THE MANOMETRIC ESTIMATION OF SMALL AMOUNTS ASPARTIC ACID

by

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The only generally accepted method for the determination of aspartic acid (AS) in proteins is its isolation as the copper salt, following precipitation of the dicarboxylic amino acids with lime and alcohol, and separation of glutamic acid as the hydrochloride (FOREMAN[1]). Like other preparative methods, this procedure necessitates large amounts of material and is liable to considerable error by losses. Painstaking labour and unusual experimental skill are required to obtain accurate analytical results. An idea of the difficulty of this method is given by the work of CHIBNALL and his colleagues[2] — the only authors who achieved a really quantitative separation of AS from protein hydrolysates, after a thorough reinvestigation of the FOREMAN procedure. The method is, naturally, of no avail for the analysis of small samples of protein, for serial determinations, and for the estimation of AS in biological objects, e.g., in studies on tissue metabolism.

The colorimetric method of FROMAGEOT and HEITZ[3], based on conversion of AS to acetaldehyde by deamination with nitrous acid and oxidation with permanganate, is non-specific and unreliable, since acetaldehyde is also formed from alanine, serine, threonine, lactic, malic, and glyceric acids, which must be removed or accounted for by separate analyses, so as to determine AS by difference (cf. BRAUNSTEIN and BYCHKOV[4]). The recently published highly sensitive procedures for microbiological assay of AS[14,5] involve the use of expensive and difficultly obtainable constituents in the synthetic nutrient media. Their specificity has not yet been studied in detail, especially with respect to nitrogen-free dicarboxylic acids.

On the search for a new approach to the micro-determination of AS in biological materials, the following statement of H. D. DAKIN suggested to us a promising analytical principle. In his paper on methyl aspartic acids[6], DAKIN writes: "As is well known, aspartic acid is converted virtually quantitatively into fumaric acid on methylation and this reaction, in the writer's opinion, offers the best available method for the estimation and determination of aspartic acid". In support of this thesis, DAKIN reports one experiment in which aliquots of an acid casein hydrolysate were subjected to methylation and crystalline fumaric acid was isolated by ether extraction, in yields corresponding to 4.70 to 4.93 per cent of AS. In 1918 DAKIN had obtained from casein a maximum yield of 4.1 per cent AS as the copper salt[13].

The purpose of the present investigation was 1. to establish whether the conversion to fumaric acid is quantitative on methylation of milligram quantities of AS in very dilute aqueous solution; 2. to explore the degree of specificity of this reaction, and 3. to adapt the reaction for the estimation of small amounts of AS in protein hydrolysates and deproteinized tissue filtrates.

The data reported below show that this aim has been satisfactorily achieved.

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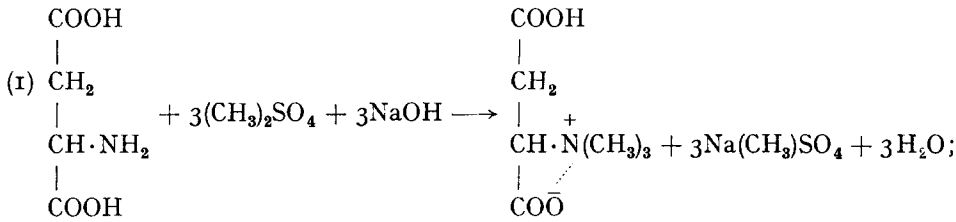
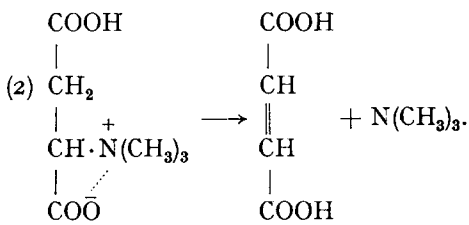
EXPERIMENTAL

Principle of the procedure

On exhaustive methylation (with methyl iodide or methyl sulphate), α-amino acids are converted into the corresponding betaines (ENGELAND[7]). The betaines of certain amino acids, especially β-amino acids, are unstable and can easily be split into αβ-unsaturated acids and trimethylamine by the action of alkalies. The betaine of AS (which is both an α- and a β-amino acid) undergoes complete decomposition at room temperature, especially in alkaline solution, to yield fumaric acid:



(1) $CH(COOH)(CH_2COOH)(NH_2) + 3(CH_3)_2SO_4 + 3NaOH \longrightarrow CH(COO^-)(CH_2COOH)(N^+(CH_3)_3) + 3Na(CH_3)SO_4 + 3H_2O;$



(2) $CH(COOH)(CH_2COOH)(N^+(CH_3)_3) \longrightarrow CH(COOH)=CH(COOH) + N(CH_3)_3.$

All *chemical* methods proposed for the determination of small amounts of fumaric acid are non-specific. Therefore we made use, for the estimation of fumaric acid, of the *enzymatic* manometric method of KREBS[8], based on reduction of fumaric acid with zinc and phosphoric acid and manometric determination of the succinic acid thus formed with succinic dehydrogenase:

(3) $HOOC\cdot CH = CH\cdot COOH + Zn + 2H^+ \longrightarrow HOOC\cdot CH_2\cdot CH_2\cdot COOH + Zn^{++};$

(4) $HOOC\cdot CH_2\cdot CH_2\cdot COOH + {}^1/_2\,O_2 \longrightarrow HOOC\cdot CH = CH\cdot COOH + H_2O.$

Conditions for the methylation of AS

The conversion of AS into fumaric acid is achieved by the alternating addition, to an aqueous solution of AS, of equal volumes of methyl sulphate and 33 per cent sodium-hydroxide in small portions (6–8 portions in the course of 1 hour) with constant shaking. Before the addition of each portion of methyl sulphate and at the end of the operation the reaction mixture should be alkaline.

Caution: Avoid inhalation of the toxic vapors of methyl sulphate and its contact with the skin.

In preliminary experiments the course of methylation of AS in pure solutions was explored by titration of the fumaric acid with permanganate in acid solution according to STRAUB[9] (1 mg fumaric acid uses 5.56 ml 0.01 *N* permanganate). The titrations were performed after extraction of the fumaric acid with ether or directly in aliquots of the diluted and acidified reaction mixture, correcting for the volume of $KMnO_4$ solution

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TABLE I

DETERMINATION OF AS IN PURE SOLUTION

FUMARIC ACID TITRATED ACCORDING TO STRAUB DIRECTLY IN THE REACTION MIXTURE (*) OR AFTER EXTRACTION WITH ETHER (**). ONE mg AS = 4.849 ml $KMnO_4$

Expt No	Volume of AS solution	Number of drops of methyl sulphate and NaOH	AS added mg	AS recovered		Notes
				mg	per cent	
1	0.5	6	0.20	0.196*	98.0	
1	1.0	6	0.20	0.197*	98.5	
1	4.0	6	0.20	0.193*	96.5	
1	6.0	6	0.20	0.194**	97.0	
2	0.5	6	1.25	1.22*	97.5	
2	4.0	6	1.25	1.21*	97.0	
2	10.0	6	1.25	1.16*	93.0	
2	10.0	6	1.75	1.61*	93.0	
3	2.0	10	0.16	0.158*	98.5	
3	2.0	6	1.0	0.974**	97.4	
4	2.0	6	1.0	0.926**	92.6	T° = 8° C
4	2.0	6	3.0	2.91*	97.0	Warmed to 20°
4	2.0	6	1.25	1.30*	102.0	,, ,, 20°
5	8.0	6	3.0	2.90**	96.7	
6	8.0	6	4.5	4.22**	95.0	
6	4.0	10	4.5	4.40*	98.0	
6	4.0	10	3.0	2.90*	96.7	
7	4.0	10	3.0	2.91*	97.0	
7	4.0	6	3.0	2.88*	96.0	
8	4.0	10	14.85	12.47**	84.0	T° below 10° C
9	6.0	10	18.5	17.91**	98.7	Warmed to 20°
9	6.0	10	18.15	16.94**	93.5	T° below 10°

reduced in reagent blanks without AS. Fumaric acid was recovered to the extent of 98–100 per cent from solutions treated with methyl sulphate and NaOH.

