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New Sakaguchi Reaction

YOSHIHARU IZUMI[1]

From the Department of Biophysics, Roswell Park Memorial Institute, Buffalo, New York

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INTRODUCTION

Many modifications (1–19) of the Sakaguchi reaction based on varying the color test reagents, such as exchanging oxine for 1-naphthol or *N*-bromosuccinimide for potassium hypobromite, have been made. No study has been undertaken in which pretreatment of the sample plays a role.

Most authors report that the results of the Sakaguchi reaction depend greatly on the relative amounts of 1-naphthol and potassium hypobromite. Amino acids react with potassium hypobromite as follows (20–22):

$$R{-}NH_2 \xrightarrow{KOBr} R{-}NHBr \xrightarrow{KOBr} RNR_2$$

Thus, the amount of potassium hypobromite available for the Sakaguchi reaction is dependent on the number and accessibility of amino groups in the sample. Earlier methods rely on using large amounts of potassium hypobromite and 1-naphthol to offset the variability introduced. Urea is usually employed to prevent side reaction of the excess potassium hypobromite. The conditions—i.e., presence of urea and of excess potassium hypobromite—and 1-naphthol increase the optical density of the blank and further complicate the color test.

The method of this report is based on acetylation of the sample before the color test. Pretreatment of the sample with acetic anhydride blocks the amino groups from the oxidation by potassium hypobromite. Thus, all potassium hypobromite added to the system is available for the color reaction and a constant ratio between 1-naphthol and potassium hypobromite is easily maintained, regardless of the number of amino groups in the sample. The quantity of reagents may be reduced to 1/10 the amount normally used. Side reactions are thus minimized and the need for large amounts of a stabilizing reagent is reduced. A small quantity of potassium nitrite is used for this purpose.

[1] Now at the Institute for Protein Research, Osaka University, Osaka, Japan.

Effects of other amino acids were studied and it was observed that a great excess of histidine, methionine, and tryptophan influenced the result.

EXPERIMENTAL

I. Reagents

The reagents used in new Sakaguchi reaction are prepared as follows:

1. Potassium hydroxide solution: 12 gm of potassium hydroxide pellets diluted to 100 ml with distilled water.
2. Acetic anhydride (ACS grade).
3. 1-Naphthol solution: 1-naphthol (Fisher certified) (0.01%) (0.00059 *M*) in 10% potassium hydroxide aqueous solution; this reagent should be prepared daily.
4. Potassium hypobromite: a convenient method is to prepare a solution of concentrated potassium hypobromite which may be stored for periods of up to 1 month at 4°C; when needed, the concentrated solution is diluted 10 times with cold 5% potassium hydroxide and kept in an ice bath. Concentrated potassium hypobromite (0.0116 *M*) is made by dissolving 0.62 ml of bromine in 100 ml of 5% potassium hydroxide in an ice bath.
5. Potassium nitrite: 0.082% aqueous solution (0.012 *M*).

II. Procedure

For simplication, the new Sakaguchi reaction will be described first, followed by an account of alternate conditions studied.

(*a*) *New Sakaguchi Reaction.* One milliliter of the sample containing from 1–30 μg arginine is placed in the test tube. The tube is cooled in an ice water bath, and 2 ml of 10% potassium hydroxide is added; 0.15 ml of acetic anhydride is then added to the mixture and dissolved by shaking the tube in the ice bath. After the mixture has remained in the ice bath for 1 hr, 1 ml of 1-naphthol solution is added. After cooling for 1 min, 0.4 ml of hypobromite solution and 0.4 ml of potassium nitrite solution are added successively with immediate shaking after each addition. The reaction mixture is allowed to stand for at least 30 min at room temperature and optical density is measured at 520 mμ at room temperature. The blank is made in the same way, using 1 ml of distilled water in place of the arginine sample.

(*b*) *Alternate Conditions Studied.* (*1*) *Variation of arginine content with fixed glycine content.* Samples containing 150 μg glycine and from 0–30 μg arginine were tested both with and without acetylation. All other conditions were exactly as those described in Section II above.

(*2*) *Effect of glycine.* Two-milliliter samples containing 10 μg arginine and from 0–40-fold molar excess of glycine were prepared. The samples were tested using the method described above and also this method without the acetylation step. In the latter procedure, potassium hydroxide and acetic anhydride were replaced by an equal volume of water.

