Studies on the Intermediary Products in the Oxidation of Tyrosine with Potassium Permanganate

Katsuya KOGA, Takao FUKUNAGA and Kyōko KANOU

(*Laboratory of Animal Biochemistry*)

On the oxidation of tyrosine by enzymes considerable amount of studies have been reported, because of the importance of tyrosine as the material of the pigment formation in the biochemical systems, the first oxidative product of tyrosine being fixed to be 3, 4-dihydroxyphenylalanine (DOPA).[1)2)] The oxidative product of tyrosine by the irradiation of ultraviolet ray was presumed to be DOPA by Arnow[3)] and that by exposure to sunlight was identified with DOPA by Obata et al.[4)] Under the irradiation of the mercury lamp Akabori et al.[5)] obtained serine, or alanine or aspartic acid. Much less studies have been done on the oxidative intermediary products of tyrosine by the chemical reagent than those mentioned above.

Previously, by one of the authors the silk fibroin (the linear protein) was treated, respectively, with the three oxidizing agents i. e. $KMnO_4$, $Ca(OCl)_2$, and H_2O_2 to obtain the different levels of degradation and then some systematical studies were performed on the changes of physical, physico-chemical and general-chemical properties.[6)] By plotting the tyrosine content in fibroin against the reduced potassium permanganate a linear relationship between the former and the latter was found on the condition that the fibroin was attacked, at the different levels of the oxidation, by the dilute potassium permanganate solution. Then it was ascertained that carboxyl group of fibroin was increased with the oxidation and organic acids were got in the filtrate after oxidation. Therefore, the occurrence of the splitting of phenol group in tyrosine was inferred but its mechanism was left unclarified.

In this paper, concerning the solution of tyrosine oxidized with permanganate aqueous solution the amino acid and several organic acids were investigated, because they were anticipated as the oxidative intermediate of tyrosine. Experimental results obtained may accelerate the elucidation of the oxidizing mechanism of fibroin.

Experimental method

1. Oxidation of tyrosine with potassium permanganate and the subsequent treatment

500 *ml.* of 0.02*M* $KMnO_4$ aqueous solution were added to 500 *mg.* of tyrosine with stirring and the reaction was allowed to continue at room temperature until potassium permanganate was entirely reduced, and thereafter the solution was filtered through the filter paper powder. For the adsorption of anions the filtrate was very slowly passed through the column of Amberlite IRA-410 (-CO_3 form), 3×17 *cm.* The passed solution was thrown away. According to Overell's report,[7)] the column was treated to elute the adsorbed

ions with 300 *ml.* of 1*N* Na_2CO_3 and after a time was treated with 300 *ml.* of 10 % NaOH. Both eluted solution were separately maintained and then firstly passed through the column of Amberlite IR-120, 6×4 *cm.* and secondly passed slowly through the same resin column, 3×22 *cm.* Those passed solutions containing free organic acids were concentrated together to about 20 *ml.* under reduced pressure and lyophilized. This was called the free acid type.

The oxidation and the subsequent procedures mentioned above were taken thrice separately, and then those mixed lyophilized powders were used as the sample for the partition chromatography of organic acid. Moreover, the procedure, completely same, was performed repeatedly. In this case, however, the solution passed through Amberlite was neutralized with 1*N* NaOH and then lyophilized. This was called the salt type. The quantities required for the neutralization were about 5.5 *ml.* of 1*N* NaOH.

2. Identification of the amino acid in the oxidized solution

The existence of amino acid in the oxidized solution was investigated by the paper chromatography. A slight quantity of the powder of the free acid type was dissolved in a little volume of water and this solution was applied to a starting line of the filter paper. Tōyō filter paper No. 50 was used throughout the paper chromatographic studies. Ethanol-ammonium hydroxide-water (40 : 2 : 8),[8] and butanol-acetic acid-water (4 : 1 : 1)[9] were separately employed as the solvent. 0.1 % ninhydrin solution in butanol-water was used as the reagent for the color development.

3. Determination of aspartic acid in the oxidized solution

500 *mg.* of tyrosine were oxidized with 500 *ml.* of 0.02*M* $KMnO_4$ and filtered. The filtrate was passed through Amberlite IR-120 column, neutralized and then concentrated under reduced pressure. The solution was filled up to 50 *ml.* This solution was used as a sample for the determination of aspartic acid. The determination followed the procedure of Darling.[10] 2 *ml.* of standard solution containing 8 *mg.* of aspartic acid were poured over the Al_2O_3-column, 5 *gm.* with dia. 8 *mm.* and washed with 60 *ml.* of water. Aspartic acid was eluted with 5 *ml.* of 3*N* KOH followed by 40 *ml.* of 0.005 *N* KOH. The elutriate was neutralized with the concentrated sulfuric acid followed the dilute acid. Precipitation which occurred was removed and the solution was concentrated.

