A Rapid and Direct Method for the Quantitative Determination of Tryptophan in the Intact Protein

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1. A method is given for the quantitative determination of free tryptophan or tryptophan in the intact protein by treating with ninhydrin in a mixture of formic acid and hydrochloric acid (reagent *b*), for 10 min at 100°C. Glycyltryptophan was used as a standard for the determination of tryptophan in the intact protein. The extinction at 390 nm was linear in the range 0.05–0.5 μ mol for free tryptophan (ϵ 7120) and 0.05–0.30 μ mol for glycyltryptophan (ϵ 15400). 2. Free tryptophan in the presence of protein may be determined by treating with ninhydrin in a mixture of acetic acid and 0.6 M-phosphoric acid (reagent *a*) for 10 min at 100°C, the extinction being linear for tryptophan in the range 0.05–0.9 μ mol. *N*-Terminal tryptophan peptides also give the typical yellow product on treatment with reagent *a*. 3. Tryptophan content of several pure intact proteins when treated with the above method gave values in good agreement with those reported by others. A mean tryptophan content of 11.25 (S.E.M. $\pm$ 0.08) μ mol/100 mg of protein was found in rat brain during development from 1 to 82 days after birth.

An acid ninhydrin reagent used for the specific spectrophotometric determination of cysteine was reported to give a yellow product (λ_{max} 370 nm) from tryptophan, 5-hydroxytryptophan, 5-hydroxytryptamine and indol-3-ylacetic acid (Gaitonde, 1967). Since the molar extinction coefficient given by indol-3-ylacetic acid was approximately three times that given by tryptophan, it appeared that the yellow product from tryptophan resulted mainly from its specific reaction with the indole ring. This finding suggested that the reaction may be applicable for the direct determination of tryptophan in the intact protein (without subjecting the protein to alkaline hydrolysis).

In this paper methods are described for the quantitative determination of free tryptophan (in the presence of proteins) and tryptophan in the intact protein on treatment with the appropriate ninhydrin reagent for 10 min at 100°C. The method reported here for tryptophan in the intact protein is new and has advantages over other methods based on u.v. spectrophotometry (Goodwin & Morton, 1946; Bencze & Schmid, 1957) and Ehrlich reaction (Spies & Chambers, 1949), since it is rapid, simple, direct and specific for tryptophan in the intact protein. Formic acid used in the ninhydrin reagent acts as a solvent for protein.

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EXPERIMENTAL

Chemicals. Formic acid (98–100%, sp.gr. 1.22), acetic acid (99.7%, sp.gr. 1.048–1.050), hydrochloric acid (35.4%, sp.gr. 1.18) and phosphoric acid (88%, sp.gr. 1.75) all of analytical grade, were obtained from BDH (Chemicals) Ltd., Poole, Dorset, U.K. Lysozyme, trypsin, albumin, histones and glycyltryptophan were obtained from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. Ribonuclease, tryptophan di- and tri-peptides and *N*-acetyltryptophan ethyl ester were products of Mann Research Laboratories, New York, N.Y., U.S.A. Insulin (80 units/ml) was supplied by Boots Pure Drug Co. Ltd., Nottingham, U.K. The source of several other chemicals used was as reported elsewhere (Gaitonde, 1967).

Preparation of rat brain protein. The fresh tissue was treated with solvents to remove lipids and acid-soluble components. Details on the preparation of the tissue protein are given elsewhere (Gaitonde & Martenson, 1970). The tissue residue and other proteins obtained commercially were analysed for protein content by the method of Lowry, Rosebrough, Farr & Randall (1951), with dry bovine serum albumin as a standard.

Dissolution of protein and peptides for analysis of tryptophan. A weighed amount of protein was dissolved in water, 0.5 M-NaOH or formic acid; if required the residue was incubated at 38°C for 16–20 h. The solution of tissue protein in NaOH was pale yellow and in formic acid purple; however, it required no significant correction for these colours. All peptides were dissolved in 0.1 M-NaOH; *N*-acetyltryptophan ethyl ester was dissolved in 25% methanolic 0.1 M-NaOH.

Ninhydrin reagents. The two ninhydrin reagents used for the specific quantitative determination of tryptophan were prepared by dissolving by repeated mixing 250 mg of ninhydrin in 10 ml of either reagent *a* (acetic acid-0.6 M-phosphoric acid, 3:2, v/v) or reagent *b* (formic acid-hydrochloric acid, 3:2, v/v).

