THE AMINO ACIDS OF ALFALFA AS REVEALED BY PAPER CHROMATOGRAPHY WITH SPECIAL REFERENCE TO COMPOUNDS LABELLED WITH S^{35}

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(WITH FOUR FIGURES)

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This investigation is essentially a collaboration between two laboratories bringing together problems in which each is interested.

The Department of Agricultural Research of the American Smelting and Refining Company has long been interested in the problems of sulphur toxicity and sulphur metabolism in plants which are economically important. In the course of this work investigations have been made by the use of S^{35}. These have shown the general distribution of the isotope in the plant body. To some extent chemical procedures have been employed to identify in the protein hydrolysate specific sulphur-containing compounds, notably cystine and probably methionine (**8, 9,** and **10**). It remained, however, necessary to obtain unequivocable evidence on the sulphur compounds into which the isotope had entered.

In the Department of Botany at Rochester, investigations have been in progress since 1946 utilizing the techniques of paper chromatography to identify the free and combined amino acids in plants (**2, 6,** and **7**). The sulphur-containing amino acids present rather special problems. Due to the small quantities commonly present and the poor reactivity of these substances with ninhydrin, they tend to be less easily detectable by the methods of paper chromatography than many of the other amino acids (**1**). Furthermore, as two-directional paper chromatograms are commonly carried out (*i.e.*, using phenol : collidine-lutidine), the sulphur-containing amino acids tend to be obscured by others which are commonly present. Methionine sulphoxide occupies the same area as γ-amino butyric acid, a newly discovered free amino acid which is widely distributed (**4, 6,** and **7**). Methionine itself is close to the leucines, and cystine can best be recognized if it is first converted to cysteic acid which occupies a position on the chromatograms well apart from other amino acids (**1**). In these circumstances the use of S^{35} combined with autoradiographs of paper chromatograms is an obvious device to aid in the identification of the sulphur-containing amino acids.

In the laboratories of the American Smelting and Refining Company alfalfa plants were treated with S^{35} in the form of sulphate. The alfalfa

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plants were grown in sand; prior to the treatment with $S^{35}O_4^=$ the shoots were removed, then the radioactive substance was added to the nutrient solution and the new growth which developed was examined. New leaves, formed after the addition of $S^{35}O_4^=$, contained much more (25 times) S^{35} than similar leaves which were already formed. Leaf extracts of various types were prepared and sent to Rochester for further examination by paper chromatographic methods.

Two fractions that were of greatest interest were the 80% alcohol extract and the alcohol-insoluble residue: the former contains the free amino acids while the latter contains the proteins. Of secondary interest was the expressed juice from frozen leaves from which the protein was removed by heat coagulation; the liquid portion is comparable to the alcohol extract.

The heat coagulum contains the globulins and albumins of the cells. The residue from pressed leaves, after washing with water, alcohol and 0.05 *N* HCl, should include the insoluble proteins which are more structurally than metabolically important. The heat coagulum together with the water, alcohol, and acid-insoluble fraction is approximately equivalent to the alcohol-insoluble fraction mentioned above since the acid- and alcohol-soluble protein was found to be negligible.

Preparation of the extracts

The free amino acids of fresh alfalfa leaves from $S^{35}O_4^=$-treated plants were extracted with 80% ethanol by grinding in a Waring Blendor. The mixture was filtered and the filtrate evaporated *in vacuo* in a desiccator at room temperature.

The alcohol-insoluble residue was suspended in water and hydrolyzed by heating with 8 *N* HCl for 20 hours. The HCl was removed and the solution concentrated *in vacuo*.

Leaves from comparable plants were frozen at $-20°$ C and clear juice was expressed from them at 15,000 pounds per square inch. The juice was heated for a few minutes in a water bath and the heat coagulum was separated in the centrifuge. The coagulum was hydrolyzed and the hydrolysate concentrated in the manner described above. The aqueous solution, free from the coagulum, was evaporated at room temperature.

The pressed residue, washed as indicated, was also hydrolyzed and the hydrolysate concentrated *in vacuo*.

Chromatography and autoradiography

These extracts were submitted to two-directional paper chromatography.