It is seen from the data of Table I that the conversion of AS to fumaric acid is practically quantitative, under the conditions recorded below, in aqueous solutions containing 0.2–20 mg AS or more in a volume of 1 to 5 ml. More dilute solutions should be evaporated. If the volume of the sample is less than 1 ml, crystallisation will occur in the course of methylation, interfering with the reaction. Methylation is effected at room temperature; at temperatures below 12–14° C the rate of reaction is slowed, and the samples should be warmed to 20–25° C in a water bath.

Methyl sulphate, followed by 33 per cent NaOH solution, are added 1–2 drops at a time at intervals of 5–10 minutes from burettes with tips drawn out so that one drop of methyl sulphate measures 0.03–0.04 ml, and one drop of alkali has the same or a slightly larger volume. The total amount of methyl sulphate should be at least 6 mols per 1 mol AS (with protein hydrolysates and other mixtures — 6 equivalents for each methylatable NH_2- or other group), but no less than 6–8 drops (0.20–0.30 ml), added over a period of

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1 hour. With the mentioned quantities of AS, this is a large excess. The reaction is usually completed by the time of addition of the last portion of alkali, but it is safer to keep the samples at alkaline reaction and room temperature for another hour.

Manometric estimation of fumaric acid

The amount of fumaric acid is determined by the procedure of KREBS et al[8]. (This method is also described in the laboratory compendium of "Manometric techniques" by UMBREIT, BURRIS and STAUFFER[15]).

KREBS[8] fills 1 ml of the succinate solution to be analysed into the side-arm, and 3 ml of succinic dehydrogenase (washed heart muscle mince) into the main compartment of WARBURG manometric vessels. We find it more convenient to place 2 or 3 ml of the assay solution in the main space, using 0.5 or 1.0 ml of a cell-free solution of the succinic oxidase system in the side-bulb. In this manner we obtain much lower enzyme blanks (0–1 μl) and better agreement of replicates. Fumaric or succinic acid carried through the methylation stage are recovered with a yield of 98–101 per cent; only in a few instances was the loss as high as 5–7 per cent.

Preparation of succinic dehydrogenase: The enzyme solution is prepared by a procedure slightly modified from the method of STOTZ and HASTINGS[11]:

Pass fresh or fresh-frozen pig or beef heart or pigeon breast muscle (20 g) twice through the LATAPI mincer (hearts of rats or rabbits may be used; with sheep heart the blanks are high). Place the mince in a dish on cheese-cloth and wash 6 to 8 times with cold water. At each washing stir the tissue with 15 volumes of water. At each washing stir the tissue with 15 volumes of water, let is soak for 8–10 minutes and press out thoroughly. To the washed muscle tissue add twice its weight of 0.1 *M* phosphate buffer (p_H 7.4), grind thoroughly with quartz sand in a mortar, keep at room temperature for 30 minutes and centrifuge 10 minutes at a speed of 1500–2000 per minute. Use 0.5–1.0 ml of supernatant in each WARBURG vessel. The washed mince can be stored in the frozen state for more than a week, but it should be washed once again before being ground with buffer.

Procedure for the manometric estimation of AS

The test solutions, containing no less than 0.2–0.3 mg AS in a volume of 2–5 ml, are methylated in the test-tubes of KUTSCHER-STEUDEL extractors, such as used by KREBS[8,15]. In the course of 1 hour 8 portions of methyl sulphate and of 33 per cent NaOH are added. Usually the total amount of each of the reagents is 8–16 drops, or 0.25–0.60 ml. At the end of the operation, one additional drop of alkali is supplied for each ml of AS solution, and the mixture is set aside for 1 hour.

The reaction mixture is then neutralised to congo-red with 10 *N* phosphoric acid and, after addition of water to make 5, 8 or 10 ml, the reagents for reduction of fumaric acid are added in amounts proportional to the volume of the mixture. A drop of caprylic alcohol or a few milliliters of ether serve to prevent frothing. When the reduction is completed, the walls of the extractor tube are rinsed with 2–3 ml of water, and the solution is acidified with more 10 *N* phosphoric acid till blue to congo. The sample is now ready for extraction and manometric determination of succinic acid. Some of the methyl hydrogen sulphate formed in the methylation operation will pass into the ether extract and increase its acidity, but providing careful neutralization this does not interfere with the enzymatic oxidation of succinic acid.

