the γ -globulin consisted very largely of the antibody-type globulin. If bovine antiserum against a pure antigen were available, end-group assay of the specific precipitate would be a satisfactory test of this idea, but so far such material has been unobtainable.

SUMMARY

1. Bovine γ -globulin has been fractionated into a series of overlapping fractions by partition chromatography.

2. *N*-Terminal amino acid assay of the fractions shows that a partial fractionation of components having the *N*-terminal acids, aspartic acid, serine and alanine, has been achieved, but the γ -globulin with *N*-terminal valine has not been fractionated.

3. Comparison of the *N*-terminal assay of β - and γ -globulins, and other evidence, has led to the suggestion that in the cow a part of the γ -globulin may be closely related to β -globulin.

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An Improved Method for the Microdetermination of Arginine by Use of 8-Hydroxyquinoline

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The colorimetric reaction of arginine with α -naphthol (Sakaguchi, 1925*a, b*) is simple, specific and sensitive but not reliable as a quantitative method. The colour yield is not reproducible nor is there linearity between colour yield and amount of arginine, mainly because of the instability of the coloured product under the usual conditions of the reaction.

There is some improvement when 8-hydroxyquinoline is substituted for α -naphthol (Sakaguchi, 1948), for the blank then remains almost uncoloured and the reagent is more stable. The reaction is similar for both reagents, which in the presence of alkaline hypobromite or hypochlorite form an intensely coloured compound with arginine. This compound is subsequently rapidly destroyed by hypobromite and the destruction is delayed, but not blocked, by addition of urea in excess. The rate of disappearance of the colour is quite different with different batches of urea, and this is the major drawback of the method with 8-hydroxyquinoline. There are other drawbacks to the original method (Sakaguchi, 1948) which hinder its quantitative application, namely the variability of the amount of hypobromite used and the formation of nitrogen bubbles on the walls of the cuvettes.

In the present work the colour formed by the reaction of arginine with 8-hydroxyquinoline has been stabilized by extraction into butanol, the addition of urea being avoided. The different variables which may interfere in the reaction have been studied and the most suitable conditions for quantitative application have been determined.

EXPERIMENTAL

Materials

8-Hydroxyquinoline (Carlo Erba). A solution (0.4%) of 8-hydroxyquinoline is made in 95% (v/v) ethanol. To 5 ml. of this solution 60 ml. of *N*-NaOH is added immediately before use.

Sodium hypobromite. The hypobromite is prepared freshly by dissolving 0.7 ml. of Br_2 in 50 ml. of 10% (w/v) NaOH. The solution is then diluted tenfold with water (final concentration of hypobromite, 0.15%).

Arginine hydrochloride (Merck). The product was stored in a desiccator for several days and its purity checked by paper chromatography and by N determination. Standard solutions at concentrations from 1 to 8 $\mu\text{g./ml.}$ were prepared each time.

Butanol (Carlo Erba). No purification was found necessary.

PROCEDURE

The following procedure, which was finally adopted, is described first, and the effects of the various alterations in the conditions employed are then discussed.

Fig. 1. Absorption spectrum of the colour formed by the reaction between arginine and 8-hydroxyquinoline.

Fig. 2. Relation of optical density to concentration of arginine ($\mu\text{g./ml.}$). ○, Readings taken immediately after the reaction; △, after 48 hr.; ●, after 72 hr.

To 5 ml. of the solution to be tested 1 ml. of the alkaline solution of 8-hydroxyquinoline is added. After a few minutes the diluted hypobromite solution (0.5 ml.) is added with shaking; 4 ml. of butanol is immediately layered over the coloured solution and at exactly 10 sec. after the addition of hypobromite the test tube is energetically shaken for 5–10 sec. After a few minutes the two phases separate; the aqueous layer is then withdrawn by a pipette with a long, fine tip and discarded, care being taken not to remove any of the solvent. The latter is completely cleared by addition of 0.2 ml. of absolute ethanol and its optical density is measured with a Beckman DU spectrophotometer at $507\text{ m}\mu$ (Fig. 1) against a blank prepared in the same manner. The blank is almost colourless. The Lambert–Beer law is followed up to an arginine concentration of $6\text{ }\mu\text{g./ml.}$ Sensitivity is high ($E_{1\text{ cm.}}^{1\%}$ 1280) and as little as $0.5\text{ }\mu\text{g.}$ of arginine/ml. may be reliably determined. The colour is stable (Fig. 2) and results are reproducible. The standard deviation, calculated several times on groups of ten samples, gave a value of $\pm 1.3\%$.