In all cases the extract was treated at the point of application on the paper with a 30% solution of hydrogen peroxide and dried. The purpose of the oxidation was to convert methionine and its sulphoxide to the sulphone and cystine to cysteic acid. Though DENT (4) stated that usually no molybdate is necessary to catalyze the oxidation of sulphur-containing amino acids if 30% hydrogen peroxide is used, he subsequently corrected

this. Apparently the stabilizer in the hydrogen peroxide affects the efficiency of the oxidation so that the sulphoxide may not be completely oxidized. However, in the use of this method during this work, it was found that these oxidations did not always proceed quantitatively so that the relative amounts of the oxidized and unoxidized products varied from chromatogram to chromatogram. It is now known that this may be remedied by use of molybdate catalyst and repeated (*e.g.*, three times) treatments with hydrogen peroxide on the paper. However, for purposes of interpretation, activity due to methionine sulphone and sulphoxide may be combined and referred to as methionine derivatives and activity present both as cystine and cysteic acid may be combined as total cystine (cf. table I).

In some cases, the initial activity of the preparation was determined by placing a thin-window Geiger counter over the point of application. Subsequently, when the chromatogram was developed, the distribution of the S^{35} could be determined by counting similarly the activity of various areas of the paper.

The chromatography was carried out by the general procedure used in this laboratory (**6**). (The samples were chromatographed with neutralized phenol saturated with water followed by chromatography at right angles with 3:1 lutidine-collidine saturated with water. No ammonia, diethyl amine or hydrogen cyanide vapors were used in the cabinets.) Any residual acidity in the sample was neutralized with ammonia. The final location of the amino acids was revealed by spraying with 0.1 to 0.2% ninhydrin in alcohol and heating the papers, after spraying, at 60° C. The qualitative identification of the amino acids was made drawing upon the general experience from other work in which the pure amino acids were chromatographed. Autoradiographs could be prepared after the papers were sprayed with ninhydrin. To do this, 14″ × 17″ Eastman No-Screen X-ray film and the chromatogram were brought into direct contact and the exposures made in a refrigerator. Tests have been made by the use of a thin cellophane barrier to verify that the blackening of the film attributed to S^{35} was not due to chemical fogging (**13**). The exposures varied with the activity of the preparation from a few days to several months. After developing the film, the positions of spots due to radioactive sulphur could be compared with the spots brought out by ninhydrin. The quantity of material required for good amino acid chromatograms using ninhydrin is of the order of 50 μgm. of amino nitrogen. With the samples used, this amount of material gave adequate autoradiographs due to S^{35}.

The soluble (free) amino acids

The distribution of alcohol-soluble amino acids on the chromatogram is shown in figure 1 A and the spots are identified in figure 1 B. The noteworthy features are as follows.

Valine, the leucines, γ-amino butyric acid, and tyrosine were all present in smaller amounts, relative to other amino acids, than in many other

