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AMINO ACID ANALYSIS

III. REDUCTION OF NINHYDRIN WITH TITANOUS CHLORIDE

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SUMMARY

In the reaction of ninhydrin with amino acids the presence of hydrindantin (reduced ninhydrin) is advantageous. This work compares the stability of a ninhydrin reagent which has been reduced with titanous chloride with that of a commercial preparation requiring direct addition of hydrindantin to the mixture. Aside from its greater stability, the advantages of the titanous chloride-containing preparation are:
(1) no necessity to filter the reagent while being used in the analyzer;
(2) the reagent is quicker and easier to prepare;
(3) no waiting period for maturation of the reagent is required; and
(4) the cost of the reagent is cheaper.

INTRODUCTION

Ninhydrin (1,2,3-indantrione monohydrate) is a difficult compound to work with. It is sensitive to light, atmospheric oxygen and changes in pH and temperature. The product obtained by reacting ninhydrin with alpha-amino acids is a coloured compound called Ruheman's purple[1]. Moore and Stein[2] reported that a linear relationship could be established between the intensity of this coloured compound and amino acid concentration, and that for optimum colour development, the reduced form of ninhydrin, hydrindantin (2,2′-dihydroxy-[2,2′-biindan]-1,1′,3,3′-tetrone) had to be present in the mixture. The inclusion of hydrindantin in the reagent mixture can be accomplished either by adding it directly, or by adding a reducing agent so as to reduce ninhydrin to hydrindantin.

Potassium cyanide and ascorbic acid have been used for the reduction of ninhydrin. Both these reductants had to be present at extremely high dilutions (0.005 *M*) at which reagent stability was difficult to maintain[3]; even a minute trace of oxygen could produce a large loss of colour development potential. Further, the presence of ascorbic acid in the reagent mixture gave rise to artifacts appearing on the amino acid chromatogram.

The most successful formulation of ninhydrin reagent was that containing

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stannous chloride as the reductant[2]. This preparation, although stable, produced a fine, white precipitate upon standing which accumulated in the reagent storage vessel and flow lines of the analyzer. To overcome the problem of precipitation within the analyzer flow lines, the use of titanous chloride as a reducing agent for ninhydrin was put forward[4,5].

This communication is concerned with providing a comparison between the stability of two ninhydrin reagent preparations, titanous chloride-reduced ninydrin reagent, used in a Beckman 119CL amino acid analyzer, and a ninhydrin–hydrindantin reagent, used in a Beckman System 6300 amino acid analyzer.

MATERIALS AND METHODS

Ninhydrin solid and methyl cellosolve were obtained from Pierce (Rockford, IL, U.S.A.), titanous chloride solution (15%, w/v) from Hopkin and Williams (U.K.), potassium acetate from Mallinckrodt (U.S.A.) and hydrated sodium acetate and glacial acetic acid from BDH (Poole, U.K.).

The acetate buffer was prepared in 5-l volumes and contained 1472 g (3 *M*) potassium acetate, 1020 g (1.5 *M*) hydrated sodium acetate and 500 ml glacial acetic acid. One litre of acetate buffer and 3 l of methyl cellosolve were used to dissolve 65 g of ninhydrin (solid), and 10 ml of titanous chloride solution (15%, w/v $TiCl_3$) were added to effect partial reduction of the ninhydrin. Oxygen-free nitrogen was bubbled through the mixture during the preparation, and upon transfer to the Beckman Model 119CL analyzer the mixture was stored under a 4-p.s.i. pressure of nitrogen.

The ninhydrin reagent for the Beckman System 6300 Analyzer is manufactured by Beckman-Spinco (Palo Alto, CA, U.S.A.) and is supplied in kit form (Cat. No. 338047). The user is required to add a pre-weighed amount of hydrindantin to a mixture of lithium acetate–dimethyl sulphoxide buffer and ninhydrin in solution.

A synthetic mixture of amino acids, Beckman calibration mixture (338088), was used for all the analytical determinations. After the System 6300 and 119CL analyzer(s) had been calibrated, all subsequent analyses were treated as unknown(s), using the previously obtained colour factors, K_f. Both models of amino acid analyzer used the same design of colorimeter.

