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

substance had to be finely powdered and heated at 100° with the NaOH. Traces of undissolved compound were removed by filtration.

Nitric acid solution. Concentrated 'chemically pure' HNO_3 was diluted with water to 2.5N.

Concentrated hydrochloric acid. The 'chemically pure' acid (sp.gr. 1.19) was used.

Tyrosine. The purity of the sample (Merck) was checked by paper chromatography with various solvents. The tyrosine was dried to constant weight and then dissolved in water by slight acidification with dilute HCl. For the test, standard solutions were made at concentrations ranging between 1 and $20 \mu\text{g./ml.}$

PROCEDURE

The following procedure was finally chosen. To 5 ml. samples of solution containing tyrosine, 1 ml. of α -nitroso- β -naphthol solution, 0.5 ml. of HNO_3 and 1.5 ml. of HCl were added. The mixture was shaken well, heated in boiling water for exactly 2 min. and then cooled immediately under running water. The time of boiling was calculated from the moment the water bath started boiling again after introduction of the test tubes. A large water bath was necessary to obtain rapid equilibration of the temperature in the test tubes.

The red colour developed was read on a Beckman DU spectrophotometer at $510 m\mu$ against a blank treated in an identical manner. The absorption curve (Fig. 1) showed a unique peak with a well defined maximum at $510 m\mu$, Beer's law being followed at concentrations ranging from 1 to $16 \mu\text{g./ml.}$ (Fig. 2).

Fig. 1. Absorption spectrum of the red colour formed during the reaction of α -nitroso- β -naphthol with tyrosine.

The value of $E_{1\%}^{1\text{cm.}}$ was 305. The results were constant and reproducible, and standard deviation, calculated four times on groups of ten samples, gave a value of $\pm 1.5\%$.

Effect of heating, and colour stability. During heating, the solution first becomes cloudy and yellow owing to the nitrosonaphthol, then the colour changes to red in the tubes containing tyrosine and disappears in the blank, which is perfectly clear after 1 min.

Colour is almost fully developed after heating for 1.5 min. and reaches its maximum at 2 min. It remains stable for 1 min. and then decreases slowly; after 9 min. the decrease in optical density is about 15% (Fig. 3A).

In cooled solutions the red colour is stable; readings taken after 3 hr. were not significantly different from those taken immediately after cooling the mixture. After 24 hr. at room temperature a decrease of 10.8% was observed (Fig. 2).

Influence of α -nitroso- β -naphthol concentration. The reaction was performed as described, but with α -nitroso- β -naphthol solutions at concentrations ranging from 0.012 to 0.1%. A steady increase in extinction coefficient was observed with increasing nitrosonaphthol concentration (Fig. 3B). Concentrations higher than 0.1% could not be used, be-

Fig. 2. The tyrosine-nitrosonaphthol reaction. Relation between quantity of tyrosine and colour yield and the stability of the colour. O, Readings taken immediately after the reaction was performed; □, readings taken 24 hr. later.

cause at this concentration the 0.1N-NaOH was saturated.

Influence of nitric acid. To 0.5 ml. of solution containing the chosen quantity (usually 50 μ g.) of tyrosine, various amounts of 2.5N-HNO₃ were added (0.5–3 ml.), the volume was made up to 5.5 ml. with water, the α -nitroso- β -naphthol solution and the concentrated HCl were added and the reaction was performed as usual. Almost no difference was observed between the tests with 0.5 and 1 ml. of HNO₃. With increasing amounts of HNO₃, however, a sharp decrease of the extinction coefficient took place (Fig. 3C).

Influence of hydrochloric acid. The procedure employed was the same as that described in the preceding paragraph, with the difference that the amount of HNO₃ remained constant and that of HCl was varied from 0.0 to 3.0 ml. Without HCl and also with 0.5 ml. the red colour did not appear; in its place a yellow colour developed, with a maximum absorption at 450 m μ . The blank also remained yellow under these conditions. With 1.0 ml. of HCl the red colour appeared but was contaminated by an orange shade; the blank was a faint yellow. With 1.5 ml. of HCl the tyrosine tube was red and the blank colourless. No important differences of extinction coefficient were observed with addition of HCl ranging between 1.0 and 2.0 ml. With larger amounts a noticeable decrease was observed (Fig. 3D).