Determination of free tryptophan or tryptophan in the intact protein. To a sample (0.5 ml) containing 0.05–0.8 μ mol of tryptophan in a test tube was added 0.5 ml of ninhydrin reagent *a* or *b*. The reagent *a* was used for free tryptophan in the presence of protein and reagent *b* was used for free tryptophan in the absence of protein or for tryptophan in the intact protein. The contents of the tube were mixed, covered with metal caps or glass marbles and heated in a boiling-water bath for 10 min. The tubes were cooled, and 3 ml of ethanol (95%) was added to each to bring the volume to 4 ml and the contents were mixed. The extinction at $\lambda_{\max}$, between 385 and 390 nm or extinction at 400 nm was measured against the reagent blank. The extinction was proportional to the amount of free tryptophan in the range 0.05–0.9 μ mol with reagent *a* and 0.05–0.5 μ mol with reagent *b* or to the amount of protein (Figs. 1 and 2). The tryptophan content of the intact protein was determined with glycytryptophan as the standard (0.05–0.3 μ mol).

Since the two ninhydrin reagent blanks give an extinction peak at 350 nm, it is important to add the exact volume of the reagent to all the samples. The pipettes calibrated with certain dyes were found unsuitable for delivering the ninhydrin reagent, since the strong acids present in the reagent dissolved small amounts of the dye in the reagent.

Procedure for the determination of tryptophan content of a protein. Since the molar extinction of the yellow product given by *N*-terminal tryptophan peptide on treatment with reagent *b* is approximately the same as that given by free tryptophan but that given by the products of *C*-terminal tryptophan peptide or peptide in which tryptophan is sandwiched between two amino acids by peptide linkages is 2.22 times that given by free tryptophan (Table 1), the following procedure may be used for the determination of tryptophan in a given sample of protein. A solution containing 5–10 mg of the

protein is treated with reagent *a*. If the reaction mixture shows no extinction peak between 385 and 400 nm, the protein contains no *N*-terminal tryptophan. The total tryptophan content of the protein is then determined by treating another portion of the protein solution with reagent *b* as described above.

The presence of an extinction peak between 385 and 400 nm in the reaction mixture with reagent *a* indicates the presence of *N*-terminal tryptophan in the protein. The *N*-terminal tryptophan content is determined by reference to 0.76 times the extinction values for known amounts of free tryptophan (Table 1). Another portion of the protein solution is then treated with reagent *b* and the observed extinction is corrected by subtracting the 0.90 times the extinction given by tryptophan equivalent to the amount of *N*-terminal tryptophan in the sample, on reaction with reagent *b*. From the net extinction so obtained, the amount of tryptophan present as *C*-terminal tryptophan or tryptophan sandwiched between two amino acids in the polypeptide chain is calculated with glycytryptophan as the reference compound.

Since 1 μ mol of tryptophan on treatment with reagent *a* and reagent *b* and dilution of the reaction mixture to 4 ml gives extinction values of 1.30 and 1.78 respectively, the *N*-terminal tryptophan content of x_a mg of protein will be:

$$\frac{E_a}{0.76 \times 1.30} = \frac{E_a}{0.988} = E_a \mu\text{mol}$$

where E_a is the extinction given by x_a mg of protein on treatment with reagent *a*. If the extinction given by x_b mg of the same protein is E_b on treatment with reagent *b* and dilution of the reaction mixture to 4 ml, its tryptophan content minus *N*-terminal tryptophan will be:

$$\frac{E_b - 0.90 \times 1.78 \times E_a \times \frac{x_b}{x_a}}{2.22 \times 1.78} = \frac{E_b - 1.6 \times \frac{x_b}{x_a} \times E_a}{3.95} \mu\text{mol}$$

If $x_b = x_a$ this will become:

$$\frac{E_b - 1.6 E_a}{3.95} \mu\text{mol}$$

Fig. 1. Extinction of the yellow product formed on treatment with different amounts of tryptophan with ninhydrin reagent *a* (●) or reagent *b* (○).

Fig. 2. Extinction of the yellow product formed on treatment with different amounts of lysozyme (●), albumin (○) and trypsin (Δ) with ninhydrin reagent *b*. Each unit scale represents 0.1 mg for lysozyme, 0.5 mg for albumin and 0.1 mg for trypsin.

