K	Low K	−Ca	Low Ca	−Mg	Low S	−Cu	Low Zn	−Mn	−Mo	−B
Aspartic acid	0·48	−0·17	−0·11	0·58	0	0·05	0·05	0·85	0·37	0·24	0·24	0·49	2·04	0·48	0·04	0·17
Threonine	0·14	−0·09	−0·13	0·05	−0·02	0·03	0	0·05	0·07	−0·08	0	0·66	0·49	0·04	0·04	−0·01
Serine + amides	1·35	−0·89	0·24	13·96	1·02	2·92	0·35	12·12	6·88	2·59	3·92	11·57	43·12	0·71	−0·06	0·27
Glutamic acid	0·89	−0·16	−0·03	0·35	0·03	−0·01	0·09	1·46	0·65	−0·14	0·04	1·08	2·11	0·71	0·66	0·43
Glycine	0·08	−0·03	−0·01	0·27	0·02	0·12	0	0·26	0·14	0·10	0·09	0·37	0·41	0·07	0·12	0·01
Alanine	1·40	−0·78	−0·45	0·52	−0·50	−0·05	0·14	0·44	−0·01	0·07	−0·91	11·0	3·13	1·27	−0·56	−0·05
Valine	0·21	−0·10	0·18	0·34	0	0·34	0·04	0·22	0·19	0·06	−0·13	0·32	0·35	0·08	0·01	−0·06
Isoleucine	0·05	−0·05	0	0·10	0·03	0·18	0·04	0·12	0·42	0·06	0·09	0·08	0·10	0	−0·05	−0·02
Leucine	0·01	−0·01	0·06	0·09	0·05	0·14	0·06	0·09	0·06	0·12	0·10	0·12	0·10	0·05	−0·01	−0·01
Tyrosine	0	−0·01	0·10	0·01	0·07	0·13	0	0·07	0·05	0·32	0·06	0·20	0·13	0·02	0·03	0
Phenylalanine	0	0	0·01	0·11	0·06	0·58	0·01	0·32	0	0·13	0	0·11	0·06	0·05	0·03	0
γ-Aminobutyric acid	0·05	0·06	0·03	0·25	0·18	0·58	0·17	0·37	0·06	0·28	0·18	0·52	0·51	0·37	0·19	0·04
Ammonia	5·51	−4·7	−1·02	19·89	14·27	38·7	11·58	38·22	4·99	11·46	10·99	15·69	36·64	7·81	1·30	7·29
Lysine	0·09	0·17	−0·02	0·15	0·06	0·1	0·04	0·07	−0·03	0·05	0·48	0·23	1·12	0·18	0·15	0·13
Histidine	0·16	0·08	0·05	0·20	0·02	0·22	−0·02	0·26	0·09	0·12	0·50	0·31	1·25	0·54	0·26	0·12
Tryptophan	0·16	−0·12	−0·14	−0·12	−0·11	0	−0·12	−0·06	−0·06	−0·06	−0·13	−0·11	0·56	−0·15	−0·09	−0·15
Arginine	0·20	−0·16	−0·10	−0·11	−0·20	−0·14	−0·17	−0·04	0·20	−0·15	−0·20	0·30	0·51	−0·16	−0·19	−0·20
Unidentified non-protein nitrogen	19·31	−10·13	−10·03	111·28	12·56	44·03	1·95	76·75	14·97	12·98	−2·46	67·88	147·25	5·39	16·82	5·02
Yield (g)	107·7	−88·3	−51·0	−82·7	−26·3	−68·7	−33·0	−54·0	−29·7	−33·7	−33·7	−79·3	−88·7	−81·7	−35·2	−70·2

represent the main nitrogen pools; but the very large increases in the "unidentified" fraction suggest that this fraction would repay further attention. In the whole table there is only one example of a large increase specifically associated with a single deficiency; this is the almost 10-fold increase in alanine caused by Cu deficiency. This might be useful for diagnosis; though care would be needed to exclude the possibility that Zn was involved, since this also produced a marked increase in alanine.

(*b*) *Multivariate Examination*

The complete system, of 64 samples associated with 18 measurements of nitrogen fractions and one of yield, was subjected to a principal component analysis. The result was dominated by a first component, accounting for 46% of the total variance in the system, which was simply a negative correlation of all fractions with yield. It is seen that the concentration of most amino acids increased, and it may be assumed that the greater the increase, the greater the impairment of protein synthesis; this component expresses the extent to which impairment of protein synthesis reduces yield.

