LOSSES OF TRYPTOPHAN DURING CHROMATOGRAPHIC ANALYSIS*

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Numerous workers using chromatographic methods for studying amino aciduria are unable to detect tryptophan in samples of physiological or pathological urine. CLARKSON AND KENCH[1] detected it in urine in cases of poisoning with heavy metals, the quantities ranging from 0 to 21 mg of amino-N according to the creatinine content of the urine. SCHÖNENBERG[2] found tryptophan in samples of urine (100 μl) of 23 out of 25 premature infants. WOODSON *et al.*[3], who used a microbiological test specific for the indole ring, detected it in quantities ranging from 9 to 56 mg/24 h. MOORE AND STEIN[4], EVERED[5], JONXIS[6], HUISMAN[7], and all others who determined amino acids by means of the ion-exchange method were not able to find tryptophan in urine. SMITH[8] on the other hand, always detected tryptophan in chromatograms of physiological urine that had been developed with isopropanol and treated with Ehrlich's reagent for location.

In our investigations[9] attention was drawn to the considerable losses of tryptophan and other amino acids with high R_F values that occur in the case of filter paper chromatograms developed with phenol systems.

The purpose of the present research was to determine the losses of tryptophan, occurring both during the chromatographic analysis proper and during the preparatory operations.

EXPERIMENTAL

Materials

Standard tryptophan solutions (Merck). Fibrinogen from bovine blood (own preparation). Samples of physiological urine.

Methods and results

The following tests were used to detect tryptophan in solution and in chromatograms:

(a) ninhydrin (0.1 % in acetone) in the cold[10],

(b) ninhydrin–copper test[11],

(c) fluorescence test (U.V.)[12],

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(d) Ehrlichs reagent (*p*-dimethylamino-benzaldehyde).

Comparative studies were carried out on the sensitivity of the individual tests.

TABLE I

SENSITIVITIES OF TRYPTOPHAN TESTS

Test	*μg/cm²*
U.V.	0.03
Ninhydrin*	0.06
Ninhydrin–Cu	0.20
p-DMB	0.06

* Detected at room temp.

The following methods were used for the quantitative determination of tryptophan in solution:

(a) SPIES AND CHAMBERS[13],

(b) FISCHL[14],

and in eluates obtained from chromatograms:

(c) SULLIVAN AND HESS[15],

(d) BORKOWSKI AND MADECKA-BORKOWSKA[16],

(e) Fluorescence test[17].

TABLE II

COMPARISON OF METHODS USED FOR THE QUANTITATIVE DETERMINATION OF TRYPTOPHAN

No.	*Authors*	*Principle of method*	*Material*	*Optimum concentration of Try in μg*	*% Error of method*	*Remarks*
1	SULLIVAN AND HESS	*p*-DMB HCl, $NaNO_3$	Solutions	10–40	5–10	
2	BORKOWSKI AND MADECKA-BORKOWSKA	Ninhydrin–Hg	Eluates	1–15	10–15	
3	SPIES AND CHAMBERS	*p*-DMB H_2SO_4	Solutions	10–100	3–5	Cl^- and SO_3^{2-} interfere
4	SULLIVAN AND HESS	*p*-DMB	Eluates from paper	13–40	10–25	
5	FISCHL	ADAMKIEWICZ-HOPKINS	Solutions	5–50	3–5	Oxidizing agents interfere

Investigations were carried out on tryptophan losses resulting from operations preliminary to the chromatographic analysis proper:

A. Hydrolysis

B. Decoloration,

C. Desalting.

A. Hydrolysis

Tryptophan losses during hydrolysis were studied in standard solutions of tryptophan and fibrinogen. The following techniques were used:

(a) acid hydrolysis with 6 *N* HCl,
(b) acid hydrolysis with 8 *N* H_2SO_4,
(c) alkaline hydrolysis with 5 *N* NaOH,
(d) enzymic hydrolysis with pepsin and trypsin. The acid and alkaline hydrolyses were carried out at 105° in sealed vials, for periods ranging from 30 min to 24 h. Tryptophan was determined by two parallel methods:
FISCHL method (indole ring),
SPIER-PASHER method (amino nitrogen)[18].

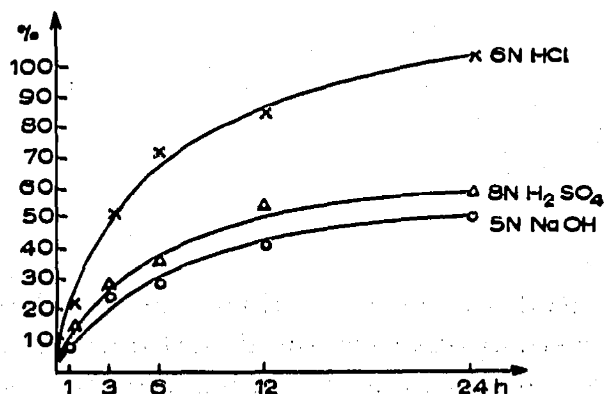

Fig. 1. Disappearance of tryptophan under different conditions of hydrolysis.