The results obtained in estimations of AS in pure solution are listed in Table II.

It will be seen that the recovery of AS usually amounts to 94–101 per cent (average 98.0 per cent); only exceptionally do manometric replicates from one methylated sample diverge by more than 5 per cent. One analyst can make 6–8 determinations with manometric measurement in duplicate in 2–$2^1/_2$ work-days.

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We, further, investigated the applicability of the method for the estimation of AS in the presence of other amino acids, especially those known to form unstable betaines, such as serine, methionine, tyrosine, cystine. These experiments showed that other amino acids, added in 5 to 10-fold excess with respect to AS, do not interfere with its

TABLE II

MANOMETRIC DETERMINATION OF AS IN PURE SOLUTION

(1 mg AS = 84.2 μl O_2)

Expt No.	AS content of		O_2 uptake, μl		Recovery of AS in total sample	
	Total sample mg	Manometric vessel mg	Observed	Theoret.	mg	per cent
1	0.25	0.18	17.2	15.6	0.27	108
2	2.0	0.25	23.6	21.05	2.24	112
	2.0	0.5	43.8	42.1	2.08	104
	4.0	0.5	44.8	42.1	4.24	106
3	4.0	0.67	54.3	56.1	3.87	96.8
	4.0	0.67	55.9	56.1	3.98	99.6
	2.85	0.4	34.0	34.6	2.81	98.5
4	4.5	0.75	60.0	63.3	4.26	94.7
	4.5	0.75	60.6	63.6	4.30	95.7
	4.5	0.75	59.4	63.3	4.22	93.8
5	4.5	0.75	59.4	63.3	4.22	93.8
	4.5	0.75	63.5	63.3	4.51	100.3
6	6.0	1.5	126.5	126.3	6.01	100.1
	6.0	0.5	40.6	42.1	5.79	96.5
	8.0	2.0	160.8	168.4	7.59	94.9
	8.0	1.0	80.3	84.2	7.63	95.2
	14.85	1.0	76.8	84.2	13.65	92.2*
7	14.85	1.0	70.2	84.2	12.47	83.3*
8	18.15	1.21	100.8	102.1	17.91	98.7
	18.15	1.21	96.3	102.1	17.14	94.6

* Methylation performed at low temperature (< 9° C).

determination (Table III). The methylation of cystine results in the appearance of malodorous decomposition products (mercaptans), which pass into the ether extract but do not affect the enzymatic oxidation of succinic acid. The low recovery in one of the experiments with added methionine was evidently fortuitious.

Similar yields were obtained in recovery experiments with AS added to protein hydrolysates (Table IV). This shows that no interfering substances are formed upon methylation of the hydrolysate.

Asparagine was the only of the tested substances that interfered with the estimation of AS. When treated with methyl sulphate, asparagine yields variable amounts of fumaric acid — from 0.2 to 0.4 mols per 1 mol of asparagine (Table V).

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TABLE III

DETERMINATION OF AS IN THE PRESENCE OF OTHER AMINO ACIDS

Added amino acid	Aspartic acid		
	Added mg	Found mg	Recovered per cent
Serine 35 mg	4.5	4.37	97.3
,, 35 ,, ...	4.5	4.11	91.5
,, 35 ,, ...	4.5	4.18	93.0
Methionine 50 mg . .	12.0	10.36	86.0
,, 50 ,, . .	12.0	11.26	93.4
Tyrosine 69 mg . . .	4.5	4.25	94.6
,, 69 ,, . . .	4.5	4.22	93.6
Tryptophane 61 mg. .	4.5	4.29	95.6
Glutamic acid 50 mg .	12.0	11.76	98.0
,, ,, 50 ,, .	4.5	4.49	99.8
Cystine 15 mg	3.0	2.70	90.0
,, 15 ,, ...	3.0	2.94	98.0
,, 10 ,, ...	3.0	2.89	96.4
,, 10 ,, ...	3.0	2.84	94.7
,, 15 ,, ...	3.0	2.84	94.7
,, 15 ,, ...	3.0	2.82	94.1