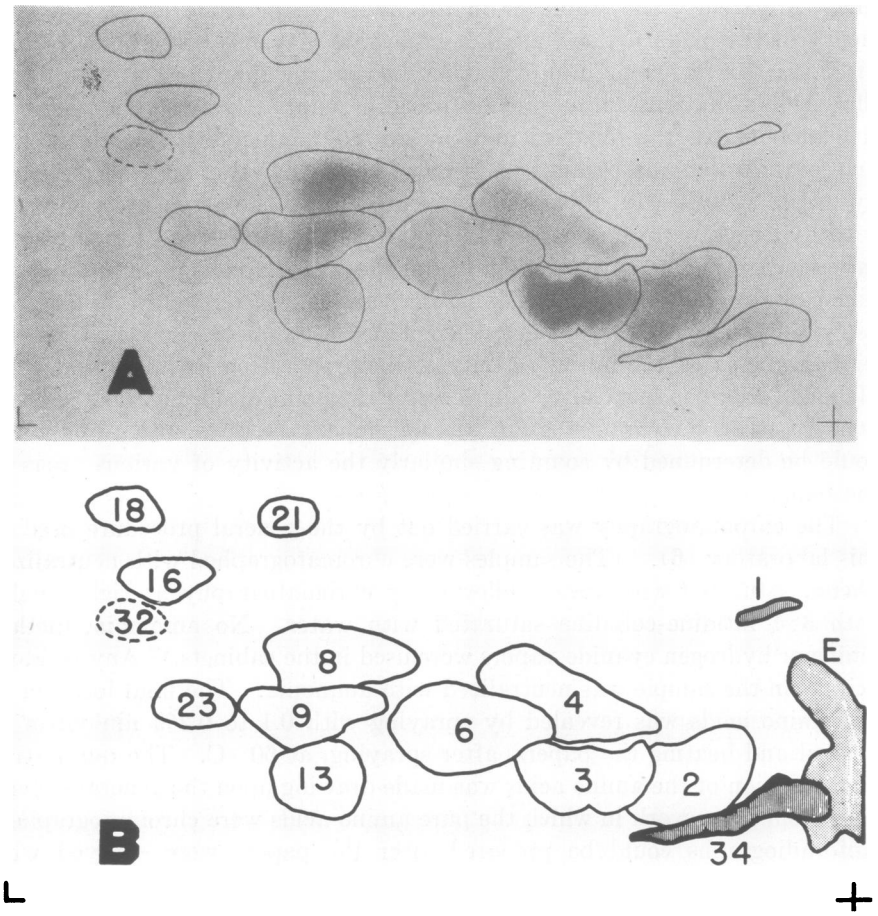

FIG. 1. Amino and S^{35} compounds in the alcohol-soluble fraction of alfalfa leaf. A. Two-directional paper chromatogram. B. Key to ninhydrin reactive and radioactive areas.

Legend: 1, cysteic acid; 2, aspartic acid; 3, glutamic acid; 4, serine; 6, asparagine; 8, alanine; 9, glutamine; 13, arginine; 16, valine; 18, leucine and isoleucine; 21, tyrosine; 23, γ-amino-butyric acid; 32, unknown; 34, glutathione; E, unknown, probably inorganic sulphur salts.

In this and in all subsequent figures the point of origin is in the lower right-hand corner (+) and the directions of movement are horizontal in phenol and vertical in collidine-lutidine. The following conventions have been adopted in the keys. All areas of activity, whether due to ninhydrin or S^{35}, have been outlined, a dashed outline indicating a spot of very low ninhydrin density. Areas showing ninhydrin reactivity only are plain, those showing both ninhydrin reactivity and radioactivity are striped vertically, and those showing radioactivity only are stippled. All compounds reacting with ninhydrin are designated with numbers corresponding to those used by DENT, STEPKA, and STEWARD (2). Compounds containing S^{35} but not reacting with ninhydrin are designated by letters.

common tissues that we have examined (**6**). The obvious presence of aspartic and glutamic acids, serine, asparagine, glutamine, and alanine was an expected and a common feature. The extensive ninhydrin reactive area (No. 34) near the point of origin was in the region in which glutathione is to be expected and added glutathione was found to superimpose upon this area. The presence of glutathione was confirmed by hydrolysis of the fraction since the spot disappeared and the amount of cysteic acid increased. β-Alanine, obscured by glutamine (No. 9 in fig. 1 B), was also revealed upon hydrolysis. The basic amino acids were represented only by arginine. Histidine, even if present, could not have been readily detected on these chromatograms since there was no ammonia in the cabinets. At least one unknown amino compound existed and occupied the position indicated by No. 32 and there was also a spot in the position known to be occupied by cysteic acid.

It will be noted that there was no evidence by ninhydrin of methionine sulphone, which usually occurs between valine and alanine, though it is not possible to eliminate methionine and methionine sulphoxide on the basis of the ninhydrin chromatogram alone as these might be obscured, as described above.

The autoradiograph showed radioactivity in the areas indicated on the key (fig. 1 B). It will be noted that methionine and its derivatives were either completely absent or present only in such small amounts that they were not detectable either by ninhydrin or by the radioactivity of S^{35}. The conspicuously radioactive components of the alcohol-soluble fraction appeared on the chromatogram in the area in which glutathione may be expected, with some evidence of cysteic acid derived from cystine. Inorganic forms of sulphur would not be expected to move far from the point of origin and so may have contributed to the conspicuous area shown (E). Hydrolysis of the alcohol-soluble fraction followed by chromatography and autoradiography furnished no evidence which was inconsistent with the presence of glutathione and cystine as the only S^{35}-containing organic substances in this fraction.

Examination of a water extract of alfalfa leaves, freed from heat-coagulable protein, gave results similar to those obtained in the alcohol extract. Valine, the leucines, tyrosine were all present but in relatively small amounts and the amino acids which appeared in the alcohol extract were also present in the water extract with the exception of arginine which was not detected.