(*3*) *Effect of potassium acetate on the color reaction.* The effect of potassium acetate was tested under the following conditions: (*a*) The standard, new Sakaguchi reaction as described above. (*b*) Acetic acid used in place of acetic anhydride in the standard, new Sakaguchi reaction. (*c*) Without acetic acid or acetic anhydride. Each color reaction was performed with a 1-ml sample containing 10 μg arginine.

(*4*) *Variation of relative quantities of potassium hypobromite and 1-naphthol.* The relative amounts of potassium hypobromite and 1-naphthol were varied to study conditions for maximizing the color value. In all cases, the starting samples contained 10 μg arginine and 150 μg glycine, and the final volumes were adjusted by addition of water. Blanks containing no arginine were prepared for each sample.

(*5*) *Variation of the absolute quantity of potassium hypobromite and 1-naphthol.* Again the starting samples contained 10 μg arginine and 150 μg glycine, and the absolute quantities of potassium hypobromite and 1-naphthol were varied, always maintaining the same ratio between them. This ratio, which had been found previously to yield maximum color value, was 6 moles of potassium hypobromite to 1 mole of 1-naphthol. In each case, the volume was adjusted by potassium hydroxide solution after the reaction had gone to completion. A blank was made for each sample.

(*6*) *Influence of other amino acids.* Two-milliliter samples containing 10 μg arginine and a 40-fold molar excess of another amino acid were subjected to the new method described in Section II.

(*7*) *Variation of sample volume with constant absolute arginine content.* The new Sakaguchi reaction was performed on samples containing 10 μg arginine introduced into the reaction in volumes varying from 2 to 5 ml. Blanks were prepared for each sample.

(*8*) *Effect of diluting with water after the color reaction.* After the new Sakaguchi reaction was performed on the sample containing 10 μg arginine, the colored solution was diluted 2–16 times with water and the optical density was measured. The blank was diluted and similarly measured.

(*9*) *Stabilization of the color reaction with potassium nitrite.* The use of potassium nitrite to stabilize the color of the solution was tested in the presence of a 30-fold molar excess of glycine and also without glycine.

(*10*) *Pretreatment with nitrous acid.* One milliliter samples, containing from 0–32 μg arginine, were treated with 0.1 ml of 10% potassium nitrite and 0.1 ml of 30% acetic acid. After 10 min at room temperature, the reaction mixtures were heated in a boiling water bath for 1 hr. The dried contents of each test tube was dissolved in 1 ml of 1-naphthol, and 0.3 ml of potassium hypobromite and 0.3 ml of potassium nitrite were added.

RESULTS AND DISCUSSION

Excellent results are obtained by the Sakaguchi reaction when the amino groups have been protected from potassium hypobromite oxidation by prior acetylation. The standard curve of the nonacetylated samples does not go through the origin, and the color yields are lower than for the acetylated samples. When the reaction is performed by the procedure described in Section II, the standard curve always starts at the origin, as shown in Fig. 1.

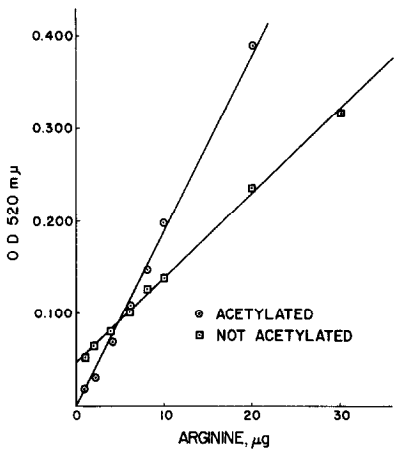

FIG. 1. Standard curve of acetylated and nonacetylated samples. Glycine content is fixed.

The color reaction of the acetylated sample is completely independent of glycine content. Without acetylation, the results are greatly influenced by the glycine content. In nonacetylated samples containing little or no glycine, the color produced in the reaction is destroyed by an excess of potassium hypobromite. With glycine in excess, potassium hypobromite reacts with glycine and is not available for the color reaction. The effect of glycine on the untreated and acetylated sample is shown in Fig. 2.

