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CYTOCHROMES PURIFIED FROM THE LARVA, PUPA AND ADULT HOUSEFLY, *MUSCA DOMESTICA* L.

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SUMMARY

Cytochrome *c* was isolated from larvae, pupae and adults of the housefly, *Musca domestica* L., and highly purified. The properties of housefly cytochrome *c* did not vary during metamorphosis. Although housefly cytochrome *c* was similar to mammalian cytochrome *c* in several properties, it differed in that it was precipitated by saturated ammonium sulphate and that it reacted with Pseudomonas cytochrome oxidase more rapidly. From larvae and pupae of the housefly, housefly cytochromes *b*-563 and *b*-555 were isolated by extraction with aqueous ammonium sulphate, whereas these cytochromes could not be isolated from adult flies. It is concluded that the housefly cytochrome system varies during metamorphosis.

INTRODUCTION

The terminal electron-transport system of the housefly was investigated by CHANCE AND SACKTOR[1], and ESTABROOK AND SACKTOR[2], and it was found to be similar to that of mammalian tissues. ESTABROOK AND SACKTOR[2] purified cytochrome *c* from the flight muscles of the housefly, and concluded that the cytochrome *c* obtained was identical with mammalian cytochrome *c*. This conclusion, however, was reached only by measuring the visible spectrum of the cytochrome *c*. From the silkworm, *Bombyx mori*, TUPPY[3] isolated cytochrome *c*, and found it to be similar to mammalian cytochrome *c* chemically, spectrophotometrically and enzymically.

It has been established by us[4] that a distinct specificity exists in the reaction of cytochrome *c*'s with cytochrome oxidase. Thus, cow cytochrome *a* rapidly oxidizes reduced cow cytochrome *c* but does not oxidize reduced Pseudomonas cytochrome *c*-551. Conversely, Pseudomonas cytochrome oxidase* rapidly oxidizes reduced Pseudomonas cytochrome *c*-551, but scarcely oxidizes reduced cow cytochrome *c*. It has also been shown that this specificity can be used to detect a delicate difference between cytochrome *c*'s. Thus, tests of the reactivity of a cytochrome *c* with cytochrome oxidases may be used to compare various cytochrome *c*'s with one another.

* As this enzyme does not correspond to any enzyme listed in Report of The Commission on Enzymes, the name, Pseudomonas cytochrome *c*-551: nitrite, O_2 oxidoreductase, is proposed for this enzyme[5].

In the present investigation, cytochrome *c* was purified from larvae, pupae and adults of the housefly, and its properties examined. Two *b*-type cytochromes were also purified and their properties are described.

PREPARATIONS AND MATERIALS

Purification of cytochrome c

About 1200 g of 3rd instar larvae were ground for 30 min with 1 kg of sand, and to the paste were added 2 l of water. The pH of the mixture was adjusted to 6.0 and $(NH_4)_2SO_4$ was added to 40% saturation. The mixture was allowed to stand overnight at 5° in a refrigerator and then filtered through a Büchner funnel with the aid of Celite. The filtrate, which showed two absorption bands around 550 and 560 mμ, was dialysed against tap water overnight. The diffusate was transferred to an Amberlite CG-50 column which had been equilibrated with 0.01 M ammonium phosphate buffer* (pH 7.0), and the cytochrome *c* was adsorbed on the column. The unadsorbed solution showed an absorption band around 560 mμ and was used for purification of *b*-type cytochromes (see below). The Amberlite CG-50 column on which cytochrome *c* had been adsorbed was washed with 0.01 M ammonium phosphate buffer (pH 7.0), and the cytochrome *c* was eluted with 1.0 M ammonium phosphate buffer. The eluate was dialysed against 0.01 M ammonium phosphate buffer (pH 7.0) and tranferred to the Amberlite CG-50 column described above. Then the cytochrome *c* was eluted with 0.3 M ammonium phosphate buffer (pH 7.0). The eluate was used as the purified cytochrome *c* preparation. Cytochrome *c* was also purified from pupae and adult flies in the same way.