The restricted set of data, this time using treatment means and excluding yield, was then subjected to a Q component analysis (i.e. an analysis whereby the configuration of the *treatments* was examined directly). The first component was dominated by Zn and, to a less extent, Cu; the second by K; and the third again by Cu. The effects of Zn and Cu were so overwhelming that the analysis was repeated with these treatments excluded. The only effect was to shift the dominance to P, K, Ca, and Mg—essentially a shift from the first to the second block of Table 2. The system does not, in fact, behave as if it were continuous; the strongly blocked structure of Table 2 continued to dominate the system even if a continuous model (such as is implied by component analysis) were imposed.

IV. Discussion

None of the nutrient deficiencies used in the present experiment produced the red striping of the leaves which had occurred in other field and pot trials and we were unable to show the cause of this symptom.

Although a relationship between a particular deficiency and free amino acid content has not been demonstrated, large changes in the free amino acid content of *S. sphacelata* occurred under the various deficiencies (see Table 3). Large changes in the free amides occurred. The free amides results include serine which is not completely separated from asparagine and glutamine during the ion-exchange chromatography. Paper chromatography showed that the increase in the "serine and amides" was due principally to increase in the amide fraction. Zinc deficiency caused the greatest increase in this fraction. P, Ca, and Cu deficiency also cause large increases. The only treatment to cause a decrease in the fraction was nitrogen deficiency. Severe nitrogen deficiency lowered the concentration of all estimated amino acids except histidine and arginine.

Severe depletion of the divalent ions of Ca, Cu, and Zn cause greatest increase in concentration in a number of amino acids. P, K, and S deficiency also caused large increases. Increase in ammonia was observed to be greatest in Zn deficiency followed

by Ca, Fe, and K deficiencies while nitrogen deficiency considerably lowered the ammonia content.

In general the absence of an element from the nutrient solution has caused greater changes in the amino acid pattern than an intermediate level of deficiency. Sulphur or zinc deficiency are exceptions but the very low yield of plant material in minus Zn made necessary the pooling of all three replicates to obtain an adequate sample size. Isoleucine and leucine are increased more in the low Ca than minus Ca treatment. Alanine increased greatly in Cu deficiency and to a lesser extent in Zn deficiency.

Qualitatively, the free amino acid pool is very similar to other grasses and plants. The present study has confirmed some of the findings of Hodgson (1964) on *S. sphacelata*. In the present experiment alanine accounted for approximately 26% of the amino acid nitrogen in the control plants whereas Hodgson found approximately 45% in control plants of the same age. Tyrosine was not detected in our control extracts, but accounted for approximately 10% of the free amino acid nitrogen in the experiment of Hodgson. Similarly he found γ-aminobutyric acid at a much higher concentration. However, the method of preparation of samples, viz. drying in a forced draught at 50°C after harvest, prior to extraction, could account for some differences. As only one harvest was taken in our experiment comparison at other stages of growth cannot be made.

The high ammonia content of *S. sphacelata* is also unusual. This had previously been reported by Birch, Dougall, and Hodgson (1964). The ammonia levels were much higher in their plant material than in this experiment. The large changes in ammonia content are not proportional to changes in the dicarboxylic amino acids, although when ammonia content increases so do the dicarboxylic amino acids. Considerable care was taken during the plant extraction procedure to prevent ammonia production from glutamine. The exception to this finding is in nitrogen deficiency where the ammonia content decreases much more than aspartic or glutamic acid. The ammonia : aspartic plus glutamic acid ratio ranges from 2·6 in the control plants to 31·8 in the minus S treatment but the ratio is 0·6 in the minus N treatment.

Although not estimated quantitatively, proline was present in all samples. Paper chromatography showed that proline appeared to be increased in several deficiencies, most markedly in Zn or Ca deficiency. β-Alanine was detected in some deficiencies, notably Zn or Cu. Ehrlich, Sakaguchi, and Pauly reagents applied to paper chromatograms did not show any production of other nitrogenous compounds in response to any of the deficiencies used.

Whereas Coleman and Richards (1956) noted that potassium-deficient barley accumulated considerable putrescine, none was detected in *S. sphacelata* under any of the treatments. Tryptophan is very often absent from the free amino acid pool and was not reported by Hodgson. In this experiment tryptophan was detected in the control plants but decreased greatly under most nutrient deficiencies. Zn deficiency was an exception in which the level of this amino acid was higher. This was not expected as yield and presumably protein synthesis were greatly depressed. Tsui (1948) suggested that in zinc deficiency protein synthesis is depressed because the synthesis of tryptophan, an auxin precursor, is inhibited.

Of all the "low" treatments, Zn caused the greatest change in amino acid levels and yield of plant material. The depression in yield equalled that of the minus N treatment. This may suggest that the requirements of *S. sphacelata* for zinc are greater than for other nutrients or that the adequate nutrient status with respect to other elements may exacerbate the effects of the zinc deficiency through interaction.