RESULTS AND DISCUSSION

Although Kirschenbaum to suggested the use of dimethyl sulphoxide (DMSO) as a solvent for ninhydrin, Moore improved the recipe so as to be able to keep more hydrindantin in solution by substituting lithium acetate for sodium acetate in the buffered reagent. It is basically Moore's formulation[7] that is used in the Beckman System 6300 amino acid analyzer (supplied by the manufacturer). Upon addition of hydrindantin to the DMSO–lithium acetate mixture, continued stirring for 3 h is required to effect solution. This delay before being able to use the reagent in the analyzer, plus its cost, are the only drawbacks found against the satisfactory use of this reagent.

Initially the Beckman System 6300 Analyzer had a design defect which allowed atmospheric oxygen to contaminate the stored ninhydrin reagent. Table I shows the results of successive analyses of the same amino acid mixture over a period of 8 days.

TABLE I

DATA OBTAINED WITH BECKMAN SYSTEM 6300 AMINO ACID ANALYZER (BEFORE MODIFICATION OF NINHYDRIN REAGENT STORAGE BOTTLE)

Amino acid	*Amount (nmoles)*				
	Day 1	*Day 2*	*Day 3*	*Day 4*	*Day 8*
ASP	5.00	5.436	4.929	4.829	4.122
THR	5.00	5.116	5.032	4.938	4.097
SER	5.00	5.289	4.820	4.745	4.090
GLU	5.00	6.119	4.910	4.848	4.172
PRO	5.00	1.824	4.948	4.917	4.500
GLY	5.00	5.684	4.902	4.845	4.069
ALA	5.00	5.571	4.521	4.490	3.868
CYS	2.50	3.942	2.047	2.141	1.713
VAL	5.00	5.322	4.704	4.596	3.989
MET	5.00	5.668	4.890	4.817	4.151
ILE	5.00	5.149	4.873	4.766	4.077
LEU	5.00	5.310	4.835	4.734	4.087
TYR	5.00	4.941	4.912	4.798	4.112
PHE	5.00	4.718	4.899	4.779	4.112
HIS	5.00	5.497	4.855	4.679	3.934
LYS	5.00	6.385	4.873	4.749	4.046
AMM*	5.00	9.677	4.955	4.801	1.996
ARG	5.00	4.472	4.611	4.361	3.800

* AMM = ammonia.

TABLE II

DATA OBTAINED WITH BECKMAN SYSTEM 6300 AMINO ACID ANALYZER (AFTER MODIFICATION OF NINHYDRIN REAGENT STORAGE BOTTLE)

Amino acid	*Amount (nmoles)*				
	Day 2	*Day 4*	*Day 7*	*Day 14*	*Day 20*
ASP	5.017	4.794	4.898	4.867	4.695
THR	5.142	5.051	5.071	5.041	4.759
SER	5.033	4.871	4.857	4.948	4.761
GLU	5.132	4.978	4.977	5.026	4.801
PRO	5.026	4.985	4.916	5.018	4.889
GLY	5.214	5.143	5.074	5.132	4.875
ALA	5.161	4.940	4.861	5.026	4.710
CYS	4.148	3.279	3.235	3.244	2.562
VAL	5.162	4.984	4.903	5.023	4.680
MET	5.049	4.867	4.792	4.774	4.564
ILE	5.085	4.916	4.792	4.892	4.637
LEU	5.017	4.862	4.752	4.841	4.628
TYR	5.095	4.992	4.903	4.948	4.745
PHE	5.033	4.930	4.850	4.946	4.744
HIS	6.332	4.757	4.814	4.490	4.534
LYS	5.029	4.892	4.818	4.927	4.698
AMM*	5.558	5.261	5.562	6.529	6.400
ARG	5.203	4.862	4.688	4.851	4.640

* AMM = ammonium.

