CHROM. 5382

Amino acid analysis: The reduction of ninhydrin reagent with titanous chloride

Reduced ninhydrin (hydrindantin) can be added to the reagent mixture used with amino acid analyzers for colour development[1] or it can be formed in the reagent mixture by the addition of stannous chloride[2,3] (reduction can also be effected with cyanide[4] or ascorbic acid[5,6]). When the hydrindantin is added directly to the reagent mixture the disadvantages are that great care must be exercised to prevent air oxidation taking place during transfer to the analyzer reservoir[1] and a delay of 12 h is recommended before the mixture is ready for use. When the hydrindantin is formed by the addition of stannous chloride, precipitation of tin salts eventually occurs in the reagent reservoir and flow lines of the analyzer. Where a piston ninhydrin pump is used to supply ninhydrin mixture this salt deposit could give rise to variations in the pumping rate. Periodic flushing of the system with water will remove this precipitate.

It has been found that commercially available titanous chloride solutions can be used successfully and more conveniently in place of stannous chloride in the preparation of reduced ninhydrin reagent for use in automatic amino acid analysis. Furthermore, no precipitate has been observed to occur in the reagent mixture upon standing.

Titanous chloride solution L.R. (15% w/v) was supplied by Hopkin and Williams Ltd., Essex, Great Britain, and contained 14.78% w/v of $TiCl_3$ (gravimetric determination using Cupferron). Ninhydrin and methyl cellosolve, 'Sequanal' grade, were supplied by the Pierce Chemical Co., Rockford, Ill., U.S.A. 'Analar' sodium acetate and potassium acetate were supplied by B.D.H., Poole, Dorset, Great Britain. 'Univar' stannous chloride was obtained from Ajax Chemicals Ltd., Sydney, Australia. The amino acid analyses were carried out on a Technicon Auto-Analyzer.

The ninhydrin reagent mixture was prepared according to SPACKMAN *et al.*[2], except that a mixed sodium/potassium acetate buffer[7] and a lower concentration of ninhydrin were used, the reduction being effected with either stannous chloride or titanous chloride. The mixture used contained 60 g of ninhydrin dissolved in a 3:1 mixture of methyl cellosolve and acetate buffer. The final volume of the reagent was 4 l and the ninhydrin was reduced with either 7.5 ml of titanous chloride solution or 1.2 g of stannous chloride. The reagent was kept under nitrogen.

When the titanous chloride is added the reagent mixture immediately changes colour from yellow to red (which fades to brown) and after an interval of 30 min is ready for use. It is worth noting the convenience of a pipette addition of titanous chloride solution over the weighing out of the stannous chloride in the preparation of the reagent mixture. Although titanous chloride, like stannous chloride, is susceptible to air oxidation[8] no significant difference could be detected in the colour development potential of a reagent mixture prepared utilizing titanous chloride solution recently acquired from the supplier or another sample, estimated to be thirteen years old, from the same supplier.

The amino acid analyses were obtained by using a two-column system. The short column contained Beckman M81 resin (height of resin, 6 cm) and the long

TABLE I

CALIBRATION CONSTANTS[a] OBTAINED WITH A SYNTHETIC AMINO ACID MIXTURE

Amino acid	*$SnCl_2$ reduction*	*Range*		*$TiCl_3$ reduction*	*Range*	
		High	*Low*		*High*	*Low*
Lys	68.5	69.0	68.0	73.6	76.5	72.6
His	63.8	65.2	61.7	66.8	68.2	64.6
Arg	67.0	68.4	64.8	65.5	66.7	64.5
Asp	57.0	58.5	55.5	53.7	55.0	51.4
Thr	61.0	62.5	60.5	59.0	61.2	57.0
Ser	63.0	65.0	61.0	61.6	63.0	60.0
Glu	65.0	66.0	63.0	64.2	65.0	63.4
Pro	15.6	16.4	14.6	15.9	16.8	15.0
Gly	63.5	66.0	62.5	63.1	66.7	61.0
Ala	65.2	66.5	64.5	64.7	66.3	63.0
½Cys	33.5	33.8	33.2	32.7	33.3	31.9
Val	64.1	66.0	62.0	63.0	64.8	62.7
Met	64.7	66.5	63.1	65.5	66.7	64.3
Ile	63.2	67.0	62.0	63.0	65.0	62.4
Leu	64.5	67.0	64.0	63.0	65.0	60.5
Tyr	62.5	64.0	61.5	62.0	62.8	61.4
Phe	62.3	63.7	60.6	61.4	62.2	60.8

[a] Average of five analyses.

column contained Technicon Chromobead Type A resin (height of resin, 57 cm). Buffer was pumped at 60 ml/h and the AutoAnalyzer was modified to allow the buffer to go directly into the mixing coil prior to the reaction bath coil (by-passing the proportioning pump). Nitrogen was pumped at approximately 50 ml/h and the ninhydrin at 60 ml/h. A double glass coil approximately 75 ft. long was used in the reaction bath and the reaction mixture took 15 min to go through the coil. The flow cells had a 15-mm light path and the absorbance of the reaction mixture was monitored at 570 nm and 440 nm.