For the hydrolysis of fibrinogen 200 mg of a preparation containing 3 % tryptophan was used. Acid hydrolysis (HCl and H_2SO_4) and alkaline hydrolysis were conducted as described above at 105° for periods of 30 min to 24 h.

Free tryptophan and protein-bound tryptophan were determined in the hydrolysates, dialysis being applied.

For enzymic hydrolysis 200 mg of fibrinogen was used; this was first subjected to the action of pepsin (pharmaceutical preparation, 20 mg) for 12 h, and to that of pancreatin (30 mg) for 48 h.

The results of these comparative studies are shown in Table III.

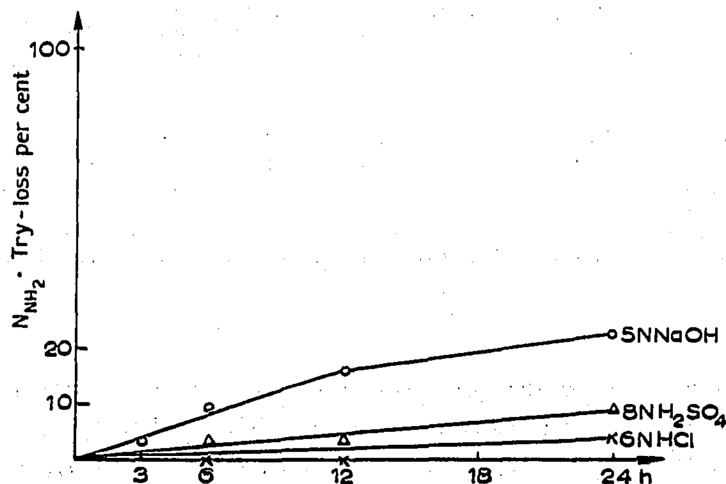

Fig. 2. Disappearance of tryptophan amino nitrogen under different conditions of hydrolysis.

TABLE III

LOSS OF TRYPTOPHAN DURING HYDROLYSIS OF PROTEINS (FIBRINOGEN)

Time (h)	6 N HCl			8 N H_2SO_4			5 N NaOH			Pepsin-trypsin
	Tryd*	Tryp*	Loss	Tryd	Tryp	Loss	Tryd	Tryp	Loss	Loss
	%			%			%			%
0.5	36	26	38	70	24	6	—	—	—	—
1.5	26	16	58	60	22	18	—	—	—	—
3	15	10	75	55	17	29	56	20	24	—
6	8	6	86	44	16	40	45	15	40	—
12	0	0	100	36	14	50	35	12	53	—
24	0	0	100	30	14	56	32	10	58	—
60	—	—	—	—	—	—	—	—	—	5–8

* d = dialysable; p = proteinic.

B. Decoloration

Coloured biological material was decolorized with active charcoal (carbo medicinalis).

In our previous investigations[19] we found that decoloration by means of charcoal is accompanied by losses of amino acids, which are greatest for cyclic amino acids, especially tryptophan. In order to determine the loss of tryptophan during decoloration with charcoal, samples of the solution being studied (5 ml) were shaken with charcoal (0.2 g). In order to reduce the adsorption of amino acids, a few drops of chloroform were added. Our comparative studies concerned standard tryptophan solutions also in the presence of other amino acids, and urine.

Tryptophan was determined before and after adsorption on charcoal by the method of FISCHL. The results of these investigations are given in Tables IV and V.

TABLE IV

LOSS OF TRYPTOPHAN DUE TO ADSORPTION ON CHARCOAL

No.	Try before ads. µg	Try after ads. µg	% Loss	Try + $CHCl_3$ before ads. µg	Try + $CHCl_3$ after ads. µg	% Loss	Try + AA* before ads. µg	Try + AA after ads. µg	% Loss	Try + AA + $CHCl_3$ before ads. µg	Try + AA + $CHCl_3$ after ads. µg	% Loss
1	27.5	0	100	27.5	8.0	71	26.0	0	100	34.0	14.0	59
2	47.5	0	100	49.0	16.0	69	44.0	0	100	43.0	17.0	61
3	37.5	0	100	35.0	11.0	70	36.0	0	100	67.0	27.0	61

* Mixture contained 20 µg of each Amino Acid (Ser, Ala, Glu, Arg, Try).