Purification of b-type cytochromes

As stated above, the solution unadsorbed by the Amberlite CG-50 column during collection of cytochrome *c* contained *b*-type cytochromes. To this solution was added $(NH_4)_2SO_4$ to 90% saturation, and the resulting precipitate was collected by filtration with the aid of Celite. The precipitate was dissolved in a minimum volume of water, and the solution obtained was dialysed overnight against 0.01 M ammonium phosphate buffer (pH 7.0). The diffusate was transferred to a DEAE-cellulose column which had been equilibrated with 0.01 M ammonium phosphate buffer (pH 7.0). Nearly all the brown pigments were adsorbed on the column, and the unadsorbed solution showed an absorption band at 563 mμ. To the unadsorbed solution was added $(NH_4)_2SO_4$ to 50% saturation and the solution was centrifuged at 10 000 × *g* for 10 min. To the supernatant further $(NH_4)_2SO_4$ was added to 90% saturation and the solution was centrifuged at 10 000 × *g* for 10 min. The precipitate obtained was dissolved in an appropriate volume of 0.2 M phosphate buffer (pH 7.0). This solution was used as the housefly cytochrome *b*-563 preparation.

* In this communication, the concentration of ammonium phosphate buffer is expressed with regard to the NH_4^+ ion. For example, 0.01 M ammonium phosphate buffer contains 0.01 gram ions of NH_4^+ per litre.

Another cytochrome was adsorbed together with the brown pigments on the DEAE-cellulose column. This cytochrome could be eluted separately from the brown pigments by 0.05 M NaCl. The eluate obtained was used as the cytochrome *b*-555 preparation.

Preparation of Pseudomonas cytochrome oxidase and cow cytochrome a

Pseudomonas cytochrome oxidase was purified from *Pseudomonas aeruginosa* by the method of YAMANAKA AND OKUNUKI[4], and cow cytochrome *a* from cow-heart muscle by the method of OKUNUKI *et al.*[6].

METHODS

Spectrophotometric determination

Spectrophotometric determinations were performed in a Cary model-14 recording spectrophotometer.

Cleavage of haem from cytochrome c

Cleavage of haem from cytochrome *c* was performed according to the method of PAUL[7] with a slight modification as previously described[8].

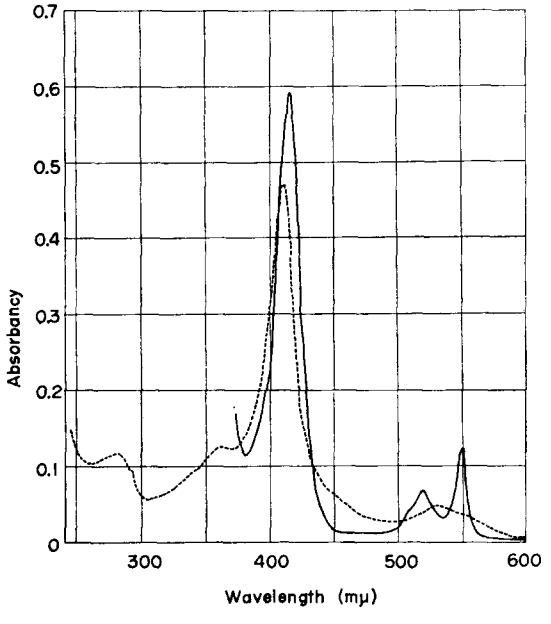

Fig. 1. The absorption spectrum of housefly cytochrome *c* from larvae. The cytochrome was dialysed against 0.02 M phosphate buffer (pH 7.0) overnight, after addition of a small amount of $K_3Fe(CN)_6$. - - - - -, oxidized; ———, reduced with $Na_2S_2O_4$.

RESULTS

Absorption spectrum of cytochrome c

As Fig. 1 shows, the absorption spectrum of housefly cytochrome *c* was identical with that of mammalian cytochrome *c*; it had maxima at 280, 360, 410 and 530 mμ in the oxidized form and at 415, 520 and 550 mμ in the reduced form. Pupal and adult cytochrome *c*'s had the same absorption peaks as those of larval cytochrome *c*.

The ratio of $A^{\text{reduced}}_{550\ m\mu}/A^{\text{oxidized}}_{280\ m\mu}$ was 1.04, which is comparable to that of crystalline mammalian cytochrome *c* (see ref. 9). Thus, the purity of the larval cytochrome *c* preparation was high, although the preparation was not obtained in a crystalline state. The same was true for adult cytochrome *c*. But the pupal cytochrome *c* preparation was still crude. The ratio of $A^{\text{reduced}}_{550\ m\mu}/A^{\text{oxidized}}_{280\ m\mu}$ was 0.394. Rechromatography on an Amberlite CG-50 column did not increase this ratio to the same value as those observed for larval and adult cytochrome *c*'s. Pupal cytochrome *c* was further purified by chromatography on a DEAE-cellulose column which had been equilibrated with 0.01 M ammonium phosphate buffer (pH 7.0). By this procedure, the colourless protein contaminating the pupal cytochrome *c* preparation was removed by adsorption on the column, and the pupal cytochrome *c* in the unadsorbed solution was considerably purified. The ratio of $A^{\text{reduced}}_{550\ m\mu}/A^{\text{oxidized}}_{280\ m\mu}$ increased to about 1. Therefore larval, pupal and adult cytochrome *c*'s are identical as judged from their absorption spectra.