TABLE IV

RECOVERY OF ADDED AS FROM CASEIN HYDROLYSATE

Expt No.	Sample contains		Found AS mg	Recovery of added AS per cent
	Casein hydrolysate ml	AS mg		
1	—	3.00	2.88	96
	1.5 (= 60 mg casein)	—	3.06	—
	1.5	3.00	5.94	96
2	—	3.00	2.88	96
	1.5 (= 60 mg casein)	—	3.01	—
	1.5	3.00	5.85	94
3	—	3.00	3.06	102
	2.0 (= 30.6 mg casein)	—	1.92	—
	2.0	3.00	4.77	95

ESTIMATION OF AS IN TISSUE EXTRACTS

For the estimation of free AS in biological fluids and deproteinised tissue filtrates, preformed fumarate and succinate must be removed. This is achieved by extracting these and other ether-soluble acids with ether prior to methylation. A less commendable alternative procedure is to determine the sum of AS, fumarate and succinate in a methylated sample and fumarate plus succinate in a non-methylated one, and to estimate AS by difference.

In Table VI the results are presented of recovery experiments with AS added to animal tissues.

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TABLE V

DETERMINATION OF AS AND ASPARAGINE

Expt No.	Added		Found AS per	
	AS mg	Asparagine (in terms of AS) mg	mg	per cent of theoretical
1	9.0	—	8.99	99.8
	—	45	7.98	17.8
	9.0	45	13.06	24.0
2	—	10	4.4	44
3	—	2.0	0.60	30
	—	3.0	1.20	40
4	—	4.0	1.44	36
	—	4.0	1.52	38

Mixtures of 2 g ground tissue (pig or beef heart), 5 ml AS solution (10–20 mg AS) and 3 ml of water were deproteinised with 2 ml of 5 per cent metaphosphoric acid at 40° C and filtered. Samples of tissue without AS and of AS without tissue were run in parallel.

Aliquot parts of the filtrate (2–4 ml) were delivered into KUTSCHER extractors, made acid to congo, filled up to about 5 ml and extracted 3 hours with ether. The funnel was then withdrawn from the extractor tube and rinsed with a little water. The ether layer in the tube was removed by suction, the aqueous solution neutralised and methylation started.

In these experiments AS was recovered to the same extent as in analyses of pure AS solutions.

TABLE VI

RECOVERY OF ADDED AS FROM HEART MUSCLE PULP

Expt No.	Amount of tissue g	Added AS mg	Found AS mg	Recovery of added AS per cent
1	2	—	0.196	—
	2	10	9.55	93.5
	—	10	9.60	96.0
2	2	—	0.16	—
	2	10	9.56	94.0
	—	10	9.75	97.5
3	2	20	20.26	100.5
	2	20	19.5	96.5
	—	20	19.5	96.5

ESTIMATION OF AS IN PROTEIN HYDROLYSATES

Since the AS content of most proteins is higher than 2 per cent, 50–100 mg of hydrolysed protein will be sufficient, as a rule, for one methylation sample. 100 mg protein correspond to 14–16 mg of nitrogen, or about 1 milli-equivalent. The methylation of this amount requires 6 millimols of methyl sulfate (mol. w. 128) = 0.77 g = 0.59 ml.

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To test the applicability of the method for the estimation of AS in proteins, acid hydrolysates of two samples of casein were analysed for AS.

2-gram portions of casein were hydrolysed 14 hours with 20 ml of 20 per cent HCl. Excess HCl was removed by repeated evaporation in vacuo with the addition of water. The hydrolysates were filtered from humin into 50 ml volumetric flasks and made up to the mark.

1.5 ml samples of hydrolysate were transferred to KUTSCHER extractors, neutralized, made up to 4 ml with water, and methylated etc., as described.