C. Desalting

Desalting of the studied material (standard tryptophan solution with mineral salts added; mixture of amino acids with mineral salts added) was conducted on the cation exchanger SDW_3 (U.S.S.R.). The results of the determination of tryptophan losses (in percentage) are shown in Table VI.

TABLE V

LOSS OF TRYPTOPHAN ADDED TO URINE DUE TO ADSORPTION ON CHARCOAL

Try in urine before adsorp. µg	*Try in urine after adsorp.* µg	*Loss* %	*Urine + Try before adsorp.* µg	*Urine + Try after adsorp.* µg	*Loss* %	*Urine + Try + $CHCl_3$ before adsorp.* µg	*Urine + Try + $CHCl_3$ after adsorp.* µg	*Loss* %
60.0	12.0	86	98.0	0	100	98.0	26.0	73
60.0	11.0	88	98.0	0	100	98.0	34.0	65

TABLE VI

LOSS OF TRYPTOPHAN DURING DESALTING ON THE CATION EXCHANGER SDW_3

Tryptophan added µg	*Tryptophan determined* µg	*Eluate*		
		Try recovered µg	*Loss of Try* µg	*Loss* %
5.0	4.0	2.7–3.0	1.0–1.3	25–32
10.0	9.5	7.0–7.5	2.0–2.5	21–26
20.0	20.0	15.5–17.0	3.0–4.5	15–22
10.0 + AA*	9.5	7.8–8.0	1.5–1.7	16–18

* Mixture contained 10 µg of each Amino Acid (Asp, Arg, Ala, Ser).

D. Loss of tryptophan during development of chromatograms

Comparative studies were carried out by developing chromatograms of tryptophan in the phenol, butanol and propanol systems. The ascending technique was applied and Whatman paper No. 1 (28.5 × 28.5 cm) was used. The results of the estimations before and after development of the chromatograms are presented in Table VII.

The correlation between the loss of tryptophan (in percentage) and its concentration and the time of development of the chromatograms in phenol is shown in Fig. 3. We failed to detect tryptophan either directly or in eluates of those areas of the filter paper where the migration of amino acids in phenol–water had taken place. The sensitive fluorescence test was used for location.

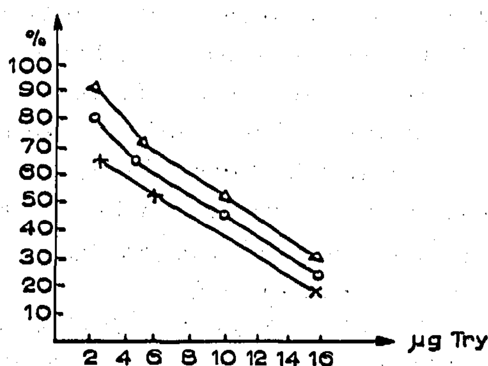

Fig. 3. Loss of tryptophan during development in phenol–water as a function of the concentration of tryptophan and the time of development. ×—×—× 6 h; ○—○—○ 12 h; △—△—△ 18 h.

TABLE VII

LOSS OF TRYPTOPHAN DURING DEVELOPMENT ON PAPER CHROMATOGRAMS

No.	Solvent	% Loss of tryptophan		
		2.6 μg	5.2 μg	10.4 μg
1	Phenol–water (7:3)	88	67	48
2	Propanol–water (7:3)	30	22	15
3	*n*-Butanol–acetic acid–water (15:2:3)	58	23	10
4	Isopropanol–ammonia–water (20:1:2)	50	15	9
5	*n*-Butanol–methyl ethyl ketone–water (2:2:1)	87	65	48

DISCUSSION AND CONCLUSIONS

The location and determination of tryptophan in biological material are always difficult and still present a controversial problem.

There may be various reasons for these difficulties: first, tryptophan solutions are labile, and numerous reagents used in chromatographic analysis exert a destructive action on tryptophan.

In our investigations we detected tryptophan using the universal tests for amino acids, such as ninhydrin alone in acetone solution and ninhydrin as copper complex, the U.V. test, and tests specific for the indole ring, such as *p*-dimethylamino-benzaldehyde (*p*-DMB). Of these tests the U.V. test is the most sensitive. In detailed investigations on the sensitivity of this test[12] we found that sensitisation by impregnating the paper with 1,2-naphthoquinone-4-sulphonate does not increase its sensitivity for tryptophan, while it does for other amino acids.