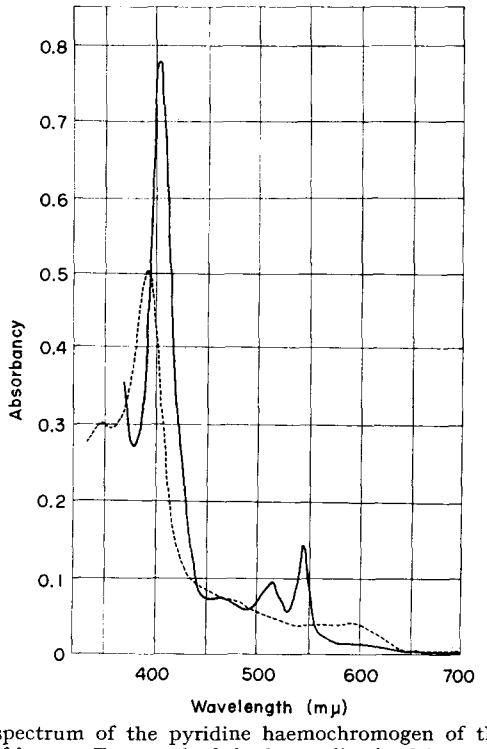

Fig. 2. The absorption spectrum of the pyridine haemochromogen of the haem isolated from housefly cytochrome *c* of larvae. To 2.0 ml of the haem dissolved in 0.2 M Na_2HPO_4 at pH 11, 0.5 ml of pyridine was added. - - - -, oxidized; ———, reduced with $Na_2S_2O_4$.

Haem of cytochrome c

As the absorption spectrum of the pyridine haemochromogen of housefly cytochrome *c* was identical with that of mammalian cytochrome *c*, it seemed likely that the haem of the cytochrome *c* was haem *c*. The haem of the cytochrome *c* was split off from the protein moiety by the Ag_2SO_4-method[7]. The absorption spectrum of its pyridine haemochromogen is shown in Fig. 2. Fig. 2. shows that the haem isolated from housefly cytochrome *c* is haematohaem, and thus the haem of the cytochrome *c* is haem *c*.

Cytochrome c content of larvae, pupae and adults

The amount of cytochrome *c* isolated from larvae, pupae and adult flies is presented in Table I. The amount of cytochrome *c* was determined assuming that the

TABLE I

CYTOCHROME *c* CONTENT OF HOUSEFLY TISSUES AT SUCCESSIVE STAGES

Stage	*Amount of cytochrome c (mg/kg of tissue)*
Larva	39
Pupa	40
Adult	128

molar extinction coefficient is the same as that of mammalian cytochrome *c* namely, $\varepsilon_{550\ m\mu} = 27.8 \cdot 10^3\ M^{-1}\ cm^{-1}$ (ref. 10).

Precipitability with ammonium sulphate

Larval, pupal and adult cytochrome *c*'s were precipitated completely even from dilute solution by saturated $(NH_4)_2SO_4$, unlike mammalian and yeast cytochrome *c*'s.

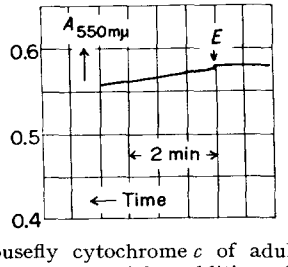

Fig. 3. Oxidation of reduced housefly cytochrome *c* of adults by Pseudomonas cytochrome oxidase. Reduced cytochrome *c* was prepared by addition of a small amount of $Na_2S_2O_4$ and subsequent dialysis against 0.04 M phosphate buffer (pH 6.5) overnight. The reaction mixture consisted of 1.1 ml of 0.04 M phosphate buffer (pH 6.5), 0.1 ml of reduced cytochrome *c* and 0.03 ml of 7.0 μM Pseudomonas cytochrome oxidase. At point E, Pseudomonas cytochrome oxidase was added. The reaction was carried out at 24.5°. Reduced larval and pupal cytochrome *c*'s were oxidized by Pseudomonas cytochrome oxidase at the same velocity as reduced adult cytochrome *c*.