On separate portions of casein determinations were made of moisture, ash content and KJELDAHL N (observing the indications of CHIBNALL[12]):

	Moisture	Ash	N content (on ash- and moisture-free basis)
Casein I, practical grade	9.70 %	3.14 %	14.4 %
Casein II, "according to HAMMARSTEN", KAHLBAUM	10.07 %	1.55 %	15.1 %

The analytical results are reported in Table VII. The nitrogen content of both casein samples (ash- and moisture-free) was lower than required for highest purity casein

TABLE VII

ESTIMATION OF AS IN CASEIN

(brackets indicate manometric duplicates from one methylated sample)

Hydrolysate	Casein in methylated sample mg	Found AS, per cent	
		on ash- and moisture-free basis	recalculated to casein containing 15.73 % N
Casein I, 14.4 % N			
Hydrolysate No. 1 . . .	60 mg	5.75	6.30
	60 ,,	6.01	6.55
	60 ,,	5.82	6.32
	60 ,,	5.92	6.46
Hydrolysate No. 2 . . .	60 mg	5.73	6.25
	60 ,,	5.66	6.18
	60 ,,	5.92	6.45
	60 ,,	5.60	6.10
	Average:	5.73	6.33 ($\mu = \pm 0.15$)
Casein II, "HAMMARSTEN" 15.1 % N			
Hydrolysate No. 1	61.2 mg	6.60	6.86
	61.2 ,,	6.62	6.88
	30.6 ,,	6.41	6.67
	30.6 ,,	6.48	6.70
	30.6 ,,	6.93	7.20
	30.6 ,,	6.74	6.97
	30.6 ,,	6.12	6.36
	30.6 ,,	5.96	6.20
	30.6 ,,	6.35	6.60
	30.6 ,,	6.35	6.60
Hydrolysate No. 2 . . .	45 ,,	6.20	6.35
	45 ,,	6.60	6.86
	Average:	6.45	6.69 ($\mu = \pm 0.29$)

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(15.73% N, CHIBNALL[12]). The analytical data have been recalculated to casein containing 15.73 per cent N.

The estimated AS content of pure casein, computed from the average of 12 manometric determinations on two hydrolysates of the "HAMMARSTEN" casein, was 6.69 per cent 6 u = ± 0.29 per cent). Since the average recovery of AS is 96–98 per cent, the actual AS content of casein is probably 0.12–0.25 per cent higher.

DISCUSSION

Few amino acids yield betaines that are unstable at room temperature; among these aspartic acid is unique in being transformed into fumaric acid on methylation. Success in developing the present manometric micromethod was due to the fortunate circumstance that formation and breakdown of the betaine of AS runs to completion in very dilute aqueous solutions. The reported data show that by this procedure AS can be estimated satisfactorily on the milligram scale in pure solutions, tissue extracts and protein hydrolysates, with the usual degree of accuracy of enzymatic manometric techniques, i.e., with a yield of 97 ± 5%.

Application of the highly specific succinic dehydrogenase for the estimation of fumaric acid, with eventual preliminary removal of preformed fumarate and succinate, imparts to the method strict specificity for AS. In pure solutions and in mixtures containing no foreign ether-soluble reducing substances after methylation, AS can be determined, in somewhat lower amounts and with the same degree of accuracy, by titration of the fumaric acid with permanganate according to STRAUB (see Table I).

AS-containing peptides were not available for testing, but it is clear from structural considerations that they cannot give rise to free fumaric acid and will not interfere with the determination of free AS, unless they undergo hydrolysis during the methylation operation.

The only substance interfering with the estimation of AS is asparagine, variable fractions of which are converted into fumaric acid upon treatment with methyl sulphate. Accordingly the manometric micromethod cannot be used directly for the estimation of AS in extracts from plant tissues containing asparagine. There should be no special difficulties, however, in effecting a preliminary quantitative separation of AS and asparagine (e.g., by electrophoretic or adsorption procedures), and upon acid or alkaline hydrolysis the amount of asparagine could likewise be determined by the manometric method.

The results obtained in determining the AS content of casein by the manometric micromethod are in good agreement with the highest estimates reported in literature, viz., with the microbiological assay values of HAC and SNELL[5] and with the most exact gravimetric analyses effected on 40–50-gram protein samples by CHIBNALL and coworkers[2] (CHIBNALL considers these analyses to be accurate within 2 per cent).

Data on the AS content of casein reported in the literature are listed in Table VIII in the chronological order.

It is seen that the present manometric procedure makes possible the rapid estimation of the AS content of proteins with a fair degree of approximation by a few replicate determinations on 50–100-milligram samples. This aim could not be achieved by the formerly published methods, excepting the microbiological assays, the specifity of which is in need of further investigation.