The amino acid calibration mixture used was prepared in this laboratory and was applied to the ion-exchange columns at a concentration of 0.1 μmoles of each amino acid per ml. The volume applied was 1 ml. The data in Table I were obtained utilizing the two methods of reducing ninhydrin described above. The figures given are the average of five analyses and were calculated by dividing the $H \times W$ factor[2] by the concentration of the amino acid in the sample. These values are referred to as calibration constants.

The results presented in Table II relate to the stability of the ninhydrin reagent mixture prepared with titanous chloride. The calibration constants in column No. 1 were obtained 24 h after reagent preparations and those in column No. 2 28 days later (reagent kept under nitrogen). From the constants shown in Table II it can be seen that no deterioration in the colour development potential of the reagent can be detected.

To determine the working range of the titanous chloride-reduced ninhydrin reagent, the amino acid arginine was selected as being the most useful for this purpose; the arginine peak is low and broad on the chromatogram while most of the other amino acids, when present in excess of 0.1 μmoles, give peak heights too large to be estimated accurately on the logarithmic scale of the recorder.

TABLE II

CALIBRATION CONSTANTS OBTAINED OVER A PERIOD OF 28 DAYS WITH TITANOUS CHLORIDE AS THE NINHYDRIN REDUCING AGENT

Amino acid	*No. 1* (24 h)	*No. 2* (28 days)	*Amino acid*	*No. 1* (24 h)	*No. 2* (28 days)
Lys	76.5	72.2	Ala	64.8	65.3
His	66.0	64.6	½Cys	33.3	32.8
Arg	65.6	65.8	Val	60.2	64.8
Asp	51.4	54.0	Met	65.8	64.3
Thr	57.3	60.8	Ile	61.6	62.1
Ser	61.0	63.0	Leu	62.4	63.5
Glu	63.4	65.0	Tyr	61.4	61.5
Pro	15.5	16.1	Phe	60.8	61.0
Gly	63.0	62.7			

Mixtures of amino acids, containing (a) 0.1 μmoles, of each amino acid, (b) 0.2 μmoles, (c) 0.3 μmoles, and (d) 0.4 μmoles were applied to the short column in consecutive analyses. The $H \times W$ factor[2] for arginine was calculated in each case and the calibration constants were found to be (a) 66.0, (b) 66.1, (c) 67.1, and (d) 62.5. These results indicate that over the most useful and accurate range of the recorder peak print-out, a linear colour development takes place and that the potential of the reagent mixture for developing colour with amino acids is far in excess of that which would be required to maintain optimum conditions for quantitative analyses.

Whenever the titanous chloride reducing agent was replaced by stannous chloride (and *vice versa*) on consecutive analyses no significant change was required in the colorimeter potentiometer settings to maintain the correct locations for the recorder baseline print-out.

The above results show that, as a reducing agent for ninhydrin, titanous chloride is superior to stannous chloride in ease of mixture preparation and in the absence of salt precipitation within the flow lines of the analyzer. Satisfactory results are also given in colorimetric analysis.

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1 S. MOORE AND W. H. STEIN, *J. Biol. Chem.*, 211 (1954) 907.
2 D. H. SPACKMAN, W. H. STEIN AND S. MOORE, *Anal. Chem.*, 30 (1958) 1190.
3 S. MOORE AND W. H. STEIN, *J. Biol. Chem.*, 176 (1954) 367.
4 E. SCHWERDTFEGER, *J. Chromatogr.*, 7 (1962) 418.
5 R. ABDEHALDEN, *Z. Physiol. Chem.*, 252 (1938) 81.
6 E. S. WEST AND R. E. RINEHART, *J. Biol. Chem.*, 146 (1942) 907.
7 D. EAKER, *Symposium on Evaluation of Novel Protein Products, Stockholm, Sweden, Sept., 1968.*
8 A. I. VOGEL, *Textbook of Quantitative Inorganic Analysis*, 3rd Ed., Longmans, London, 1962, p. 329.

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