BBA 45945

LIGHT-SCATTERING STUDIES ON RABBIT BRAIN MICROSOMES

III. COUPLING OF CYTOCHROME b_5 AND RELATED OXIDATIVE ACTIVITIES WITH MICROSOMAL CONTRACTION

KOHTARO KAMINO AND AKIRA INOUYE

Department of Physiology, Kyoto University School of Medicine, 606, Kyoto (Japan)

(Received December 31st, 1969)

SUMMARY

Addition of NADH or NADPH to a microsomal suspension in 0.25 M sucrose caused a significant decrease in light scattering, a decrease which was enhanced by the further addition of cytochrome *c*. In the presence of vitamin K_3, the NADPH-induced change was also enhanced; the NADH-induced change, however, remained unaffected. From measurements of the dissymmetry coefficient and of the angular light-scattering pattern, these light-scattering changes were found to be due most probably to shrinkage of the microsomal vesicles. The action of NADH or NADPH in causing such a microsomal shrinkage was not affected by amylobarbitone, antimycin A, rotenone, cyanide or ouabain but was depressed by *p*-chloromercuribenzoate and *N*-ethylmaleimide. From these results it is suggested that cytochrome b_5 and related oxidative activities in microsomes couple, in some way, with volume changes in the microsomal vesicles.

INTRODUCTION

In our previous report we presented methods for the quantitative estimation of volume changes in microsomal vesicles by means of light-scattering measurements, and it was demonstrated by using these methods that the Boyle–Van 't Hoff relation was applicable to the osmotic behavior of brain microsomes[1]. Later we also reported volume changes of microsomal vesicles induced by ATP and some nucleotides[2].

It has been well established by light-scattering studies that there exists a close correlation between mitochondrial swelling and contraction and the process of oxidative phosphorylation, the so-called high-amplitude turbidity changes. PACKER AND RAHMAN[3] attempted light-scattering studies on the effects of cytochrome *c*, NADH and NADPH on liver microsomes, but they failed to establish the presence of light-scattering changes of a microsomal suspension corresponding to the high-amplitude changes observed for mitochondria.

On the other hand, INOUYE AND SHINAGAWA[4,5] demonstrated the presence of cytochrome b_5 and related electron transfer systems in brain microsomes which were summarized as shown in Fig. 1. Here, fp_1, fp_2 and fp_3 represent three flavoproteins,

NADH–cytochrome b_5 reductase, NADPH–cytochrome b_5 reductase and NADPH–cytochrome *c* reductase, respectively. However, the physiological significance of these oxidative activities in brain microsomes remains unknown.

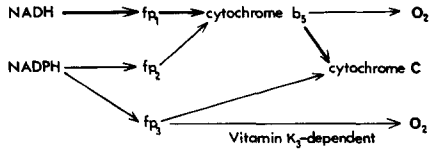

Fig. 1. Scheme of electron transfer pathways in brain microsomes.

As stated in our previous report[2], though only in a preliminary form, NADH and NADPH were found to cause a slight but significant decrease in light scattering from microsomal vesicles. Expecting, therefore, to find a clue to the physiological role of microsomal electron transfer systems, we examined their correlation with volume changes in microsomal vesicles of rabbit brain.

METHODS

Essentially the same methods as those employed in our previous studies[1,2] were used in this investigation.

Microsomal suspensions were usually prepared from rabbit brain homogenized in 0.25 M sucrose as described. In some experiments, however, brains were homogenized in 10 vol. of 0.125 M KCl solution containing 20 mM Tris–HCl buffer (pH 7.3); the salt solution was used only for homogenization and washing, the final microsomal pellets being suspended in buffered 0.25 M sucrose and stored in the cold before use.

Angular scattering measurements, the determination of the dissymmetry coefficient and a protein assay were carried out as described previously. In the present studies, I_{45} and Z with a wavelength of 578 mμ were routinely employed where I_{45} and Z were, as in our previous report[1], defined as

$$I_{45} = \frac{\text{deflection of galvanometer at } 45°}{\text{deflection of galvanometer at } 0°}$$

$$Z = I_{45}/I_{135}$$

The true dissymmetry coefficient $[Z]$ was the value of Z extrapolated to zero concentration of microsomes. To check the influence of light absorption due to added substances, the light-scattering intensity at 436 mμ was also examined.

The nicotinamide nucleotides (NADH, NADPH, NAD^+ and $NADP^+$) used were obtained from the Sigma Chemical Co.

RESULTS

Effects of NADH and NADPH on light scattering

When NADH or NADPH (10^{-4} M) was added to microsomes suspended in 0.25 M sucrose, the intensity of light scattered at 45° (I_{45}) or 90° (I_{90}) showed a rapid decline, attaining a steady level within 2–3 min. On the addition of cysteine (10^{-4} M) or ascorbate (10^{-4} M), a similar effect was observed: the order of magnitude of such

an effect was as seen in Fig. 2, NADH $\simeq$ NADPH $>$ ascorbate $>$ cysteine, while NAD^+ or $NADP^+$ showed no effect.

Such an effect of NADH or NADPH was enhanced about 2-fold by further addition of cytochrome *c* (10^{-6} M), whereas addition of cytochrome *c* alone caused almost no significant change in light scattering (Fig. 2).