Composition of protein hydrolysates

The amino acids obtained on hydrolysis of the alcohol-insoluble fraction include those to be expected from protein hydrolysis (see figs. 2 A and B). Noteworthy features, however, are the presence of unknown ninhydrin reactive substances in the positions 31A and 33: these showed no evidence of radioactivity. No. 31A is presumably identical with No. 31 in figure 3.

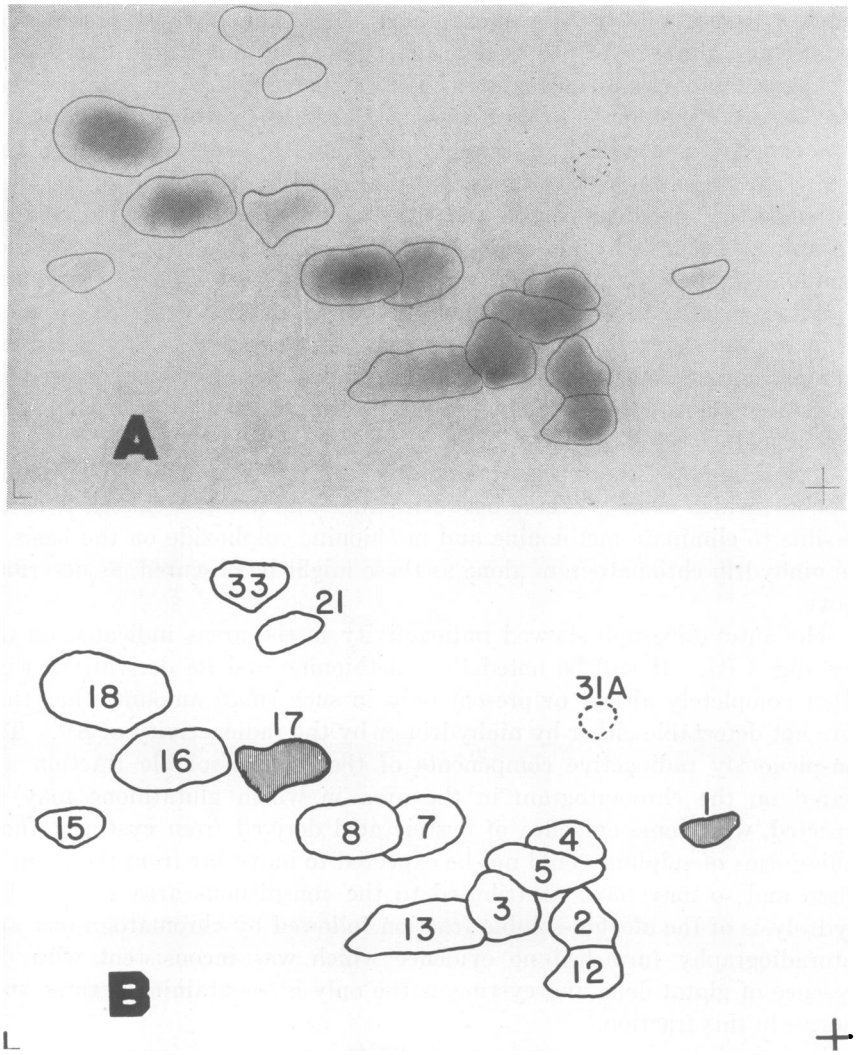

FIG. 2. Amino and S^{35} compounds in the hydrolysate of the alcohol-insoluble fraction of alfalfa leaf. A. Two-directional paper chromatogram. B. Key to ninhydrin reactive and radioactive areas.

Legend: 1, cysteic acid; 2, aspartic acid; 3, glutamic acid; 4, serine; 5, glycine; 7, threonine; 8, alanine; 12, lysine; 13, arginine; 15, proline; 16, valine; 17, methionine sulphone; 18, leucine and isoleucine; 21, tyrosine; 31A, unknown; 33, unknown.

The identity of 12 as a distinct spot from 2 was confirmed by the addition of lysine to the extract followed by chromatography. Thirteen was similarly confirmed. The slow movement of both lysine and arginine in the phenol direction in all these chromatograms may be attributed to the fact that chromatography was carried out in the absence of ammonia in the cabinet.

No. 33 appears to be chromatographically identical with a substance observed by R. M. Zacharius (working with Steward and Thompson) in hydrolysates of an alcohol-insoluble fraction from carrot.