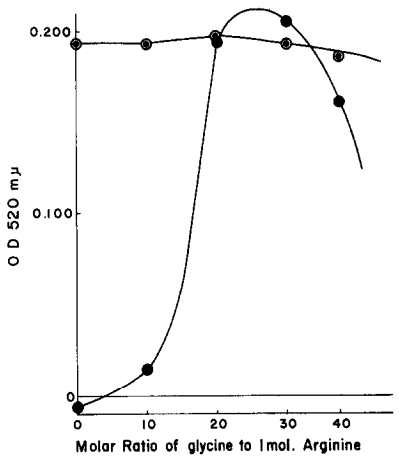

FIG. 2. Effect of glycine: (⊙)acetylated, (●)not acetylated.

Since potassium acetate is produced during the acetylation, its importance in the reaction was tested. Data recorded in Table 1 show

TABLE 1
EFFECT OF POTASSIUM ACETATE ON COLOR REACTION

No.	Conditions	OD
1	Normal (acetylated with acetic anhydride)	0.190
2	Acetic acid substituted for acetic anhydride	0.052
3	Water used in place of acetic acid or acetic anhydride	−0.006

that the highest color yields are obtained when the samples are acetylated with acetic anhydride. Substitution of acetic acid results in considerably lower color values. It can be concluded, therefore, that the acetylation and not the potassium acetate is necessary for the success of the reaction.

Using the acetylated sample, the optimum ratio is easily found. The color yield for the sample is greatly influenced by the relative amounts of potassium hypobromite and 1-naphthol, as shown in Fig. 3. The best conditions are obtained with a ratio of 6 moles of potassium hypobromite to 1 mole of 1-naphthol. Maintenance of this ratio is critical, as shown by the sharp peak of the graph.

The absolute quantities of 1-naphthol and potassium hypobromite do not influence the reaction as much as the relative amounts of the reagents, as shown in Fig. 4. This graph also indicates that an excess of these reagents interferes with the color yield.

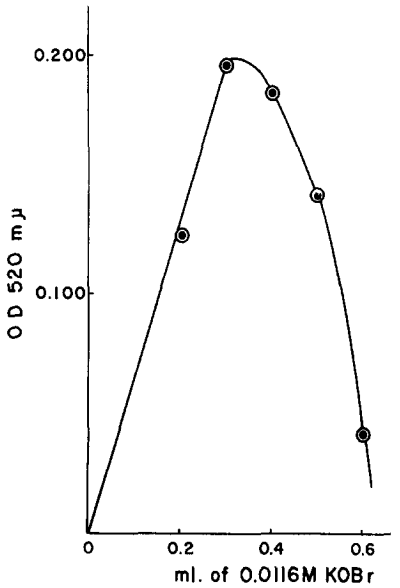

FIG. 3. Effect of varying relative quantities of potassium hypobromite and 1-naphthol. The test was performed on the 1.0 ml of 0.00059 *M* 1-naphthol solution.

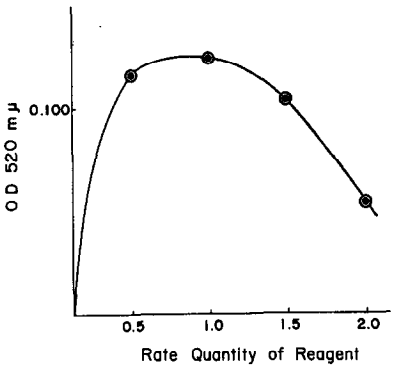

FIG. 4. Effect of varying absolute quantities of potassium hypobromite and 1-naphthol. Tests were performed on sample containing 10 μg arginine with 0.5, 1.0, 1.5 and 2.0 times as much 1-naphthol and KOBr solution as used in method described in Section II.

The effects of several other amino acids on this reaction were tested: A great excess of histidine, methionine, and tryptophan inhibits the color test, as shown in Tables 2 and 3. These amino acids have residues such as thioether, phenol, etc. which can reduce potassium hypobromite, but which cannot be blocked by acetylation. Aspartic acid and cystine

TABLE 2

INFLUENCE OF 40-FOLD MOLAR EXCESS OF OTHER AMINO ACIDS ON COLOR YIELD OF NEW SAKAGUCHI REACTION

Amino acid	Color yield[a]
Arginine (alone)	100
Alanine	108
Aspartic acid	119
Cystine	122
Glutamic acid	112
Glycine	97
Histidine	4.1
Hydroxyproline	101
Lencine	103
Lysine	108
Methionine	66
Phenylalanine	110
Proline	112
Serine	112
Threonine	99
Tryptophan	13
Tyrosine	92
Valine	102