Specificity of the reaction. As already observed by Thomas (1944) and by Udenfriend & Cooper (1952), the reaction is positive for tyramine as well as for tyrosine. At the same molar concentration tyramine gave an extinction coefficient 24 % higher than tyrosine, which is not far from the 30 % found by Thomas. On the other hand, *p*-hydroxyphenyl-ethanolamine at equimolar concentrations gave only 21 % of the colour given by tyrosine.

Other substances tested were tryptophan, tryptamine and 5-hydroxytryptamine. With these substances the reaction was almost negative.

Application of the reaction to protein hydrolysates

Hydrolysis experiments were performed both with whole serum and with protein precipitated from serum by heating at pH 4.28. The protein from 1 ml. of serum was dissolved in 2 ml. of 5N-NaOH and heated in a sealed tube at 100° for different times. The solution was neutralized with N-HCl and diluted to 50 ml. with water, and coloured products were removed with a few milligrams of active carbon. A similar procedure was used for whole serum (1 ml. of 10N-NaOH to 1 ml. of serum). Tyrosine was determined on 0.5 ml. samples of diluted hydrolysates made up to 5 ml. with water.

After hydrolysis for 1 hr. the solution was clear, and from this time up to 10 hr. results were almost

Fig. 3. Effect of the following variables on the colour yield in the reaction of tyrosine with nitrosonaphthol: A, Time of heating; B, nitrosonaphthol concentration; C, amount of HNO₃; D, amount of HCl.

Table 1. Recovery experiments

To 1 ml. of serum different amounts of tyrosine (1–10 mg.) were added. Hydrolysis was then performed with 1 ml. of N-NaOH for 2 hr. at 100°. After neutralization the solutions were diluted to 100 ml. with water. The reaction was performed on 0.5 ml. of the final solution made up to 5 ml. with water.

Tyrosine added to 1 ml. of serum (mg.)	Total tyrosine content (mg.)	Tyrosine recovered (mg.)	Recovery (%)
0	4.426	—	—
1	5.442	1.016	101.6
2	6.327	1.901	95.0
4	8.360	3.934	98.4
6	10.490	6.064	101.0
8	12.459	8.033	100.4
10	14.490	10.064	100.6

constant but with a slight tendency to decrease; a hydrolysis time of 2 hr. was chosen as optimum. Alkaline hydrolysis was preferred because it is more rapid; acid hydrolysis required about 10 hr. to give similar results. Hydrolysis was necessary only in order to obtain clear solutions, and the reaction may be used on intact proteins (Thomas, 1944).

Recovery experiments. To 1 ml. of serum different amounts of tyrosine, ranging from 1 to 10 mg., were added, before hydrolysis. The usual procedure for hydrolysis was then followed. Quantitative recoveries were obtained (Table 1).

DISCUSSION

The experimental conditions employed in the present method result in stabilization of the red colour developed by the reaction between α -nitroso- β -naphthol and tyrosine. The most important difference between the present method and that of Thomas (1944) is the use of 0.1N-NaOH, instead of ethanol, for the solution of nitrosonaphthol. The

reaction may be more readily standardized because the time of heating is not so critical as in the method of Thomas. Further, the time of heating is sufficiently long to allow equilibration between the boiling-water bath and the content of the test tubes, so that differences in wall thickness are not important. It is also unnecessary, since the colour is stable, to take readings within 8 min. of completion of the reaction. The reaction is thus easier to handle and more reproducible than the earlier method.

The present method is also preferable to that of Udenfriend & Cooper (1952) since it avoids extraction of the excess of nitrosonaphthol by ethylene dichloride. Recoveries also seem to be more complete by our method.