Table 1. *Molar extinction coefficients of the reaction products of different indole compounds on treatment with reagent a and with reagent b*

Compound	Reagent a		Reagent b	
	ϵ_{385}	$\epsilon/\epsilon_{\text{Trp}}$	ϵ_{390}	$\epsilon/\epsilon_{\text{Trp}}$
Tryptophan	5200	1.00	7120	1.00
5-Hydroxytryptophan	*	—	5120	0.74
Tryptamine	*	—	16600	2.33
5-Hydroxytryptamine	*	—	14800	2.08
Indol-3-ylacetic acid	*	—	12400	1.74
5-Hydroxyindol-3-ylacetic acid	*	—	11800	1.66
5-Hydroxytryptophol			12400	1.74
<i>N</i> -Terminal tryptophan peptides				
Trp-Gly	4880	0.94	6240	0.90
Trp-Lys	3640	0.70	6320	0.89
Trp-Tyr	3240	0.62	6240	0.88
Trp-Glu	3920	0.75	6520	0.92
Trp-Leu	4120	0.79	6720	0.94
<i>C</i> -Terminal tryptophan peptides				
Gly-Trp	400	0.07	15400	2.16
<i>N</i> -Acetyl-Trp ethyl ester	320	0.06	17450	2.45
Tryptophan 'sandwich' peptides				
Lys-Trp-Lys	240	0.05	13800	1.94
Gly-Trp-Gly	400	0.07	15000	2.11
Glu-Trp-Glu	240	0.05	17000	2.39
Leu-Trp-Leu	320	0.06	16200	2.28

* The molar extinction coefficients of these compounds obtained under somewhat different conditions were reported by Gaitonde (1967).

Hence the total tryptophan content of $x_a (= x_b)$ mg of protein will be:

$$\frac{E_b - 1.6 E_a}{3.95} + E_a \mu\text{mol}$$

RESULTS AND DISCUSSION

Tryptophan gave a yellow product on reaction with the two ninhydrin reagents: the molar extinction coefficient of the product on treatment with reagent *a* (5200) was lower than with reagent *b* (7120). The molar extinction coefficient of the product decreased when acetic acid was replaced by formic acid (3120) in reagent *a* and when formic acid was replaced by acetic acid (3920) in reagent *b*. The extinction of the yellow product of free tryptophan, lysyltryptophyl-lysine, lysozyme, trypsin and albumin reached a maximum value on heating the reaction mixture for 10 min in a boiling-water bath: the extinction showed a deviation of $\pm 2\%$ from the mean on increasing the time from 10 to 20 min. The yellow product formed in the presence of reagents *a* or *b* was stable for 3–4 h; it lost 13–15 and 7–8% of the extinction respectively in 24 h. Phenylalanine gave no yellow product with either reagent. Tyrosine gave a molar extinction coefficient of 640 with reagent *a* and 400 with reagent

b; these values are equivalent to 13 and 6% of the molar extinction coefficients of the product of tryptophan on treatment with the two respective reagents. This indicates that reagent *b* is to be preferred to reagent *a* for the determination of free tryptophan in the presence of tyrosine. No yellow product was formed from tyrosine if 0.6M-phosphoric acid was replaced by 6.0M-phosphoric acid in reagent *a*, but this decreased the molar extinction coefficient for tryptophan from 5200 to 3760.

Other indoles besides tryptophan also gave a yellow product on treatment with reagents *a* and *b* (Gaitonde, 1967). The molar extinction coefficient of the yellow product formed on treatment with reagent *b* was 7120 for tryptophan, 5120 for 5-hydroxytryptophan and a mean value of 13600 for other indoles (Table 1). These findings raised the possibility that the molar extinction coefficient of tryptophan in the intact protein may be different depending on whether treatment with reagent *b* involved partial hydrolysis of protein into (i) *N*-terminal tryptophan, (ii) *C*-terminal tryptophan or (iii) complete hydrolysis into free tryptophan. Several tryptophan peptides were therefore tested with reagents *a* and *b* separately. A yellow product was formed on treatment with reagent *a* only from *N*-terminal tryptophan dipeptides (Table 1). This

indicated that the free amino group of tryptophan was essential for the formation of the yellow product in the presence of reagent *a* and that reagent *a* did not hydrolyse the peptide bonds. The *N*-terminal tryptophan peptide gave a mean extinction coefficient of 76% of that given by free tryptophan (Table 1).