As outlined below, a slightly different procedure, namely 0.3% hypobromite in place of 0.15%, was used when the arginine content of protein hydrolysates was being measured.

Variables which affect the reaction

8-Hydroxyquinoline concentration. The concentration of 8-hydroxyquinoline in 95% (v/v) ethanol was varied between 0.05 and 1.6%. To 5 ml. of reagent solution 60 ml. of N-NaOH was added and the reaction was performed as usual, by using a series of concentrations of arginine for each 8-hydroxyquinoline concentration. The optical

Fig. 3. Effect of variations in concentration of 8-hydroxyquinoline on the arginine reaction. (A) Optical densities plotted against arginine concentration. The curves show results with different concentrations of 8-hydroxyquinoline. ▲, 0.05; ○, 0.1; □, 0.2; ■, 0.4; △, 0.6; ●, 0.8; ×, 1.6%. (B) Extinction coefficient ($E_{1\text{ cm.}}^{1\%}$) plotted against concentration of 8-hydroxyquinoline.

density of the butanol solution increased rapidly relative to that of the blank until the concentration of 8-hydroxyquinoline reached 0.6%; subsequently it decreased (Fig. 3). The decrease was very sharp at a concentration of 1.6%, because of the appearance of an intense colour in the blank and because of a change in the shade of the colour with a decrease of the absorption at 507 m μ . When the concentration of 8-hydroxyquinoline was 0.6% the blank was slightly coloured; therefore a concentration of 0.4% was chosen for routine use, although at this concentration the extinction coefficient was slightly lower.

Variation of time interval between addition of 8-hydroxyquinoline and of hypobromite. No differences in colour yield were observed when this interval was varied between 1 and 20 min.

Variations in concentration of hypobromite solution. The concentration of the hypobromite solution was changed by adding increasing amounts of water to the stock solution. Concentrations ranging from 0.075 to 0.9% were used. Other variables were kept constant as described in the standard method. The hypobromite concentration (see Fig. 4) had an important influence on the reaction. A sharp increase in colour yield was observed when the hypobromite concentration was increased from 0.07 to 0.15%; when the concentration was increased still further the extinction coefficient began to decrease.

Variation of time between addition of hypobromite and shaking with butanol. The hypobromite was introduced in the tubes and mixed as usual; the butanol was immediately layered over the aqueous layer and the tubes were shaken exactly 10, 15, 20,

25, 35 and 60 sec. after the addition of hypobromite. The highest value, as expected, was obtained at the shortest interval (Fig. 5). However, the effect of delaying the shaking was not very marked, and from 10 to 15 sec. there was only a 3% decrease in the extinction coefficient.

The 10 sec. interval was convenient and could be carefully controlled: further, a difference of 1 or 2 sec. did not make any appreciable difference to the results.

Effect of temperature. As the α -naphthol-diacetyl reaction used by Rosenberg, Ennor & Morrison (1956) is affected by variations in temperature, we examined the effect of this factor on the results obtained by the present method. Test solutions and reagents were set aside at different temperatures until they had reached equilibrium

Fig. 4. Effect of variations of concentration of hypobromite on the arginine reaction. (A) Optical densities plotted against concentration of arginine. The curves show results with different concentrations of sodium hypobromite. $\times$, 0.075; Δ , 0.15; $\bullet$, 0.22; $\blacksquare$, 0.30; $\square$, 0.37; $\circ$, 0.60; $\blacktriangle$, 0.90%. (B) Extinction coefficients ($E_{1\text{cm}}^{1\%}$) plotted against concentration of sodium hypobromite.