The ninhydrin–copper reagent is especially useful for locating tryptophan in a mixture of amino acids with similar R_F values, since it produces a characteristic blue ring round the brown spot, which ring quickly disappears[11]. The Ehrlich test (*p*-DMB), which is specific for the indole ring, is useful for locating tryptophan in amino acid mixtures with non-desalted material. The sensitivity of this test does not differ from that of the ninhydrin reaction. Several different methods were used for quantitative estimations:

The methods of SULLIVAN AND HESS, SPIES AND CHAMBERS and the method of FISCHL were applied to determine tryptophan in solutions. Of these methods that of FISCHL seems to be the best. It can be used for a broad range of concentrations, the results are approximately constant, the technique is simple and brief, the percentage of error slight and the components which usually accompany the studied substances in the biological material have no marked influence on the results of the determination of the amino acid composition. It should be stressed, however, that traces of impurities in acetic acid (except the unavoidable glyoxal) and in sulphuric acid used for analysis influence the results very distinctly.

To determine tryptophan in eluates we found convenient the ninhydrin method modified by BORKOWSKI and MADECKA-BORKOWSKA. In addition we sometimes used a method based on the fluorescence test and adapted by us to quantitative estimations[17].

Our previous investigations showed that the losses of tryptophan during chromatographic analysis are greater than those of other amino acids. It seemed therefore advisable to investigate the loss of tryptophan occurring during the various preliminary operations and during the chromatographic analysis proper.

In the first place we investigated the behaviour of tryptophan under the conditions of hydrolysis of proteins, both acid and alkaline.

Two methods were used: that of SPIER AND PASHER (amino nitrogen) and of FISCHL (indole ring).

As shown in Figs. 1 and 2 the changes in amino nitrogen after hydrolysis for 24 h are slight; the indole ring on the other hand undergoes a change. The character of the curves suggests the possibility of a condensation of indole rings. It may be also of interest to observe that changes in the indole ring are greatest in the case of hydrolysis with hydrochloric acid; they are smaller when sulphuric acid is used and slightly smaller in alkaline hydrolysates.

The reverse can be said of amino nitrogen. The greatest losses occur during hydrolysis with caustic soda, the smallest when hydrochloric acid is used. The same hydrolysis reactions were applied to bovine fibrinogen prepared in our department. Application of the FISCHL method to the determination of free and bound tryptophan in dialysates made it possible to follow the two processes occurring during fractional hydrolysis: splitting off of tryptophan and its decomposition.

It was found (Table III) that after acid hydrolysis for 3 h tryptophan had been destroyed to an extent of almost 75 %; hydrolysis with sulphuric acid destroys only 20 % of the tryptophan. After hydrolysis for 24 h with hydrochloric acid, the loss of tryptophan is 100 %; with sulphuric acid, it is 60 %. The results obtained with alkaline hydrolysis resemble those obtained on hydrolysis with sulphuric acid.

The common practice of decolorizing biological material by adsorption on active charcoal cannot be recommended when amino acid analysis is contemplated. Detailed investigations (Tables IV and V) proved that decoloration is especially harmful to tryptophan. In experiments carried out with a standard tryptophan solution, with a mixture of amino acids containing tryptophan and with urine samples to which tryptophan had been added, the losses of this amino acid fluctuated between 60 and 100 %. Desalting on ion exchangers also caused losses of tryptophan that were greater than those of other amino acids.

As far as the development of chromatograms is concerned the choice of suitable solvent systems with regard to tryptophan is especially important. Comparative investigations carried out with different solvent systems showed that the phenol systems are especially harmful to tryptophan. When the concentration of tryptophan is low, the losses in these systems are as high as 90 %. Considerable losses of tryptophan occur also in systems containing ketone compounds (Table VII).

The dependence of tryptophan losses in phenol systems on the time of development and concentration of the tryptophan are shown in Fig. 3. Even with the most sensitive tests, *e.g.* the fluorescence test, it was not possible to detect traces of tryptophan adsorbed on the filter paper. Those parts of the paper that had been traversed

by the migrating amino acids were tested for the presence of tryptophan, both immediately and after elution. In spite of repeated efforts not even traces of tryptophan could be discovered in the paper. The supposition that the indole ring is decomposed in the presence of phenol seems to be justified.

The general conclusion is that special care must be taken when mixtures containing tryptophan are analysed chromatographically. If the original biological material is subjected to hydrolysis the smallest losses, less than 10 %, occur when the time-consuming but safer enzymic method consisting in the successive application of two enzymes, pepsin and trypsin, is applied. Both acid and alkaline hydrolysis produce losses of tryptophan greater than 50 %. Decoloration on charcoal should be avoided; the error which results from desalting on ion exchangers should be taken into account. Phenol systems as well as those containing ketones should not be used for the development of chromatograms of amino acid mixtures containing tryptophan.

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SUMMARY

Detailed investigations revealed the occurrence of considerable losses of tryptophan during acid hydrolysis (HCl or H_2SO_4) and alkaline hydrolysis, during decoloration of the material on active charcoal (carbo medicinalis), during desalting on ion exchangers, and during the development of chromatograms in phenol systems or in the presence of ketone compounds.

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