In contrast, all the tryptophan peptides gave a yellow product on treatment with reagent *b*. (i) All the *N*-terminal tryptophan dipeptides gave molar

extinction coefficients similar to that given by free tryptophan (Table 1). (ii) The *C*-terminal tryptophan dipeptide glycyltryptophan, *N*-acetyltryptophan ethyl ester and tryptophan 'sandwich' tripeptides gave molar extinction coefficients of, respectively, 2.16, 2.45 and 2.18 (mean values) times that given by free tryptophan. In view of these findings it is possible that the peptide bond between the carboxyl group of tryptophan and the amino group of the next residue is split on treatment with reagent *b*, but evidently that between the amino group of tryptophan and the carboxyl group of the previous residue is resistant to such treatment, at least until the course of the reaction has departed from that with free or *N*-terminal tryptophan.

Reagent *a* gave no measurable amount of the yellow product from lysozyme (0.5mg), a protein that is rich in tryptophan content. However, reagent *b* gave the typical yellow product of tryptophan when treated with a solution containing lysozyme, albumin, trypsin and brain proteins. The extinction was directly proportional to the amount of protein (Fig. 2). Insulin and ribonuclease, reported to contain 11.3% and 6.8% of tyrosine respectively (Tristram & Smith, 1963), gave no typical yellow product: these findings give evidence that reagent *b* does not react with tyrosine in the protein and that it reacts specifically with the indole ring in the protein. Tryptophan added to a solution of lysozyme was completely recovered when treated with reagent *a* or *b* (Fig. 3).

The method is applicable to mixtures of tissue proteins as well as pure proteins (Table 2). The tryptophan content of rat brain protein showed no

Fig. 3. Optical spectra of the yellow products of tryptophan (0.25 μ mol, curve A), lysozyme (0.5 mg, curve B) and a mixture (curve C) of tryptophan (0.25 μ mol) and lysozyme (0.5 mg) on treatment with reagent *b* as described in the text. The line O indicates the spectrophotometer setting for zero extinction. The vertical line between 360 and 370 nm is an artifact resulting from the change of tungsten to deuterium light-source.

Table 2. *Tryptophan content of some proteins after treatment with ninhydrin reagent b*

Values given in square brackets represent μ mol of tryptophan in the protein equivalent to 100 mg of bovine serum albumin used as reference protein for the determination of protein content of samples by the method of Lowry *et al.* (1951). References: ^a Canfield (1963); ^b Barman & Koshland (1967); ^c Viswenatha, Lawson & Witkop (1960); ^d Tristram & Smith (1963); ^e Smythe, Stein & Moore (1963); ^f Smith (1966).

Protein	Source of protein	Wt. of protein used for analysis (mg)	Tryptophan content (μ mol/100 mg of protein)	
			Present work	Reported by Others
Lysozyme	Egg white	0.05–0.60	43.2 [26.6]	42.0 ^a , 39.0 ^b
Trypsin	Bovine pancreas	0.5–1.0	18.5 [16.1]	18.3 ^b , 16.5 ^c
Albumin	Bovine serum	1.0–5.0	3.9	2.9 ^d
Ribonuclease	Bovine pancreas	1.0–5.0	0.0	0.0 ^a , 0.4 ^b
Insulin	Bovine-porcine	1.82 (40 units)	0.7	0 ^d
Histone, arginine-rich	Calf thymus	5.0	0.4 [0.3]	0.0 ^f , ^d
Histone, lysine-rich	Calf thymus	5.0	0.9 [0.8]	
Rat brain			[11.3]	

significant changes during postnatal development from the first day until 82 days after birth: the mean value for this period was 11.25 (S.E.M. $\pm$ 0.08) μ mol/100mg of protein (2.1% of the anhydro amino acid).

REFERENCES

- Barman, T. E. & Koshland, D. E., jun. (1967). *J. biol. Chem.* **242**, 5771.
- Bencze, W. L. & Schmid, K. (1957). *Analyt. Chem.* **29**, 1193.
- Canfield, R. E. (1963). *J. biol. Chem.* **238**, 2691.
- Gaitonde, M. K. (1967). *Biochem. J.* **104**, 627.
- Gaitonde, M. K. & Martenson, R. E. (1970). *J. Neurochem.* **17**, 551.
- Goodwin, T. W. & Morton, R. A. (1946). *Biochem. J.* **40**, 628.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- Smith, M. H. (1966). *J. theor. Biol.* **13**, 261.
- Smythe, D. G., Stein, W. H. & Moore, S. (1963). *J. biol. Chem.* **238**, 227.
- Spies, J. R. & Chambers, D. C. (1949). *Analyt. Chem.* **21**, 1249.
- Tristram, G. R. & Smith, R. H. (1963). *Adv. Protein Chem.* **18**, 227.
- Viswanatha, T., Lawson, W. B. & Witkop, B. (1960). *Biochim. biophys. Acta*, **40**, 216.