As far as specificity is concerned our method does not differ from those of Thomas (1944), Giral (1944) and Udenfriend & Cooper (1952). The only compound which could seriously interfere appears to be tyramine, which is commonly absent from protein hydrolysates.

SUMMARY

1. An improved method for the quantitative determination of tyrosine by the Gerngross-Voss-Herfeld (1933) reaction is described. Optimum experimental conditions were selected.

2. The solution of α -nitroso- β -naphthol was made in aqueous 0.1N-sodium hydroxide. The exclusion of ethanol from the reaction allowed stabilization of the colour.

3. Linearity of colour response was obtained with solutions containing between 1 and 16 μ g. of tyrosine/ml.

4. The method can be used to estimate the tyrosine content of proteins.

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The Metabolism of the Trichloronitrobenzenes in the Rabbit

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In the rabbit the labile nitro group of pentachloronitrobenzene and of 2:3:4:6- and 2:3:5:6-tetrachloronitrobenzene is replaced by an acetylcysteyl group with the formation of a mercapturic acid (Betts, James & Thorpe, 1955b; Bray, Hybs, James & Thorpe, 1953), whereas in certain of the dichloronitrobenzenes mercapturic acid formation occurs by replacement of a labile chlorine atom (Bray, James & Thorpe, 1957). It was, therefore, of interest to examine the metabolic fate of the trichloronitrobenzenes with particular reference to mercapturic acid formation and its relationship to the lability of the chlorine atoms and nitro groups present. A preliminary account of the metabolism of three of these compounds has been given (Bray, James & Thorpe, 1955). In this paper a detailed account of the fate of the six isomers in the rabbit is presented.

MATERIALS

Melting points are uncorrected.

The trichloronitrobenzenes were prepared according to the method of Holleman & Haefen (1921). The melting points of the isomers were: 2:3:4-, 53°; 2:3:5-, 44-45°; 2:3:6-, 87-88°; 2:4:5-, 57°; 2:4:6-, 69°; 3:4:5-, 70-71°. The

corresponding anilines were prepared by reduction of the nitro compounds with Sn and HCl in ethanol; melting points agreed with those given in the literature. 2:3:4-Trichloroaniline was acetylated to give 2:3:4-trichloroacetanilide, m.p. 131° [Beilstein & Kurbatow (1879) gave 120-122°] (Found: N, 5.8. Calc. for $C_8H_5ONCl_3$: N, 5.9%). 4-Bromo-3:5-dichloronitrobenzene, m.p. 88°, was prepared by the method of Flürschheim & Simon (1908).

2:4:5:2':4':5'- and 3:4:5:3':4':5'-Hexachloroazoxybenzene, m.p. 170° and 226° respectively, were prepared by refluxing in absolute ethanol for 0.5 hr. the corresponding trichlorophenylhydroxylamines, which were obtained by the method of Haworth & Lapworth (1921) (Found for 2:4:5-isomer Cl, 52.4. $C_{12}H_4ON_2Cl_6$ requires Cl, 52.6%).

2:4:6-Trichloro-3-nitrophenol, m.p. 69-70°, was prepared by the method of Dacomo (1885) (Found: N, 5.7. $C_6H_3O_2NCl_3$ requires N, 5.8%). Dacomo gave no analysis. Reduction with sodium dithionite gave 3-amino-2:4:6-trichlorophenol, m.p. 95° as found by Dacomo. (This compound, prepared by a different method, was characterized as the acetyl derivative by Jacobs, Heidelberger & Rolf, 1919.) 4-Amino-3:5-dichlorophenol, m.p. 154°, was prepared by the reduction with sodium dithionite of 3:5-dichloro-4-nitrophenol obtained by the method of Hodgson & Wignall (1927).

2:3:6:2':3':6'-Hexachlorodiphenyl disulphide was synthesized from 2:3:6-trichloroaniline by the method of Jondorf, Parke & Williams (1955). The yield was 1% of colourless