Nitrogen in this solution was determined by Kjeldahl method and from the nitrogen value the quantity of aspartic acid was calculated. Recovery of aspartic acid was 87.4%. Using 2 *ml.* of sample solution the same procedure was performed. Aspartic acid value obtained was multiplied 1.149 on the basis of recovery value.

4. Separation of organic acids by partition chromatography

Experimental method proposed by Phares et al.[11] was followed. As mentioned previously, the freeze-dried substances (powders) obtained from 1.5 *gm.* of tyrosine were used as the sample. An outline of the procedure was as follows.

Celite 545 (100–200 *mesh*) was used as the support for the immobile phase. The partition columns employed were glass tube, 45×1.0 *cm.* inside diameter. The moist Celite prepared at a ratio of 8 *ml.* of 0.5*N* H_2SO_4 to 10 *gm.* of Celite was slurried with the

organic phase and packed into the column by tamping. The sample was dissolved with 3 to 3.5 *ml.* of 25% H_2SO_4 under cooled condition. Enough Celite was added to this solution to make the same appearance as that of the original mixture of Celite and 0.5*N* H_2SO_4 used in packing. This mixture was transferred on the top of Celite column with a little amount of solvent. Organic eluting agents were equilibrated with 0.5*N* H_2SO_4 prior to use. Elution was started with 10% butanol in chloroform (CB-10), continued to 300 *ml.* fraction and followed with 35% butanol in chloroform (CB-35). The effluent was collected with a fraction collector set to be 2 *ml.* of a fraction size and the organic acids were determined by titration with 0.02*N* NaOH in ethanol using brom thymol blue as indicator.

5. Identification of each component separated

Effluents corresponding to each component were got together and the solvent was evaporated and an adequate amount of water was added on the residues. Then the solution containing the sodium salts of organic acids was treated with activated charcoal for the exception of indicator. The solution considered to be containing the sodium salt of volatile acid was concentrated under reduced pressure. While, the other component solutions containing unvolatile acid were separately passed through Amberlite IR-120 column to get the free acid and concentrated. These concentrated solution were kept in a refrigerator. Individual acid components were identified by comparing with the elution diagram of Phares et al.,[11] and also by the paper chromatography, the absorption spectrum of the indole compound of organic acid,[12] the application of specific reaction and the melting point of crystals.

Developing solvents for the paper chromatography of organic acids were as follows: ethanol-ammonia-water (40 : 2 : 8),[8] phenol-formic acid-water (30 *gm* : 10 *ml* : 10 *ml*),[13] butanol-acetic acid-water (4 : 1 : 1),[9] butanol-formic acid-water (4 : 1 : 2),[14] n-propanol-conc. aqueous ammonia (60 : 40).[15] Especially, the component considered to be keto-acids was chromatographed as its 2,4-dinitrophenylhydrazone derivative using the solvents proposed by Cavallini et al.,[16] such as butanol-ethanol-water (5 : 1 : 4), butanol saturated with 3% ammonia and butanol saturated with water.

Results and discussion

It was shown in the previous report[17] that 5*ml.* of 0.02 *M* tyrosine solution consume 25 *ml.* of 0.02 *M* $KMnO_4$ solution by titration procedure at room temperature. This fact indicates that 500 *mg.* of tyrosine consume 680 *ml.* of 0.02 *M* $KMnO_4$. A ratio of tyrosine to potassium permanganate in the present studies was taken so that a trace of tyrosine might remain after oxidation. As shown in Fig. 1-a, paper chromatograms of amino acids developed regarding the oxidizing substance of tyrosine gave three spots. Comparing with standard, a large spot was identified with aspartic acid (ref. Fig. 1-b), other small ones, serine and tyrosine respectively. 273 *mg.* of aspartic acid were estimated in the oxidizing substance of 500 *mg.* of tyrosine. This value points out the production of 0.75 *mol.* of aspartic acid from 1 *mol.* of tyrosine. Elution diagrams of organic acids obtained by partition chromatography were shown in Fig. 2, 3, 4 and 5. Partition chromatography of the salt type gave six or seven components, in which three components

were especially noticeable. Three components were marked respectively A, B and C, as shown in Fig. 2, 3, 4 and 5.