IV. Acknowledgments

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Appendix

AN IMPROVED AMINO ACID ANALYSER

By R. D. Court

The analysers in most general use are based on the continuous-flow system of Moore, Spackman, and Stein (1958) in which the developed colour is continuously read and recorded so that integration of the curve is required for estimation. Another type of analyser developed by Simmonds (1958) is based on the automated fraction collection principle when each fraction is treated individually and presented to the photometer. Estimation of the result follows from addition of the absorbance readings. This analyser was further developed by Inglis (1964). However, with plant extracts which contain widely differing concentrations of free amino acids in the one sample, the absorbance readings of this analyser are often beyond the recordable range. The considerable time required for a single analysis is another limitation. By combining the continuous-flow system and the fraction-collection system of Simmonds it is possible to obtain discrete absorbance readings, increase buffer flow through the column to 30 ml/hr, and estimate amino acids in the range 0·03–2 μM.

*Method**

The analyser that has been modified is the Paton model (Paton Industries, Adelaide, S.A.) which is based on that of Simmonds and Rowlands (1960). The 150 by 0·9 cm and 50 by 0·6 cm columns have been retained. Aminex 150S resin (Bio-Rad Laboratories, Richmond, California) is used for all analyses. Modifications made to the analyser include:

(1) Replacement of each heating vessel for the fraction–reagent mixture by a coil of Teflon tubing 4 m in length through which the column effluent and introduced ninhydrin reagent flow continuously.

(2) Dilution of the emergent heated mixture with ethanol–water (1 : 1 v/v) continuously and the collection of this diluted mixture in a tube for 4 min.

(3) Dilution of the photometer sample in the cuvette after the initial absorbance reading to permit a second reading.

A flow diagram of the amino acid analyser is shown in Figure 2.

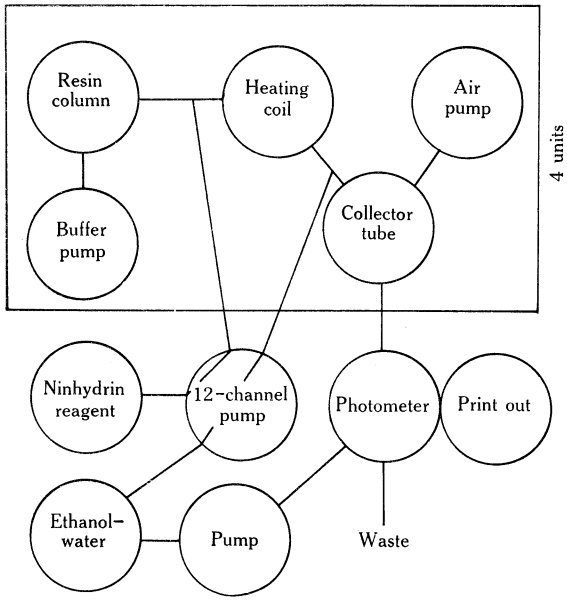

Fig. 2.

Discussion

A Technicon Autoanalyser pump introduces the ninhydrin reagent into the column effluent at a rate of 1 ml per 4 min and ethanol–water to the heating-coil effluent at a rate of 2 ml in 4 min. This is collected in a tube for 4 min then transferred to the photometer and the absorbance read and recorded automatically. The solution in the cuvette is then diluted 1 in 3 with ethanol–water, mixed by means of an aquarium pump connected to the base of the photometer cuvette, and the absorbance read and recorded.

* Full details available from author.

By using the coil-heating method the equivalent of three fractions from a column are heated simultaneously. This allows the cycle time of the analyser to be reduced from 10 to 4 min and have a heating time of 12 min for optimal colour development. Eight absorbance readings per cycle allow only 30 sec for each reading. This is now the limiting factor in the whole operation. The concentration of hydrindantin in the reagent of Moore and Stein (1954) was reduced to 1·5 g/l without reduction of colour development. A base-line stability of $\pm 0{\cdot}003$ absorbance may be obtained. In the range of column loading 0·1–1·0 μM of each amino acid recoveries of $100 \pm 8\%$ are obtained, as indicated in the following tabulation:

Amino acid	Standard deviation (%)	Amino acid	Standard deviation (%)
Cysteic acid	5	Valine	4
Aspartic acid	5	Isoleucine	5
Threonine	7	Leucine	5
Serine	7	Tyrosine	6
Asparagine	7	Tryptophan	4
Glutamic acid	6	Lysine	3
Glycine	4	Histidine	4
Alanine	4	Arginine	8

The above results were obtained from three separate analyses at four different resin column loading levels. In the range 0·03–2·0 μM a standard deviation of 10% may be obtained overall.