The analyzer was calibrated with a loading of 5 nmoles of each amino acid (2.5 nmoles for cystine) as shown in day 1, and the colour factors (K_f values) obtained for each amino acid from the analysis were used for all subsequent analyses. By day 2 it is apparent that the K_f values determined 24 h earlier are no longer adequate, as nearly all the amino acids (arginine excepted) are yielding amounts "in excess" of 5 nmoles. (Cystine increased from 2.5 to 3.94 nmoles.) In the case of glutamic acid and proline, adjacent peaks on the chromatogram, the integrator malfunctioned attributing some of the proline peak to that of glutamic acid. The increased colour development capacity of the reagent at day 2 was brought about either by a trace of oxygen contamination or by the normal maturation of the newly prepared reagent. But by day 8 the continued leakage of oxygen into the reagent had markedly decreased the colour development potential of the reagent, in the case of arginine by approximately 24%. The reaction of arginine with ninhydrin reagent appears to be particularly sensitive to the presence of oxygen, although colour development is noticeably effected with all the amino acids, the extent of decrease varying from one amino acid to the other. Of relevance in this context is the report that different amino acids react with ninhydrin with varying degrees of ease[3]. The K_f values in Table III reflect this difference. For example, glycine reacts quickly (high K_f value), while alanine reacts only half as rapidly. In fast analyzers such as the Model 119CL and the System 6300 the ninhydrin reagent and the amino acids are heated only briefly to develop Ruheman's purple; in the older analyzers, *e.g.*, the Beckman Model 120B, the reactants were heated at 100°C for 15 min to develop the colour, and the K_f values for glycine and alanine thus obtained were of the same order of magnitude.

A number of mechanisms have been proposed for the reaction of ninhy-

TABLE III

DATA OBTAINED WITH BECKMAN 119CL AMINO ACID ANALYZER

Amino acid	*K_f value, day 1*	*Amount (nmoles)*				
		Day 6	*Day 10*	*Day 18*	*Day 20*	*Day 23*
ASP	795	20.687	20.420	20.452	20.433	20.722
THR	878	20.501	20.362	20.335	20.330	20.711
SER	893	20.480	20.653	20.100	20.042	20.397
GLU	974	20.473	20.363	20.615	20.582	21.011
PRO	277	20.338	20.407	19.667	19.589	20.166
GLY	1499	20.125	20.380	20.029	19.846	20.364
ALA	706	20.625	20.159	20.060	19.942	20.481
CYS	1796	9.994	10.149	10.477	10.363	10.645
VAL	682	20.680	20.624	19.568	19.549	20.126
MET	1156	20.519	20.246	21.955	21.891	22.530
ILE	726	20.432	20.787	19.926	19.810	20.532
LEU	1056	20.332	20:529	20.427	20.297	20.913
TYR	1249	20.205	20.519	19.815	19.718	20.521
PHE	1095	20.268	20.490	20.416	20.191	21.007
HIS	2232	20.137	20.385	20.723	20.384	22.740
LYS	1290	20.007	20.828	18.919	19.577	20.203
AMM*	302	18.954	17.865	17.040	17.638	20.309
ARG	1126	20.446	20.817	19.536	19.492	20.227

* AMM = ammonia.

drin[2,6,8,9], but it must be accepted that at least four mechanisms (if you count that involving ammonia) occur. These involve: (1) the normal amino acids, which include the neutral amino acid, aromatic and basic amino acids; (2) the imino acids; (3) cystine and (4) ammonia, which reacts with hydrindantin only.

The sharp fall-off in the concentration of ammonia at day 8 (Table I) is the result of the integrator using a reserve K_f value, because the elution time was outside the programmed limits set for ammonia.Hence, in this case, the integrator divided by a larger number than necessary in the calculation (refer to K_f values in Table III):

$$\text{concn. NH}_3 = \frac{\text{Peak area}}{K_f}$$

The increase of ammonia concentration at day 2 is more difficult to explain; it is nearly double the expected concentration of 5 nmoles loaded onto the analyzer column. This result could be considered as an artifact or it could indicate that an equilibrium had been reached in the ninhydrin reagent at day 2 that was most conductive for reaction with the ammonia molecule.

Table II shows the results obtained under standard conditions with the System 6300 analyzer after a modification had been carried out. A larger bore tubing was installed between the pump and the ninhydrin in-line filter, and a separate gas line and regulator was inserted exclusively for the ninhydrin storage vessel. Previously the inert gas had entered the vessel from a manifold system. As can be seen a much improved performance by the reagent is effected. For example, in the case of arginine there was a fall-off of only 7% after the elapse of 20 days, while before the modification had been carried out an 18% loss in colour development had occurred after the passing of 7 days. Another factor that could have contributed to the increased stability of the ninhydrin reagent was the replacement of oxygen-free nitrogen by argon (which had not been suggested by the manufacturer) as the inert gas for filling the ninhydrin reservoir as reagent was withdrawn. The use of argon was found to be satisfactory, and a cylinder of argon (8 m^3) was depleted over a period of 4 months, thus, adding only a marginal increase to the cost of operating the analyzer. Also, with improved stability of the ninhydrin reagent, fewer instrument recalibration analyses have to be carried out during a long series of protein hydrolysate analyses to maintain accurate amino acid concentration determinations.