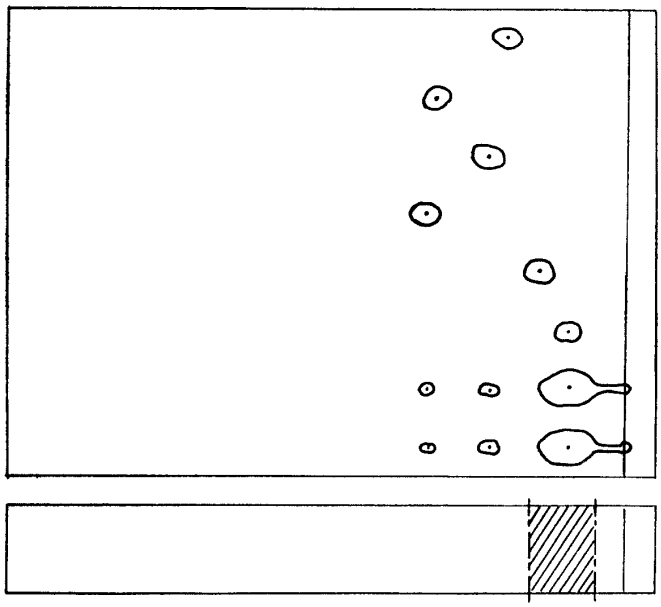

Fig. 1-a. Paper chromatograms of the oxidizing substance of tyrosine and several amino acids using EtOH-NH_4OH-H_2O (40 : 2 : 8)
Under-figure was as follows : Filter paper developed at the same time as the above was not sprayed with colour reagent, and the part corresponding the largest in the spots of sample of above-figure was cut out, extracted with water. Numerals in parentheses are Rf values.

Fig. 1-b. Paper chromatograms of the extract of oblique part in Fig. 1-a and aspartic acid
BuOH-acetic A.-H_2O (4 : 1 : 1) was used as solvent.

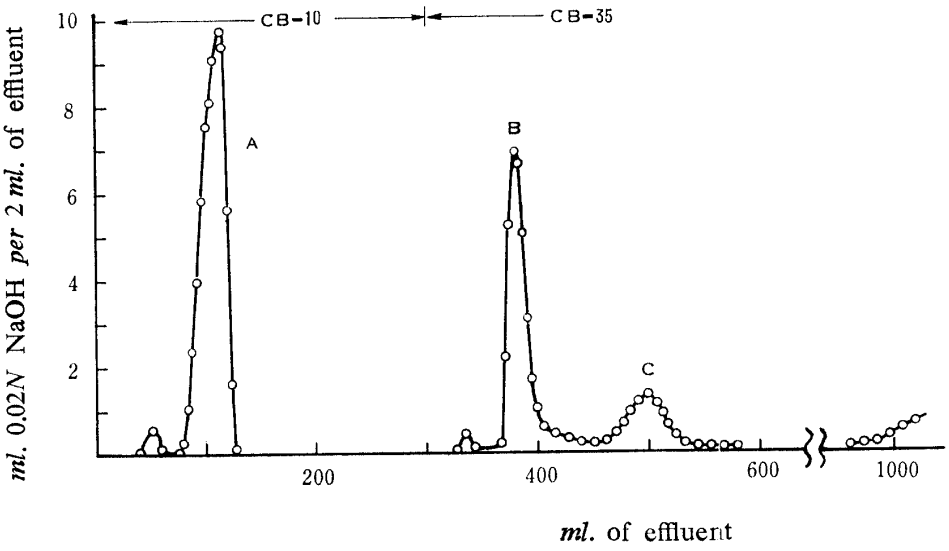

Fig. 2. Separation of organic acids concentrated in the salt type after Na_2CO_3-eluting
CB-10; 10% butanol in chloroform, CB-35; 35% butanol in chloroform. Na_2CO_3-eluting was done for Amberlite IRA-410 column, by which the organic acids had been adsorbed. Separation of organic acids was performed by the partition chromatography with Celite 545 column. Organic acids shown in the figure were derived from 1.5 *gm.* of tyrosine.