If a WARBURG respirometer is not available, the present method can easily be adapted to the determination of AS on the centigram scale in any type of exactly calibrated gasometric apparatus allowing the measurement of oxygen uptakes of the order of 1 milliliter.

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TABLE VIII

THE AS CONTENT OF CASEIN

Authors	Date	Analytical method	AS content of casein, per cent
FOREMAN[1]	1914, 1919	Preparative isolation as the copper salt	1.7
DAKIN[13]	1918	Isolation as Cu··-salt	3.5–4.1
DESNUELLE[3]	1938	Method of FROMAGEOT & HEITZ[3]	4.1
DAKIN[6]	1941	Isolation of fumaric acid after methylation	4.70–4.93
BAILEY, CHIBNALL, REES & WILLIAMS	1943	Improved FOREMAN procedure. Actually isolated as Cu··-salt	6.10–6.14
BAILEY, CHIBNALL, REES & WILLIAMS[2]	1943	Same, with calculated correction for solubility of calcium salt	6.68
STOKES & GUNNES[14]	1945	Microbiological assay witt Lactobacillus delbrückii LD5	5.7–6.4
HAC & SNELL[5]	1945	Microbiological assay with Leuconostoc mesenteroides	7.03
BRAUNSTEIN, NEMCHINSKAYA & VILENKINA	1946	Manometric estimation of fumaric acid after methylation of AS	6.3–7.2 (Average: 6.7)

SUMMARY

A strictly specific micromethod has been developed for the estimation of aspartic acid (AS), based on its conversion into fumaric acid on treatment with methyl sulphate and alkali (DAKIN[6]). This conversion is rapid and quantitative in very dilute aqueous solutions of AS. The fumaric acid is reduced with zinc and phosphoric a id and the resulting succinic acid determined in the WARBURG apparatus with the aid of succinic dehydrogenase (KREBS[8]). Quantities of AS ranging from 0.2–0.5 mg upwards can be estimated in pure solutions, deproteinised tissue extracts and protein hydrolysates, with yields of 97 $\pm$ 5 %. In pure solutions AS can be determined by methylation and titration of the evolved fumaric acid with permanganate according to STRAUB[9].

The method fails in the presence of asparagine, variable fractions of which (25–40 %) are converted to fumaric acid on methylation.

Protein samples of 50–100 mg are sufficient for the manometric estimation of AS in protein hydrolysates. The manometric method has been applied to the estimation of the AS content of casein. The analytical results, recalculated to maximally purified casein (15.73 % N) indicate an AS content of 6.69 per cent ($\mu = \pm$ 0.29 per cent). This estimate is in good agreement with the most exact gravimetric determinations of CHIBNALL and coworkers[2] and with the values obtained in microbiological assays (HAC AND SNELL[5]).

RÉSUMÉ

Nous avons élaboré une micro-méthode strictement spécifique pour le dosage de l'acide aspartique (AS), basée sur la conversion de cet acide en acide fumarique par le traitement avec le sulfate de méthyle et l'alcali (DAKIN[6]). Cette conversion est rapide et quantitative dans des solutions aqueuses de AS très étendues. L'acide fumarique est réduit par le zinc et l'acide phosphorique, et l'acide succinique résultant de cette réduction est déterminé dans l'appareil de WARBURG à l'aide de la déhydrogénase succinique (KREBS[8]). Des quantités de AS variant entre 0.2 et 0.5 mg et plus peuvent être déterminées dans des solutions pures, des extraits de tissus déprotéinisés et des hydrolysates de protéines, avec des rendements de 97 $\pm$ 5 pour cent). Dans des solutions pures AS peut être déterminé, par méthylation et titrage de l'acide fumarique dégagé, avec le permanganate, selon la méthode de STRAUB[9].

La méthode ne donne pas de résultats en présence de l'asparagine, des fractions variables de celle-ci (25–40 %) étant converties en acide fumarique par méthylation.

Des échantillons de protéines de 50–100 mg suffisent pour la détermination manométrique de AS dans les hydrolysates de protéines. La méthode manométrique a été appliquée pour la détermination de la teneur en AS de la caséine. Les résultats analytiques, recalculés pour la caséine purifiée au maximum (15–73 % N) indiquent une teneur en AS de 6.69 % ($\mu = \pm$ 0.20 %). Cette évaluation concorde parfaitement avec les déterminations gravimétriques les plus exactes de CHIBNALL et de ses collaborateurs[2], etc avec les valeurs obtenues dans les essais microbiologiques (HAC et SNELL[5]).