In the operation of the System 6300 analyzer, the pumping rates of buffer and ninhydrin reagent are preset to deliver a 2:1 ratio. This ratio has been maintained throughout the history of analyzer development by Beckman instruments and has proved successful with the ninhydrin formulations used with this company's instruments. But to use successfully ninhydrin reagent reduced by titanous chloride, this arbitrarily set ratio between buffer and ninhydrin pumping rates must be changed to 2:1:3. The results shown in Table III were obtained using the pumping rate of 40 ml/h for buffer delivery and 26 ml/h for ninhydrin reagent in a Beckman Model 119CL analyzer.

Also in this analyzer the ninhydrin reagent contained a more concentrated buffer; 3 *M* in potassium ion and 1.5 *M* in sodium ion. This was done to prevent even slight pH changes occurring when the ion-exchange column effluent, containing

hydrochloric acid, mixed with the ninhydrin reagent. No significant deviation in chromatogram baseline setting was observed from the use of the more concentrated buffer. The use of hydrated and anhydrous sodium acetate was also employed in some preparations of the buffer to find out whether the anhydrous form caused any baseline disturbance. There had been a belief that the hydrated form was a purer commercial product[10]. Apart from the anhydrous form being more difficult to dissolve, there did not appear to be any other drawback to its use. One 5-l preparation of this buffer contained 500 g of the anhydrous form and 180 g of the hydrated sodium acetate and was found to be satisfactory. The instrument was calibrated immediately upon trnasfer of newly prepared ninhydrin reagent (4 l) to the analyzer. All subsequent analyses were determined as unknowns with the integrator using the preset K_f. As can be seen there is no fall-off in colour development throughout the period over which the analyses were carried out, and this remained so until the storage vessel had been emptied. There are slight variations in some amino acid concentrations on a day-to-day basis, but other factors, not the ninhydrin reagent, are the cause of these fluctuations. The only component of the analysis mixture whose recorded concentration does vary excessively is ammonia, and this decrease in detectable amounts could be attributed to a fall in the level of hydrindantin in the reagent mixture with time. This decrease in ammonia concentration is in accord with the observation that ammonia can only react with hydrindantin to produce colour[3]. But if this explanation is correct, the decrease in hydrindantin level in the reagent mixture does not seem to have any effect on the efficiency of the reaction of amino acids with the reagent.

It has been established that, providing there has been no variation in pumping rates and no error made in preparing the reagent mixtures, each subsequent batch of samples will not necessitate recalibration of the analyzer; the K_f values for individual amino acids will remain unchanged. Further, all the components and products resulting from reaction of the titanous chloride-reduced reagent are soluble. Therefore, no elaborate filtration system need be installed in the analyser to prevent particulate matter from entering the reaction coil and colorimeter as is the case in the System 6300 analyzer. In the routine replacement of this filter, it is difficult to avoid spilling DMSO. Plastic gloves provide no protection against this substance.

There is one drawback to using titanous chloride-reduced ninhydrin reagent; it is not quite as sensitive in the reaction with amino acids as is the direct addition of hydrindantin. However, the loss in sensitivity is more than compensated for by the increased stability of this reagent. For a research worker engaged in isolating and purifying proteins before subjecting them to analysis, the titanous chloride-reduced reagent is ideally suited. With the analyzer standing unused for a number of days while sample preparation is taking place, no decrease in colour development potential of the reagent mixture occurs, thus, recalibration is not required. In the case where an analytical service is provided and the analyzer is operating continually, the DMSO–hydrindantin mixture as used in the System 6300 would be suitable as recalibration of the analyzer on a daily basis could be included without difficulty, if so desired. Hence, the selection of which ninhydrin formulation to use rests on the individual demands of the laboratory where the amino acid analyses are carried out. But what must also be taken into consideration is the cost effectiveness of the titanous chloride-reduced ninhydrin reagent; it is a fraction of the cost of the DMSO–hydrindantin preparation that is available commercially.

The use of titanous chloride in the preparation has been suggested elsewhere[10], and it has been successfully in the analysis of materials from diverse sources[11–14].

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