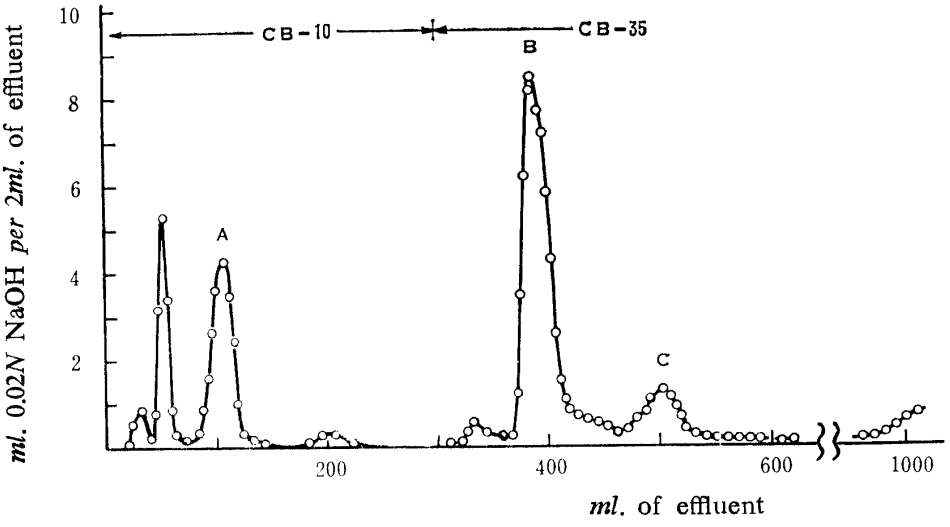

Fig. 3. Separation of organic acids concentrated in the salt type after NaOH-eluting of the residues of organic acids obtained by Na_2CO_3-elution CB-10; 10% butanol in chloroform, CB-35; 35% butanol in chloroform. Explanation for the figure was the same as that for Fig. 2.

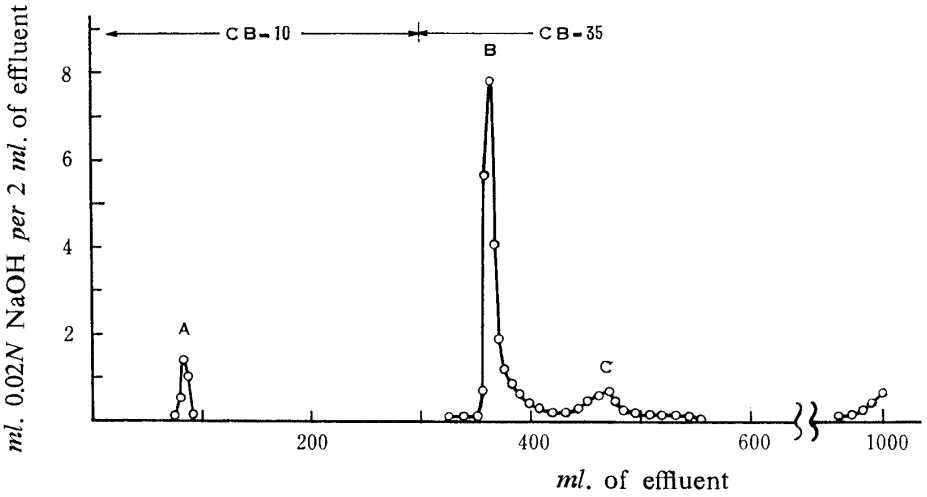

Fig. 4. Separation of organic acids concentrated in the free acid type after Na_2CO_3-eluting
CB-10; 10% butanol in chloroform, CB-35; 35% butanol in chloroform. Explanation for the figure was the same as that for Fig. 2.

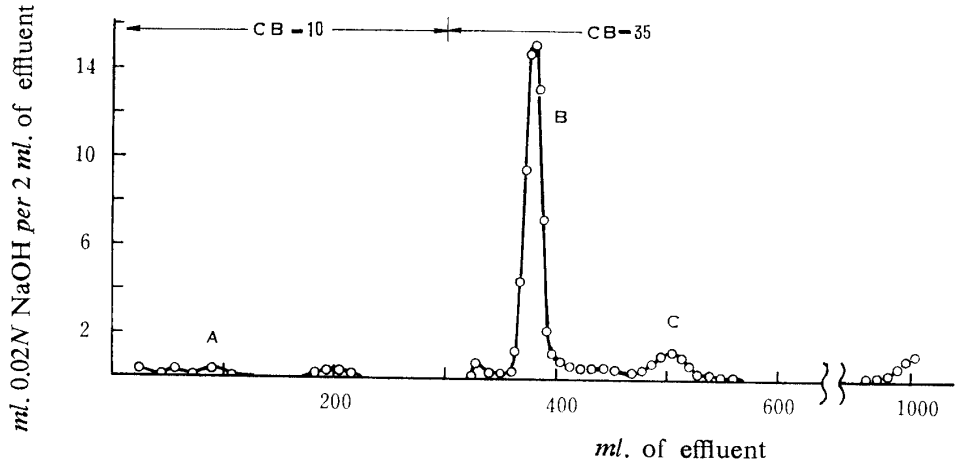

Fig. 5. Separation of organic acids concentrated in the free acid type after NaOH-elution of the residues of organic acids obtained by Na_2CO_3-elution CB-10; 10% butanol in chloroform, CB-35; 35% butanol in chloroform. Explanation for the figure was the same as that for Fig. 2.

It was observed by authors that an appropriate quantity of organic acid could be eluted from Amberlite IRA-410 column with 10% sodium hydroxide even after the acid adsorped by the column had been eluted with 1*N* sodium carbonate. The appearance just-mentioned was also observed on the partition analysis of the free acid type. These facts bring it into emphases that the use of only 1*N* sodium carbonate performed by Overell[7] was not enough to permit the entire elution of acids to take place.