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ZUSAMMENFASSUNG

Eine streng spezifische Mikromethode zur Bestimmung von Asparaginsäure (AS) wurde entwickelt. Diese Methode beruht auf der Umsetzung dieser Säure in Fumarsäure bei Behandlung mit Methylsulfat und Alkali (DAKIN[6]). Diese Umsetzung vollzieht sich schnell und quantitativ in sehr verdünnten wässerigen AS-Lösungen. Die Fumarsäure wird mit Zink und Phosphorsäure reduziert und die entwickelte Bernsteinsäure wird mit Hilfe von Bernsteinsäure-Dehydrogenase (KREBS[8]) im WARBURG-Apparat bestimmt. AS-Mengen zwischen 0.2–0.5 mg und mehr können in reinen Lösungen, enteiweissten Gewebeextrakten und in Eiweiss-Hydrolysaten, mit Ausbeuten von 97 ± 5 %, bestimmt werden. In reinen Lösungen kann AS durch Methylieren und Titrieren der entwickelten Fumarsäure mit Permanganat nach STRAUB[9] bestimmt werden.

Die Methode versagt bei Anwesenheit von Asparagin, da wechselnde Mengen davon (25–40 %) bei der Methylierung in Fumarsäure umgesetzt werden.

Eiweissproben von 50–100 mg genügen für die manometrische Bestimmung von AS in Eiweiss-Hydrolysaten. Die manometrische Methode wurde zur Bestimmung des AS-Gehaltes von Kasein angewandt. Die analytischen Resultate, umgerechnet auf Kasein mit maximaler Reinheit (15,73 % N) ergeben einen AS-Gehalt von 6.69 % (± 0.29 Prozent). Diese Bestimmung stimmt gut mit der genauesten gravimetrischen Bestimmung von CHIBNALL und seinen Mitarbeitern[2], sowie mit den bei mikrobiologischen Bestimmungen (HAC UND SNELL[5]) erzielten Werten überein.

REFERENCES

[1] F. W. FOREMAN, *Biochem. J.*, 8 (1914) 463, 481; 13 (1919) 378.
[2] K. BAILEY, A. C. CHIBNALL, M. W. REES, AND E. F. WILLIAMS, *Biochem. J.*, 37 (1943) 360; CHIBNALL, REES, WILLIAMS, *Ibid.*, 37 (1943) 372.
[3] CL. FROMAGEOT AND P. HEITZ, *Microchim. Acta*, 3 (1938) 52; P. DESNUELLE, *Enzymologia*, 5 (1938) 37.
[4] A. E. BRAUNSTEIN AND S. M. BYCHKOV, *Biochimia*, 8 (1943) 234.
[5] L. R. HAC AND E. E. SNELL, *J. biol. Chem.*, 159 (1945) 291.
[6] H. D. DAKIN, *J. biol. Chem.*, 141 (1941) 945.
[7] R. ENGELAND, *Ber. d. chem. Ges.*, 42 (1909) 2962.
[8] H. A. KREBS, D. H. SMYTH, AND E. A. EVANS, *Biochem. J.*, 34 (1940) 1041.
[9] F. B. STRAUB, *Z. physiol. Chem.*, 236 (1935) 42.
[10] H. A. KREBS, *Biochem. J.*, 31 (1937) 2095.
[11] E. STOTZ AND A. B. HASTINGS, *J. biol. Chem.*, 118 (1937) 479.
[12] A. C. CHIBNALL, A. C. REES, AND E. F. WILLIAMS, *Biochem. J.*, 37 (1943) 354.
[13] H. D. DAKIN, *Biochem. J.*, 12 (1918) 290.
[14] J. L. STOKES AND M. GUNNES, *J. biol. Chem.*, 157 (1945) 651.
[15] W. W. UMBREIT, R. H. BURRIS, AND J. F. STAUFFER, *Manometric Techniques and Related Methods for the Study of Tissue Metabolism*, Minneapolis, 1946.

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