In this protein hydrolysate of alfalfa leaves, valine, the leucines, phenylalanine (not shown in fig. 2 A and B, but identified in a chromatogram of a larger amount of the fraction), and tyrosine were prominent. This stands in marked contrast to the result on the alcohol-soluble fraction. Also, γ-amino-butyric acid, which was present in small amount in the soluble fraction, did not occur in the products of acid hydrolysis of the insoluble fraction. This is in accord with our general experience with this substance (**6** and **7**).

The basic amino acids were present in relatively large amounts in the protein hydrolysate. Lysine was easily detectable in the hydrolysate, though it did not occur free; arginine, on the other hand, was present both in the free and the combined state.

Methionine sulphone from methionine, which did not occur in quantity in the free state, appeared definitely in the protein hydrolysate and cysteic acid from cystine was also much more prominent. The autoradiograph confirms these two compounds as shown by figure 2 B, Nos. 17 and 1.

The only other S^{35}-containing compound to be found in the hydrolysate was observed in a chromatogram of four times the amount of material used in the preparation of figure 2. This was a ninhydrin nonreactive compound occurring at a position between and above serine (No. 4) and threonine (No. 7). This substance may be identical with the spot at C, figure 4, to be considered below.

Alfalfa leaves were fractionated in ways described above, yielding the following distinctive fractions:

(I) The heat-coagulable, water-soluble protein.
(II) A water-, alcohol-, and dilute-acid-insoluble fraction.

Fraction (I) was a small one, accounting for only about 2.6% of the total N. Fraction (II) was a relatively large one, accounting for about 70% of the total.

Tracings from autoradiographs and paper chromatograms of these are illustrated in figures 3 (Fraction I) and 4 (Fraction II). As in the case of figure 2, the amino acids expected to arise from protein hydrolysis appeared on the chromatograms. Three hitherto undetected ninhydrin-reacting substances appeared in the positions marked 29, 30, and 31 in figure 3. None of these unknown substances, however, contained sulphur as determined by radioactivity. No. 29 may be due to lysine since lysine hydrochloride occasionally gives two spots in positions similar to Nos. 12 and 29. Presumably No. 29 is due to the free acid and No. 12 is due to its salts. The light blue color and the shape of spot No. 30 were distinct from that of the other spots on the chromatogram, making No. 30 in all probability something other than an amino acid, possibly an ammonium salt of some other

type of acid. It should also be noted that hydroxyproline occurred in the hydrolysate of Fraction (II) (see fig. 4) although it was absent from Fraction (I) (see fig. 3). Since there is no evidence for γ-amino butyric acid in the alcohol-insoluble hydrolysate (fig. 2), all the ninhydrin reactivity at No. 14, figures 3 and 4, is ascribed to methionine sulphoxide. (The hydrogen peroxide treatment without added catalyst was not sufficient for complete oxidation of the methionine.)

The S^{35}-containing compounds which arose from hydrolysis of the two protein fractions mentioned were as follows. Methionine sulphone and sulphoxide were conspicuous (Nos. 17 and 14 of figs. 3 and 4). Cysteic acid from cystine was also definite (No. 1 of figs. 3 and 4). An unknown,