In elution diagrams of the concentrates of the free acid type, component A was slightly observed. Consequently, component A was ascertained to be a volatile acid and on the basis of the eluted position it was presumed to be formic acid. Rf values

of component A and several volatile fatty acids, and the colors of their spots on paper chromatograms were represented in Table I. The value of component A was coincident with that of formic acid. The appearance of CO_2-evolution from the component A (sodium salt) observed by Br-oxidation method[18] was also similar to that from the formate. CO_2-evolution from the acetate, propionate and butyrate was not observed at all.

Table I Rf values of sodium salts of component A and several volatile fatty acids, and colors of their spots on paper chromatograms

	Compt. A.	Formate	Acetate	Propionate	Butyrate	Pyruvate
Rf	0.14	0.14	0.14	0.18	0.26	0.22
Color of spots	yellow	yellow	yellowish brown	yellowish brown	yellowish brown	white →blue

Solvent; Ethanol-1*M* $NaHCO_3$ (50 : 1, v/v)
Color developing reagent ; 0.1*N* $AgNO_3$—5*N* NH_4OH (1 : 1, v/v)

It has been proposed by Bergerman and Elliot[12] that under suitable conditions, oxalic acid and indole react to form a red-, of pink-coloured compound which conforms to Beer's law. In their work a number of organic acids were tested for color development. Formic acid was found to produce a red colored compound. However, acetic, propionic, tartaric, citric acids in amounts of 10 *mg. per ml.* were found not to produce any color when treated with indole. Therefore, authors measured the absorption spectra of the indole-formic acid compound using Hitachi EPU-2A spectrophotometer. Absorption maximum was observed at 485 *mμ* of wave length as given in Fig. 6. The same maximum value as the above was ascertained on the curve of the test sample. After the sodium salt aqeous solution of component A had been concentrated and kept in a refrigerator, the crystallization was observed. The crystal obtained by filtering, washing with ethanol and vacum-drying exhibited the hygroscopic quality after it had been placed for a while in the air. From a number of experimental results mentioned above, component A was identified with formic acid.

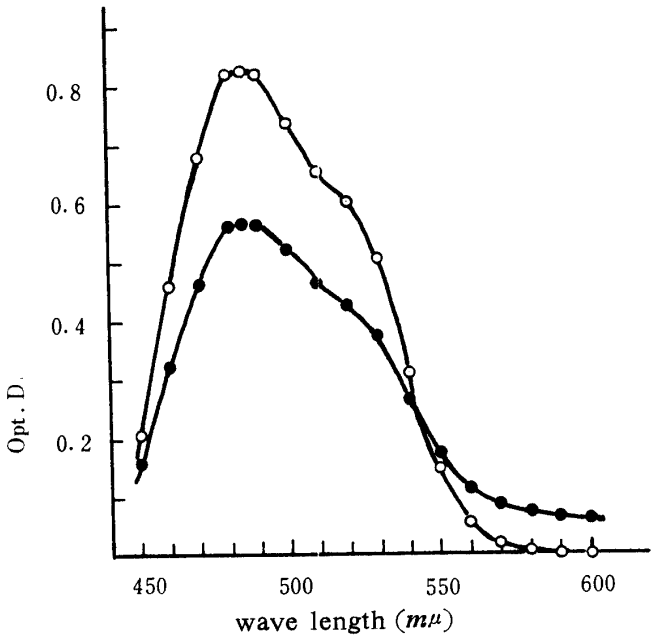

Fig. 6. Absorption spectra of indole-formic A. and indole-component A compounds
-o-o- Indole-formic A. compound
-•-•- Indole-component A compound

Component B was presumed to be oxalic acid on the basis of the eluted position.

Comparison between Rf

value of component B and that of standard oxalic acid was shown in Table II. The values of these two compounds developed with three kinds of solvent independently were quite

Table II Rf values of component B and oxalic acid

Solvents	Component B	Oxalic A.
Ethanol-NH_4OH-H_2O 40 : 2 : 8 (v/v)	0	0
Butanol-acetic A.-H_2O 4 : 1 : 1 (v/v)	0.60—0.66	0.60—0.66
Phenol-formic A.-H_2O 30*gm* : 10*ml* : 10*ml*	0.63—0.64	0.63—0.65