Fig. 5. Effect of the variation of the interval between addition of hypobromite and extraction with butanol. (A) Optical densities plotted against concentration of arginine. Extraction after: Δ , 10 sec.; $\bullet$, 15 sec.; $\blacksquare$, 20 sec.; $\square$, 25 sec.; $\circ$, 35 sec.; $\blacktriangle$, 60 sec. (B) Extinction coefficients ($E_{1\text{cm}}^{1\%}$) plotted against time intervals.

Fig. 6. Effect of the temperature variations on the arginine reaction. (A) Optical densities plotted against concentration of arginine. Reaction performed at: Δ , 14.6°; $\blacktriangle$, 21.8°; $\circ$, 37.0°; $\bullet$, 60.0°. (B) Extinction coefficients ($E_{1\text{cm}}^{1\%}$) plotted against temperature.

and the reaction was then performed by the standard method.

As shown in Fig. 6, an increase in temperature resulted in a decrease in colour yield, probably because of increased action of hypobromite on the pigment. At the usual laboratory temperatures the decrease in colour yield is about 0.5% per degree.

Colour stability. The rapid loss of colour is one of the major drawbacks of the original Sakaguchi reaction. Urea, even in very large excess, does not prevent the destruction of colour, and important differences have been observed from one batch of urea to another. It appeared that the protection afforded by urea was inversely proportional to its purity. With an old sample of urea, which gave opalescent solutions, good protection was obtained, and a decrease of only 1% in colour density after 10 min., and 7% after 60 min. With a sample of purer urea the corresponding decreases were 9 and 24% respectively, and with a highly purified specimen there were losses of 31 and 63% (Fig. 7A). With the pure urea, loss of colour varied with the arginine concentration, and thus no linear relationship existed between concentration and optical density at any of the different times studied (Fig. 7B). In view of these difficulties we avoided the use of urea by extracting the colour immediately after the reaction had been performed, thus leaving the hypobromite in the aqueous phase.

Of a series of solvents tested only butanol proved successful. Measurements of colour density were made immediately after extraction and at different

times up to 72 hr. After 16 hr. there was a decrease of 3%, after 48 hr. one of 6.7% and after 72 hr. one of 10.2%.

Application of the reaction to protein hydrolysates

Experiments were performed both on whole serum and on precipitates of serum protein. To prepare the latter, 1 ml. of 0.1M-acetate buffer at pH 4.28 was added to 0.5 ml. of serum and the mixture boiled for 5 min.; the precipitate was collected and dried at 100° *in vacuo*. The protein was hydrolysed in 6N-HCl (1 ml.) in a sealed tube for different times at 100°. After hydrolysis the product was neutralized with NaOH and diluted to 100 ml. with water. With whole serum, the same procedure was followed except that 0.5 ml. of 12N-HCl was used. In four experiments the average yield of arginine increased by 50% between 2 and 8 hr.; with longer times of hydrolysis there was a slight and probably not significant decrease. As an average of arginine determinations on proteins of sixteen human sera a value of 7.5 mg. of arginine/16 mg. of total nitrogen was found.

Recovery experiments were also done under these conditions. Pure arginine was added to the serum before hydrolysis. After hydrolysis for 8 hr., arginine determinations were made at dilutions of 1:2000 and 1:1000. Quantitative recoveries were obtained, as indicated in Table 1.

With protein hydrolysates, 0.3% hypobromite was used in place of 0.15%, even though the extinction coefficient obtained was lower ($E_{1\text{cm.}}^{1\%} = 1160$).

Fig. 7. Reaction of arginine with 8-hydroxyquinoline by the urea method, instead of extraction with butanol. (A) Effect of different batches of urea on colour stabilization. Extinction coefficients ($E_{1\text{cm.}}^{1\%}$) plotted against time. O, Urea (Ciba); Δ , urea (Carlo Erba); $\square$, urea (Merck). (B) Influence of the concentration of arginine on the variation of colour with time, for urea (Merck, purissimum). Concn. of arginine ($\mu\text{g./ml.}$): O, 2.5; Δ , 5.0; $\square$, 10.0.