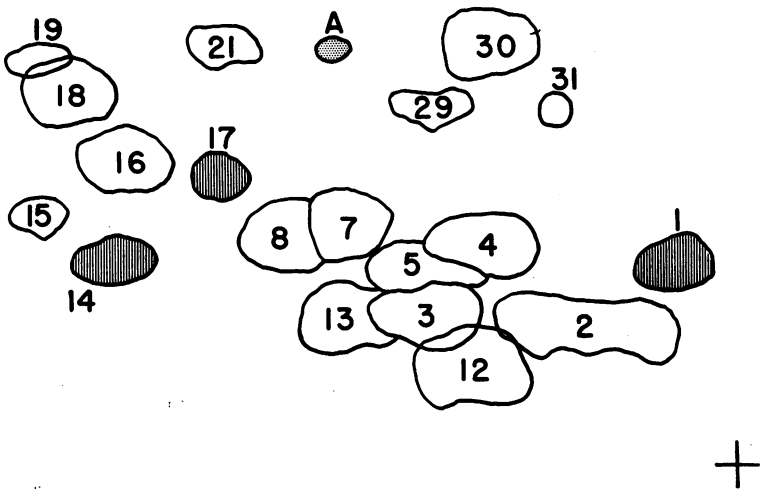

FIG. 3. Key to amino and S^{35} compounds in the hydrolysate of the coagulum from the water-soluble fraction of alfalfa leaf. Legend: 1, cysteic acid; 2, aspartic acid; 3, glutamic acid; 4, serine; 5, glycine; 7, threonine; 8, alanine; 12, lysine; 13, arginine; 14, methionine sulfoxide; 15, proline; 16, valine; 17, methionine sulfone; 18, leucine and isoleucine; 19, phenylalanine; 21, tyrosine; 28, hydroxyproline; 29, unknown, probably due to lysine; 30, unknown, probably salts; 31, unknown; A, B, C, and D, unknowns.

ninhydrin-unreactive, S^{35}-containing compound appeared in very small amount at position A of both figures 3 and 4. Three other S^{35}-containing compounds appeared in figure 4 but not in figure 3. These were B, C, and D. These spots also were very weak and represented only a small percentage of the total radioactivity. They were found in these fractions because these autoradiographs were exposed over a long period of time. All these substances (A, B, C, and D) may be organic sulphur compounds in view of their mobility. There is clearly a great variety of substances that could be considered as possibilities (5).

Some consideration was given to the possibility that one or other of the unknown spots of figures 3 or 4 might be due to taurine (a decarboxylation product of cysteic acid). However, none of these satisfy all the require-

TABLE I

THE S^{35} ACTIVITY OF THE AREAS ATTRIBUTABLE TO CYSTINE AND METHIONINE DERIVATIVES AND GLUTATHIONE ON PAPER CHROMATOGRAMS OF ALFALFA LEAF FRACTIONS. DATA SHOW ACTIVITY MEASURED AS A PERCENTAGE OF TOTAL PLACED ON THE CHROMATOGRAM.

	Cysteic acid	Activity in region of cystine	Total cystine	Glutathione	Methionine sulphoxide	Methionine sulphone	Total methionine	Total* activity recovered
Water extract free of protein	70	...	70	7	...	...	...	77
Hydrolysate of alcohol-insoluble fraction**	15	2	17	...	9	42	51	68
	9	...	9	...	15	35	50	59
	7	4	11	...	50	...	50	61
Hydrolysate of heat coagulum	30	12	42	...	12	...	12	54
Hydrolysate of water-alcohol- and acid-insoluble fraction**	2	10	12	...	52	...	52	64
	...	12	12	...	30	...	30	42

* Total cystine + total methionine + glutathione.

** Replicate chromatograms.

ments either as to position, to radioactivity, or to ninhydrin reactivity. It may be stated here that all attempts to find taurine in these alfalfa preparations have failed and it should, therefore, be concluded that it does not normally occur either as a metabolite or as a decomposition product of the extracts.

The extent to which the sulphur-containing compounds may be accounted for in terms of cystine and methionine derivatives and glutathione, is clearly shown in table I. It should be noted that owing to absorption of the weak S^{35} radiation by the paper, the recovery values in table I are necessarily low. If equal amounts of activity are spread over unequal areas of paper, the more concentrated spot will show a larger count than the others. From such experiments it is estimated that the values in table I may be as much as 20 to 30% low.

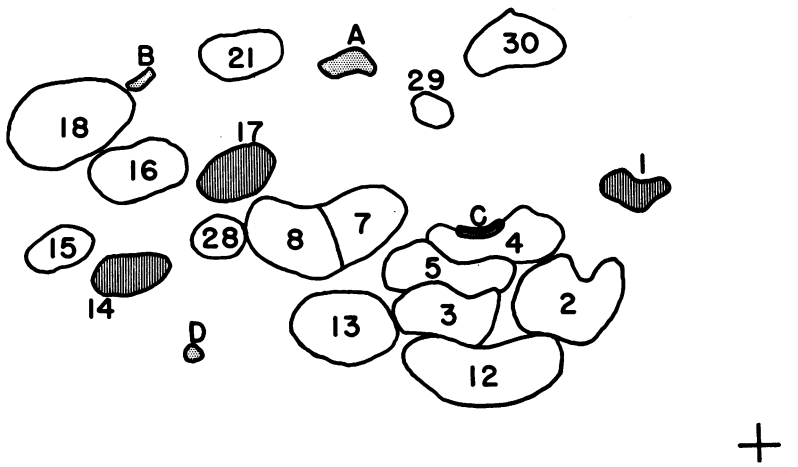

FIG. 4. Key to amino and S^{35} compounds in the hydrolysate of the water-, alcohol-, and dilute-acid-insoluble fraction of alfalfa leaf. Legend same as for figure 3.