Color developing reagent ; 0.03% methyl red in 0.05*N* borate buffer (*pH* : 8.0)

equal to each other. Subsequently, the absorption spectra of the compound of oxalic acid or component B and indole were respectively measured. As shown in Fig. 7, the maximums in both absorption curves were ascertained to have been lying together at 526 *mμ* of wave length. The crystallization was observed after the aqueous solution of component B which had been converted to the free acid was concentrated and kept in a refrigerator. The crystal was air-dried and then its reductivity was measured by the titration method using 0.01*N* $KMnO_4$. Consequently, it was ascertained that 10 *mg.* of the crystal reduce 15.85*ml.* of 0.01*N* $KMnO_4$. 10*mg.* of oxalic acid ($+2H_2O$) require theoretically 15.86 *ml.* of 0.01 *N* $KMnO_4$. Additionally, melting point of the crystal was 95.5°*C*, just coincident with that of oxalic acid. From those results, component B was identified with oxalic acid.

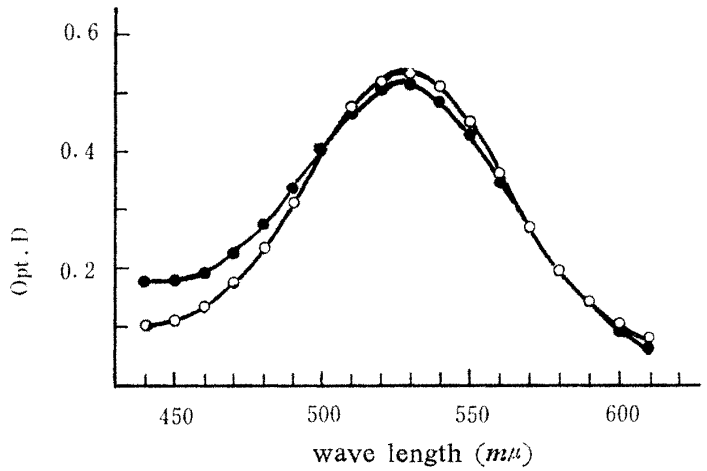

Fig. 7. Absorption spectra of indole-oxalic A. and indole-component B compounds
-o-o- Indole-oxalic A. compound
-•-•- Indole-component B. compound

The eluted position of component C was closed to that of malic acid referring with the diagram of Phares et al.[11] Component C was therefore chromatographed, running together with malic acid, but Rf values in both spots were not coincident. As shown in Table III, Rf value of the test sample was also different from the values of the other acids : tartaric, succinic and maleic acid. The same test sample solution was spotted on the filter paper and sprayed with 0.1% semicarbazide and 0.15% sodium acetate, and heated to 110°*C*. The spot exhibited the fluorescence in the ultraviolet light. Therefore, component C was recognized as a certain keto-acid. Subsequently, paper chromatog-

Table III Rf values of component C and several fatty acids

Solvents / Acids	Ethanol-NH_4OH-H_2O 40 : 2 : 8 (v/v)	*n*-Propanol-NH_4OH 60 : 40 (v/v)	Butanol-formic A.-H_2O 4 : 1 : 2 (v/v)
Component C	0.18, 0.40, 0.52	0.31, 0.43, 0.52	0.43, 0.52
Malic	0.37	0.38	0.50
Citric			0.43
Tartaric			0.34
Succinic			0.74
Maleic			0.68

Color developing reagent; 0.03 % methyl red in 0.05*N* borate buffer (*pH* : 8.0)

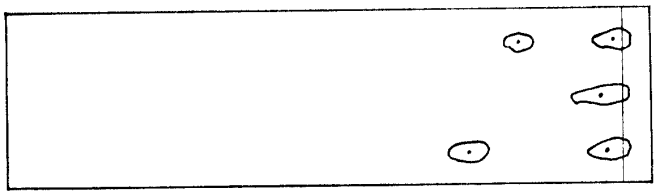

Fig. 8. Paper chromatograms of component C and free keto-acids
Solvent ; Ethanol-NH_4OH-H_2O (40 : 2 : 8)
Detection ; Spraying with 0.1 % semicarbazide and 0.15 % sodium acetate, heating and fluorescence-exhibition
Numerals in parentheses are Rf values.

raphy for keto-acids was done using the several solvents. Those chromatograms were shown in Fig. 8, 9-a, b and c. The chromatogram separated with ethanol-ammonia-water (40 : 2 : 8) showed that component C coincides with neither oxalacetic nor keto-glutaric acid. (ref. Fig. 8) The chromatogram of 2,4-dinitrophenylhydrazone of keto-acid represented that the test sample differs evidently from pyruvic acid. (ref. Fig. 9-a) The sample was separated into two spots using butanol saturated with 3 % ammonia, while oxalacetic acid was separated into three spots as shown in Fig. 9-b.