Reactivity with cytochrome oxidases

As Figs. 3 and 4 show, reduced housefly cytochrome *c* was very slowly oxidized by Pseudomonas cytochrome oxidase, whereas it was rapidly oxidized by cow cytochrome *a*. As judged by these enzymic properties, there was no difference between larval, pupal and adult cytochrome *c*'s. The turnover number for the reaction with Pseudomonas cytochrome oxidase was 2.0 per minute at 24.5° and pH 6.5, and that for the reaction with cow cytochrome *a* was 52 per minute at 19° and pH 6.5.

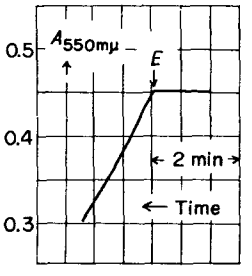

Fig. 4. Oxidation of reduced housefly cytochrome *c* of pupae by cow cytochrome *a*. For details of the preparation of reduced cytochrome *c* see the legend to Fig. 3. The reaction mixture consisted of 1.5 ml of reduced cytochrome *c* and 0.03 ml of 5.2 μM cow cytochrome *a*. At point E, cow cytochrome *a* was added. The reaction was carried out at 19°. Reduced larval and adult cytochrome *c*'s were oxidized by cow cytochrome *a* at the same velocity as reduced pupal cytochrome *c*.

Properties of cytochrome b-563

As Fig. 5 shows, the absorption spectrum of cytochrome *b*-563 had peaks at 418 and 529 mμ in the oxidized form, and peaks at 430, 531 and 563 mμ, with a shoulder at 407 mμ in the reduced form. The ratio of $A^{\text{reduced}}_{430\ m\mu}/A^{\text{reduced}}_{563\ m\mu}$ was 5.2.

In order to make the pyridine haemochromogen from cytochrome *b*-563, 3.0 ml of pyridine and 0.5 ml of 2 N NaOH were added to 3.0 ml of the cytochrome preparation. Although cytochrome *b*-563 was fairly pure, as judged from the visible spectrum shown in Fig. 5, the preparation contained much colourless protein which was precipitated on addition of pyridine and NaOH. After being vigorously shaken the mixture of the cytochrome preparation and pyridine was centrifuged at 5000 $\times$ *g* for 10 min. The pyridine layer was separated, a small amount of $Na_2S_2O_4$ was added followed by a few drops of water to dissolve the $Na_2S_2O_4$, and the absorption spectrum was measured. The absorption spectrum of the pyridine haemochromogen of cytochrome *b*-563 is shown in Fig. 6. It had absorption peaks at 417, 521 and 556 mμ. Evidently the haem of cytochrome *b*-563 is protohaem.

The evidence described above indicates that cytochrome *b*-563 is similar to mammalian cytochrome *b* (see ref. 10), except that the former can be easily extracted with aq. ammonium sulphate without the aid of any detergent, unlike the latter[11]. Cytochrome *b*-563 could be reduced very slowly by lactate in the presence of yeast lactate dehydrogenase (EC 1.1.2.3)[12] under air, but the reduction stopped when about 30% of the cytochrome *b*-563 had been reduced. CO did not affect the absorption spectrum of reduced cytochrome *b*-563 at pH 7.0.

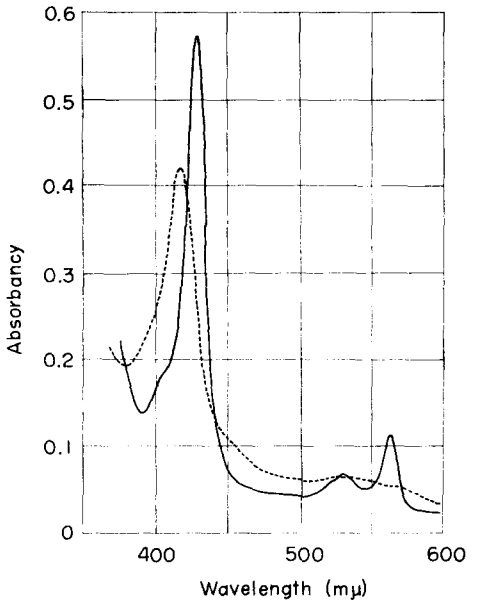

Fig. 5. The absorption spectrum of housefly cytochrome *b*-563 from larvae. The cytochrome was dialysed against 0.01 M phosphate buffer (pH 7.0) after addition of a small amount of $K_3Fe(CN)_6$. - - - - -, oxidized; ———, reduced with $Na_2S_2O_4$.