Discussion

The amino acids found both "free" and in proteins in alfalfa leaves are similar to those found in other tissues (**6**). No other comparable analyses of amino acids of alfalfa leaves have been found. Legume roots have been found by VIRTANEN to excrete aspartic acid, β-alanine (**11**) and glutamic acid (**12**) so there are presumably large quantities of these in the roots. It is of interest, therefore, that β-alanine is found in the leaf extracts; indicating that it is not peculiar to the root nodules. ZELITCH, BURRIS, and WILSON (**14**) and HUNT (**3**) have each reported a range of amino acids in legume roots similar to that described in this study.

In regard to the sulphur compounds, it is surprising that there were only two alcohol-soluble sulphur compounds—cystine (as cysteic acid) and glutathione, and that there was no indication of methionine or any of its oxidation products. In the hydrolysates, however, methionine (as its oxidation

products) was present. Even when the samples were not treated with hydrogen peroxide, it was impossible to determine whether the methionine occurred in the plant only as methionine since it may have been oxidized in the course of chromatography (**1**). It is of interest to note from table I that methionine predominated over cystine in the insoluble proteins. The reverse was true in the heat coagulum.

Clearly, there are not many organic sulphur compounds in leaves other than the sulphur amino acids. This is borne out by the chromatograms which were scanned with a Geiger counter and the areas of activity compared with the ninhydrin-reactive regions (see table I). As much as 70 to 75% of the activity placed on the paper could be accounted for in terms of ninhydrin-reacting substances (Nos. 1, 14, 17, and 34 of figs. 1 B, 2 B, 3 and 4). Considering the number of possible sulphur compounds which might occur, it is somewhat surprising that so much of the S^{35}, as shown by both the scanned chromatograms and autoradiographs, was present in so few compounds. However, some of the radioactivity due to immobile substances and previously attributed to inorganic forms of sulphur may have been due to sulphonic acids or other polar organic molecules.

It was of interest to note again (**6**) that γ-amino butyric acid did not occur in the protein but was found only in the free state.

Summary

1. The free and combined amino acids of alfalfa leaves have been examined by the methods of paper chromatography and autoradiography for S^{35} which was introduced into the plants in the form of sulphate via the roots.

2. Aspartic and glutamic acids, alanine and serine were the conspicuous amino acids which were detected free in alfalfa, and the amides glutamine and asparagine were also prominent.

Valine, the leucines, and tyrosine were all readily detectable though they occurred in smaller amounts, relative to the others, than in many other tissues (*e.g.*, potato tuber). The only free basic amino acid detected was arginine.

γ-Amino butyric acid occurred, though it was not as conspicuous in alfalfa as in many other plants.

The free sulphur-containing compounds consisted of cystine (detected as cysteic acid) and substances, most probably glutathione, which yielded cystine on hydrolysis.

Methionine and its oxidation products, the sulphoxide and sulphone, were not detectable in the free state.

3. The combined amino acids of alfalfa were examined after hydrolysis of (a) an alcohol-insoluble fraction, (b) a heat coagulum of a water extract, and (c) a water-, alcohol-, and acid-insoluble fraction. These amino acids were those commonly found in proteins; valine and the leucines were more prominent in the hydrolysates than in the free state; glycine, lysine, threonine, methionine, and proline were only detected in the protein hydrolysates.

Hydroxyproline appeared only in the hydrolysate of the water-, alcohol-, and acid-insoluble fraction. Phenylalanine was detected only in the hydrolysate of the heat-coagulable protein.

Certain unidentified, sulphur-free, but ninhydrin-reactive substances occurred in the acid hydrolytic products of alcohol-insoluble material.

The prominent and identifiable S^{35}-containing compounds in these hydrolysates were methionine sulphone and sulphoxide from methionine and cysteic acid from cystine. Certain sulphur compounds, present in small amount in the hydrolytic products, were detected by autoradiographs of chromatograms. None of these satisfied the requirements for taurine.

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