Two spots in the former gave the Rf values conforming respectively to the two in three spots in the latter. It could be ascertained in this figure that the sample differed also with keto-glutaric acid. The chromatogram developed by butanol saturated with water gave the appearance resembling the one described above. (ref. Fig. 9-c) Namely, three spots of the test sample gave the Rf values closed to the three in four spots of oxalacetic acid hydrazones which were clearly separated, and also differed from that of keto-glutaric acid. From those experiments, component C was ascertained not to be identified with a particular keto-acid. Cavallini et al. obtained very well-shaped and well-separated spots of the hydrazones of the following keto-acids: α-keto-glutaric, oxalacetic, pyruvic and acetacetic acids by paper chromatography. However, it was noticed by Seligson and Shapiro[19] that pyruvic acid gave rise to two hydrazones. Isherwood and Cruickshank[20] described in their paper that pyruvic acid gave three hyorazones, α-keto-glutaric acid, only the slower-moving one and oxalacetic acid gave four spots, two of them being the pyruvic hydrazones produced by decarboxylation of the oxalacetic acid hydrazone.

From the paper electrophoresis of keto-acid hydrazones, Tauber[21] presumed the existence

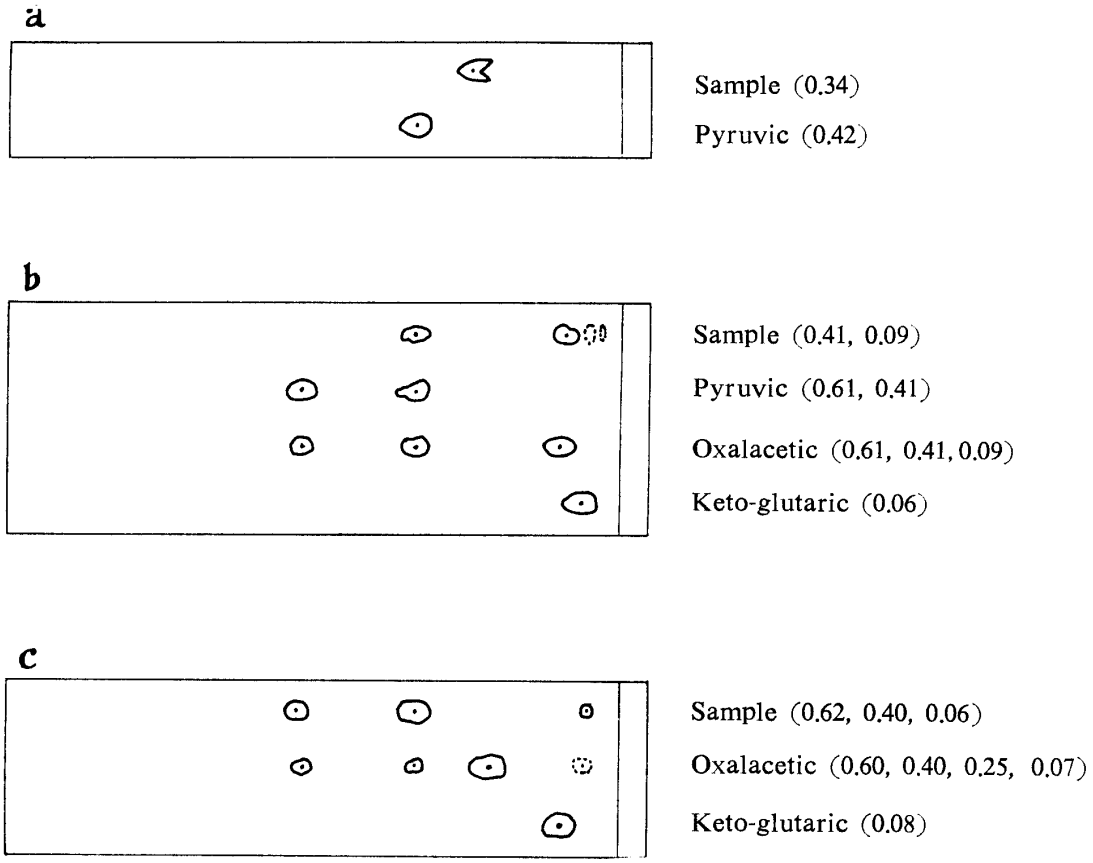

Fig. 9. Paper chromatograms of 2, 4-dinitrophenylhydrazones of component C and several keto-acids
Solvent ; (a) Butanol-ethanol-water (5 : 1 : 4)
(b) Butanol saturated with 3 % NH_3
(c) Butanol saturated with water

Table IV The quantities of the amino acid and organic acids separated as intermediary products in the oxidation of tyrosine