[a] Color yield =

$$\frac{\text{OD of new Sakaguchi reaction on sample containing arginine and other amino acid} \times 100}{\text{OD of new Sakaguchi reaction on arginine alone}}$$

TABLE 3

INFLUENCE OF HISTIDINE, METHIONINE, AND TRYPTOPHAN ON COLOR YIELD OF NEW SAKAGUCHI REACTION

Mole number of amino acid to arginine	40	20	10	5	25	1.25
Histidine	4.1	47	72	93	100	101
Methionine	66	100	92	104	104	92
Tryptophan	13	55	97	94	91	98

considerably increase the color yield. Alanine, glutamic acid, lysine, phenylalanine, proline, and serine also increase the color yield to about 10% above the value for arginine alone. Cystine does not effect the color reaction because the disulfide group in cystine is easily destroyed with potassium hydroxide before the color reaction takes place.

These results also show that arginine determination is not absolutely reliable as a method for protein analysis, especially if many histidine residues are present.

Specific color yield is independent of arginine concentration and not effected by dilution after the reaction.

Addition of nitrite after a 10-min color reaction stabilized the color yield, as shown in Fig. 5.

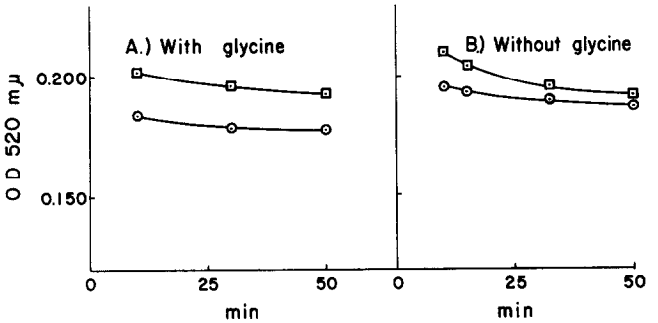

FIG. 5. Stabilization of color reaction with potassium nitrite: (⊙)with potassium nitrite, (⊡)without potassium nitrite.

The color test performed on samples pretreated with nitrous acid gives results similar to those obtained with acetylated samples, as shown in Fig. 6. This fact underlines the importance of inhibiting the amino

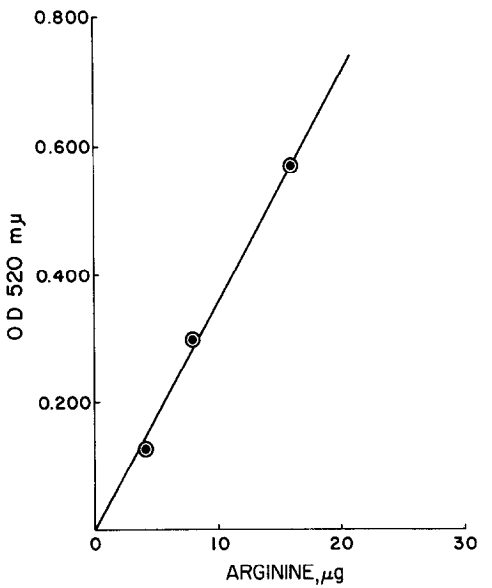

FIG. 6. Color yield of Sakaguchi reaction on sample treated with nitrous acid before the reaction. Final concentration is 2.3 times greater than the concentration used in Fig. 1.

groups to prevent oxidation by potassium hypobromite in the Sakaguchi reaction. Although the nitric acid method may be used, it is more practical to use the acetylation method described in Section II, because it is less complicated and far less time consuming.

SUMMARY

The Sakaguchi reaction for arginine has been improved by pretreatment of amino acid mixtures with acetic anhydride to block amino groups. The advantages of this method are: (*1*) the color produced by the Sakaguchi reaction is stabilized; (*2*) optimum conditions for the Sakaguchi reaction are easily maintained; (*3*) a reliable standard curve can be produced. Some amino acids containing reduced groups (histidine, methionine, tryptophan, tryrosine) remain and inhibit the Sakaguchi reaction.

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