Table 1. Recovery of arginine added to protein hydrolysates

To 1 ml. of serum different amounts of pure arginine (base), 1.65–6.61 mg. were added. Hydrolysis was carried out as described in the text and the neutralized product made up to 100 ml. After shaking with a few milligrams of decolorizing carbon and filtering, the arginine reaction was performed on 0.5 and 1 ml. of the filtrate, with hypobromite at a concentration of 0.3% ($E_{1\text{cm.}}^{1\%} = 1160$).

Arginine added (mg./ml. of serum)	Total arginine content (mg.)	Arginine recovered (mg.)	Recovery (%)
Reaction on 0.5 ml. of filtrate			
0	4.30	—	—
1.65	5.95	1.65	100.0
3.30	7.59	3.29	99.7
4.96	9.20	4.90	98.8
6.61	10.96	6.66	100.7
Reaction on 1 ml. of filtrate			
0	4.30	—	—
1.65	6.00	1.70	103.0
3.30	7.60	3.30	100.0
4.96	9.35	5.05	101.8
6.61	10.95	6.65	100.6

instead of 1280). This hypobromite concentration appeared the most convenient at the hydrolysate concentrations indicated. Values lower than those expected were obtained in recovery experiments with the 0.15 % hypobromite. With this concentration quantitative recoveries could be obtained by further dilution of the hydrolysates (dilution at 1:4000–1:5000).

DISCUSSION

The procedure described permits quantitative application of the Sakaguchi reaction. The greatest advantage of the method is the stability of the coloured product in butanol. The sensitivity, already high in the original method, is further increased, since the volume of the final coloured solution is reduced by the extraction.

For concentrations of arginine ranging between 0.5 and 6 $\mu\text{g./ml.}$ there is a linear relationship between quantity and colour yield and there is good reproducibility. The variability of the older method introduced by variability in the quality of urea, by nitrogen-bubble formation, by condensation on cuvettes and by rapid decrease of colour intensity is avoided. These factors are so important that each constitutes a serious obstacle to the quantitative application of the older methods. The empirical variations of hypobromite concentrations from one test to the other, suggested by Sakaguchi (1948), are also avoided. This is particularly important, as variations in hypobromite concentrations have a considerable effect on the colour yield.

The α -naphthol-diacetyl reaction in the presence

of propanol, performed by the method of Rosenberg *et al.* (1956), is undoubtedly an improvement over the original Sakaguchi (1925*a, b*; 1948, 1950, 1951) method, but it is less sensitive than the present method and less reproducible. In the present method the colour develops immediately, and then shows no significant change for a long time; the influence of temperature is also limited.

SUMMARY

1. The colour formed by the reaction of arginine with 8-hydroxyquinoline in the presence of hypobromite is stabilized by extraction with butanol, and the use of urea avoided.

2. A study of variables that may interfere in the reaction is presented, and the optimum conditions for performing the test are established.

3. The amount of hypobromite necessary to avoid interference by protein hydrolysates is determined.

4. The reaction allows quantitative determination of arginine between 0.5 and 6 $\mu\text{g./ml.}$; the colour is stable for at least 12 hr. Results are constant and reproducible (standard deviation $\pm 1.3\%$).

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Colorimetric Determination of Tyrosine

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The colour reaction between α -nitroso- β -naphthol and tyrosine was discovered by Gerngross, Voss & Herfeld (1933). Despite its sensitivity and its specificity, it was not used by these authors as a quantitative method, because of the instability of the colour formed. Attempts to find conditions which allowed stabilization of the red colour were made by Thomas (1944) and Giral (1944), but, under the conditions proposed by these authors, stability was far from satisfactory. Udenfriend & Cooper (1952) proposed the transformation of the unstable red colour into a stable yellow one, by heating in nitric acid solution and subsequently extracting the excess of reagent by ethylene di-

chloride. Good stabilization was obtained by this method.

By a systematic investigation of the different variables of the α -nitroso- β -naphthol reaction with tyrosine, we found that it was possible to stabilize the red colour and to use a simplified procedure which avoided extraction by ethylene dichloride.

EXPERIMENTAL

Materials

α -Nitroso- β -naphthol (Carlo Erba). The reagent was recrystallized from ethanol and dissolved in 0.1*N*-NaOH to give a 0.1 % solution. To obtain complete solution the