	0.02 *N* NaOH (*ml.*)[a]	Quantities (*gm.*)	Molar number	Index (*mol.*)	
Initial tyrosine	—	1.50	8.27×10^{-3}	1.00	
Asp. A.		0.82	6.16×10^{-3}	0.75	
Formic A.	187	0.17	3.74×10^{-3}	0.45	
Oxalic A.	220	0.20	2.19×10^{-3}	0.27	0.96 or (0.90)
Keto A.	54	—	1.08×10^{-3} * (0.54×10^{-3})**	0.13 (0.065)	
Other A.	44	—	0.88×10^{-3}	0.11	

(a) Titration values for neutralizing each acid fraction
* Monocarboxylic acid
** Dicarboxylic acid

of the isomer of α-keto-glutaric dinitrophenylhydrazone. As inferred by other workers, because of the formation of stereo-isomer or even of polymer of keto-acid hydrazones,

chromatographic separation and identification of keto-acids seemed very difficult when they were in their mixture. Actually, from the experiments performed by authors, component C was incapable of being identified with a particular keto-acid.

The amino acids and organic acids separated as the intermediary products in the oxidation of tyrosine were represented together in Table IV. The production of 0.75 *mol.* of aspartic acid from 1 *mol.* of tyrosine shows evidently the splitting of the phenol ring of tyrosine and the production of a trace of serine may perhaps be attributed to a partial oxidation of aspartic acid. Authors have obtained three kinds of organic acid in which formic acid is the largest, oxalic acid middle, keto-acid the smallest, comparing as to the molar number. Total amount of the production of organic acid was about 1 *mol.* This result shows that organic acids produced by the splitting of phenol ring of tyrosine could be recovered sufficiently.

Earland and Stell[22] have assumed from the indicator behavior of fibroin oxidized with permanganate solution that the tyrosine is oxidized to a quinone structure, being of an obscure structure. Sitch and Smith[23] oxidized the fibroin or tyrosine separately with hydrogen peroxide. They made many attempts to demonstrate the presence of an ortho-quinone in the oxidized substance, but the attempts were not successful. They described that the reduction of the oxidized substance failed to give the green color which is characteristic of dihydric phenols. Kuga[24] prepared the silks degradated at different levels by hydrogen peroxide-oxidation and performed the qualitative test for tyrosine, dopa and indole compound. Like Arnow's presumption,[3] he emphasized, from the experimental results, that tyrosine residue in silk fibroin was converted, at first, to dopa residue. Tashiro[25] described in his paper that the phenol radical of *p*-hydroxyphenyl compound was oxidized by sodium metaperiodate, but dopa was not produced and phenol ring was not cut. He identified *p*-hydroxyphenylacetaldehyde and *p*-hydroxyphenyl acetic acid as the products in the oxidation of tyrosine. It may be undoubted that the conversion of tyrosine to dopa took place under the weak oxidation such as the photo-oxidation. However, it was shown in the previous report[6] that dopa was rapidly oxidized when it was treated with potassium permanganate solution. Moreover, no existence of dopa in the oxidized solution was ascertained.

From the results obtained by the present study, it is evident that when tyrosine is oxidized with potassium permanganate aqueous solution the phenol ring of tyrosine is split. And subsequently, aspartic acid, very slight serine, formic, oxalic and keto acids are given as the intermediary products. These results permit the authors to presume that the tyrosine residue in fibroin will take the similar splitting when fibroin is to be treated with potassium permanganate.

Summary

Tyrosine was oxidized with dilute potassium permanganate aqueous solution at the room temperature. The oxidized solution was filtered and treated with Amberlite IRA-410 and then with Amberlite IR-120 columns. Being regarded as both types of the free acid and sodium salt, the solutions were separately concentrated under the reduced pressure and lyophilized. Those lyophilized powders were employed as the sample for seeking after the amino acids and several organic acids, which were anticipated as the oxidized intermediary products of tyrosine. The existence of aspartic acid and of very

slight serine in the oxidized substance was ascertained by the paper chromatography. Especially, 0.75 *mol.* of aspartic acid were derived from 1 *mol.* of tyrosine.

The separation of organic acids was performed by the partition chromatography with Celite 545 column. Three components were chiefly noticeable in the elution diagrams. Individual acid component separated was identified by comparing with those known, and also by the paper chromatography, the absorption spectra of the indole-organic acid compound, the application of specific reaction, and the melting point of crystal.

As the result, three components were identified with formic, oxalic and keto acids respectively. About 1 *mol.* of organic acids in total was obtained from 1 *mol.* of tyrosine. Comparison was made as to the molar number, with the ascertainment that formic acid was the largest, oxalic acid, middle, keto-acid, the smallest.

From the identification of the oxidized intermediary products, it was fixed that when tyrosine is oxidized with potassium permanganate aqueous solution the phenol ring of tyrosine is split.

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