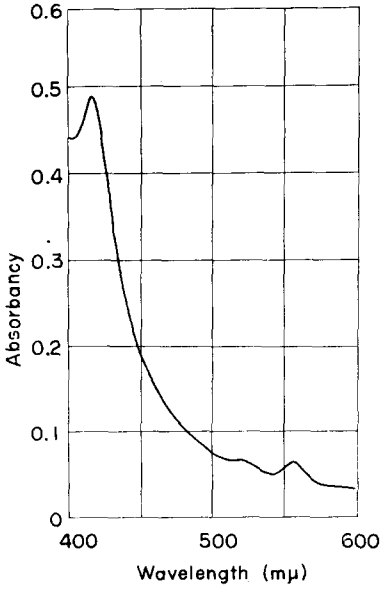

Fig. 6. The absorption spectrum of the pyridine haemochromogen of housefly cytochrome *b*-563 of larvae. For details of the preparation of the pyridine haemochromogen see the text.

Properties of cytochrome b-555

The absorption spectrum of cytochrome *b*-555 is shown in Fig. 7. It had a peak at 414 mμ in the oxidized form, and peaks at 424, 526 and 555 mμ with shoulders at 400 and 560 mμ in the reduced form. The ratio of $A^{\text{reduced}}_{424\ \text{m}\mu}/A^{\text{reduced}}_{555\ \text{m}\mu}$ was 6.2. The pyridine haemochromogen of this cytochrome was prepared in the same way as that of cytochrome *b*-563, and it was found to be identical with that of cytochrome *b*-563. Thus, the haem of cytochrome *b*-555 is also protohaem. It may seem that the cytochrome *b*-555 preparation is composed of two cytochrome components, as judged from the shape of the α-band. However, the cytochrome *b*-555 preparation has not been separated into two components so far. It is probable that cytochrome *b*-555 has an asymmetric α-band.

The two *b*-type cytochromes described above could also be extracted from

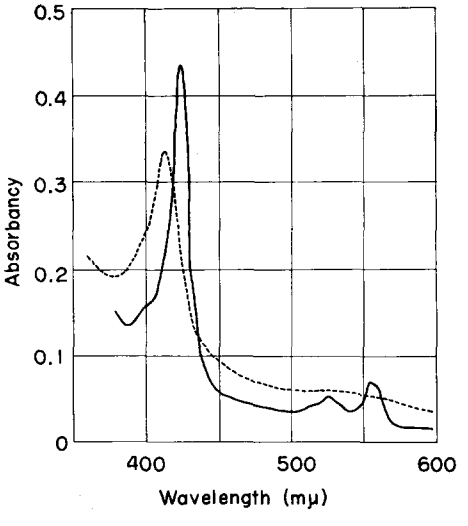

Fig. 7. The absorption spectrum of housefly cytochrome *b*-555 from larvae. The experimental conditions were the same as for Fig. 5. - - - - -, oxidized; ———, reduced with $Na_2S_2O_4$.

pupae, although in less amount than from larvae. They could not be extracted from adult flies.

It was hard to obtain preparations of the *b*-type cytochromes that were completely free of melanin pigments, and the yield of these cytochromes was low. It was also difficult to obtain large quantities of source materials. Therefore, further purification of the *b*-type cytochromes and determination of their properties were not undertaken.

DISCUSSION

From the findings of ESTABROOK AND SACKTOR[2] on purified housefly cytochrome *c* and those of TUPPY[3] on purified silkworm cytochrome *c*, it is currently thought that insect cytochrome *c* is identical with mammalian cytochrome *c*. In this investigation,

various properties of highly purified housefly cytochrome *c* were studied. With regard to its absorption spectrum and its haem, housefly cytochrome *c* was identical with mammalian cytochrome *c*. This confirms the results of ESTABROOK AND SACKTOR[2]. The fact that reduced housefly cytochrome *c* was rapidly oxidized by cow cytochrome *a* seems to support the idea that housefly cytochrome *c* is identical with mammalian cytochrome *c*. However, it is noteworthy that housefly cytochrome *c* was completely precipitated by saturated $(NH_4)_2SO_4$ even from dilute solution, unlike mammalian cytochrome *c*, although it was highly purified as judged from its ultraviolet absorption spectrum. The velocity with which reduced housefly cytochrome *c* was oxidized by Pseudomonas cytochrome oxidase was greater than that at which reduced cow cytochrome *c* was oxidized[4]. It is therefore concluded that housefly cytochrome *c* is a different chemical entity from mammalian cytochrome *c*.

It was of interest to see whether cytochromes vary in quantity or quality during the developmental stages of the housefly, because it is known that the cytochrome content of the silk worm, *Platysamia cecropia* L., varies greatly during metamorphosis. Thus, cytochromes *b* and *c* are only detectable in the somatic muscles of diapausing pupae while all the tissues of larvae and adults show a complete cytochrome system[13]. Larval, pupal and adult cytochrome *c*'s of housefly were found to be identical with one another. But the cytochrome *c* content varied greatly during metamorphosis: larvae and pupae contained almost the same amount of cytochrome *c*, whereas adult flies contained more than three times as much cytochrome *c* as larvae and pupae. It has been reported that the cytochrome *c* content of *Platysamia cecropia* L. is very low in diapausing pupae[13], but pupal houseflies have the same amount of cytochrome *c* as larve.

It is noteworthy that cytochromes *b*-563 and *b*-555 could be extracted from larvae and pupae in a water-soluble state without the aid of a detergent. This was not so for adults. Two kinds of *b*-type cytochromes have been solubilized from etiolated mung-bean seedlings without the aid of a detergent[14]: cytochromes *b*-561 and *b*-555 were highly purified by chromatography on a DEAE-cellulose column. Housefly cytochromes *b*-563 and *b*-555 are similar to mung-bean cytochromes *b*-561 and *b*-555, respectively, in their absorption spectra. It is interesting that housefly cytochrome *b*'s and mung-bean cytochrome *b*'s have both been isolated from young developing organisms although housefly is an animal and mung bean a plant.

It is not yet known whether cytochromes *b*-563 and *b*-555 are the same entities as the *b*-type cytochromes (cytochromes *b* and b_5)[2] in the adults, which are so tightly bound to particulate material that they cannot be extracted by aqueous solution.

From the facts described above, it is evident that there is a difference in the cytochrome systems of larvae and pupae and of adults. Thus, even though the configuration of cytochrome molecules does not vary, the cytochrome system varies with the developmental stage of housefly.

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REFERENCES

1 B. CHANCE AND B. SACKTOR, *Arch. Biochem. Biophys.*, 76 (1958) 509.
2 R. W. ESTABROOK AND B. SACKTOR, *Arch. Biochem. Biophys.*, 76 (1958) 532.
3 H. TUPPY, *Z. Naturforsch.*, 12b (1957) 784.
4 T. YAMANAKA AND K. OKUNUKI, *Biochim. Biophys. Acta*, 67 (1963) 379.
5 T. YAMANAKA, S. KIJIMOTO AND K. OKUNUKI, *J. Biochem. (Tokyo)* 53 (1963) 416.
6 K. OKUNUKI, I. SEKUZU, T. YONETANI AND S. TAKEMORI, *J. Biochem. (Tokyo)*, 45 (1958) 847.
7 K-G. PAUL, *Acta Chem. Scand.*, 4 (1950) 239.
8 T. YAMANAKA AND K. OKUNUKI, *Biochim. Biophys. Acta*, 67 (1963) 407.
9 B. HAGIHARA, I. MORIKAWA, I. SEKUZU AND K. OKUNUKI, *J. Biochem. (Tokyo)*, 45 (1958) 551.
10 R. K. MORTON, *Rev. Pure Appl. Chem.*, 8 (1958) 161.
11 I. SEKUZU AND K. OKUNUKI, *J. Biochem. (Tokyo)*, 43 (1956) 107.
12 T. YAMANAKA, T. HORIO AND K. OKUNUKI, *J. Biochem. (Tokyo)*, 45 (1958) 291.
13 D. G. SHAPPIRIO AND C. M. WILLIAMS, *Proc. Roy. Soc. (London)*, *Ser. B*, 147 (1957) 218.
14 H. SHICHI AND D. P. HACKETT, *J. Biol. Chem.*, 237 (1962) 2955.