Effect of the addition of vitamin K_3 on NADPH-induced changes in light scattering

SATO *et al.*[6] reported that liver microsomes contain an NADPH oxidase requiring certain naphthoquinones as cofactor. This oxidase was purified by NISHIBAYASHI *et al.*[7] and was shown to be identical with microsomal NADPH–cytochrome *c* reductase, previously isolated by several workers[8,9]. INOUYE AND SHINAGAWA[4] demonstrated that NADPH was hardly oxidized aerobically in the presence of brain microsomes and that the addition of a catalytic amount of vitamin K_3 (2-methyl-1,4-naphthoquinone) to this aerobic system induced an NADPH–oxidase activity. We therefore examined the effects of vitamin K_3 on NADPH-induced changes of light scattering in brain microsomes.

Upon the addition of vitamin K_3 alone, hardly any change in I_{45} was observed. Moreover, NADH-induced changes in light scattering were also unaffected by the addition of vitamin K_3 (Fig. 3). In the presence of vitamin K_3, however, NADPH-induced changes in light scattering were enhanced becoming 2–2.5 times higher.

These results strongly suggest the coupling of microsomal naphthoquinone-dependent NADPH–cytochrome *c* reductase activity with the light-scattering changes observed.

Effects on light scattering of light absorption due to added drugs

Since NAD(P)H, vitamin K_3 and, in particular, cytochrome *c* show noticeable absorption spectra in the range of 340–580 mμ, it is necessary for the interpretation of the light-scattering changes to take into consideration the effects of light absorption caused by adding these compounds.

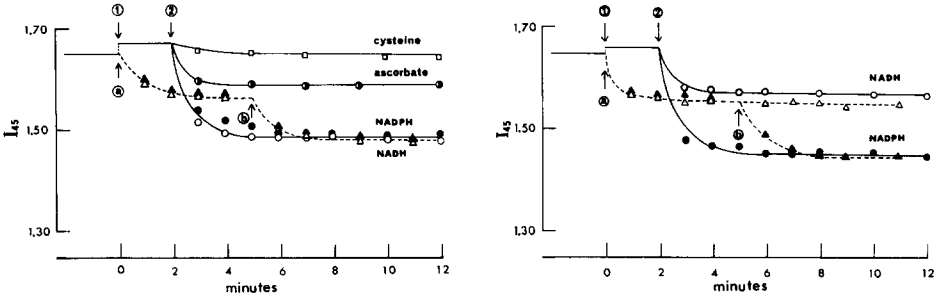

Fig. 2. Effect of cytochrome *c* and NAD(P)H on light scattering from a brain microsomal suspension. (A) At arrow 1, cytochrome *c* (10^{-6} M) was added. At arrow 2, NADH (○), NADPH (●), ascorbate (◐) or cysteine (□) was further added (10^{-4} M). (B) At arrow a, NADH (△) or NADPH (▲) was added (10^{-5} M). At arrow b, cytochrome *c* (10^{-6} M) was further added. The observations A and B were carried out on aliquots of the same microsomal sample (187 μg protein per ml). Ordinate: I_{45} at 578 mμ. Abscissa: time in min.

Fig. 3. Effect of vitamin K_3 and NADPH on light scattering from a brain microsomal suspension (187 μg protein per ml). (A) At arrow 1, vitamin K_3 (10^{-6} M) was added. At arrow 2, NADH (○) or NADPH (●) was further added (10^{-4} M). (B) At arrow a, NADH (△) or NADPH (▲) was added. At arrow b, vitamin K_3 was further added. Ordinate, I_{45} at 578 mμ.

As described previously, the value of I_{45} was always referred to I_0, so that the effects of light absorption due to added substances would be canceled out to a great extent. However, at 368 mμ, for instance, addition of NADH caused a large galvanometer deflection due to its intense light absorption, which made it difficult to follow changes in the light scattering.

To minimize such effects, 578 mμ was used as an inert wavelength out of 4 wavelengths available. Indeed, in the range of concentration used in this study, none of the added drugs showed marked absorption at 578 mμ. Taking into account the absorption spectra of these substances, a wavelength of 436 mμ also appeared appropriate. At this wavelength the results described in previous sections could be confirmed. In spite of the use of the inert wavelength, addition of cytochrome *c* (Fig. 2) or vitamin K_3 (Fig. 3) often caused a slight elevation in the level of I_{45}, which was always found to be negligible for semiquantitative analysis.

TABLE I

COMPARISON OF LIGHT-SCATTERING CHANGES INDUCED BY NICOTINAMIDE NUCLEOTIDES WITH THOSE INDUCED BY HYPERTONIC SUCROSE

Sucrose concn. (M)	$(I_{45})_{\lambda=436}/(I_{45})_{\lambda=578}$	*Compounds added to 0.25 M sucrose solution*	$(I_{45})_{\lambda=436}/(I_{45})_{\lambda=578}$
0.25	2.67 ± 0.02	Cyt.*c*(10^{-6} M) alone	2.66 ± 0.03
0.50	2.69 ± 0.02	Cyt.*c*(10^{-6} M) + NADH (10^{-4} M)	2.68 ± 0.02
0.75	2.66 ± 0.01	Cyt.*c*(10^{-6} M) + NADPH (10^{-4} M)	2.67 ± 0.01
1.00	2.68 ± 0.01		
1.50	2.67 ± 0.03	Vit.K_3(10^{-6} M) alone	2.68 ± 0.03
2.00	2.66 ± 0.02	Vit.K_3(10^{-6} M) + NADPH (10^{-4} M)	2.68 ± 0.02

For the light-scattering changes due to osmotic contraction of the microsomal vesicles, the ratio of I_{45} at 436 mμ to that at 578 mμ remained nearly constant for a microsomal concentration of 80–180 μg protein/ml (Table I). If the measured values of I_{45} in the presence of these substances were not seriously affected by light absorption, this ratio would remain at nearly the same value as that in sucrose alone. As shown in Table I, this was found to be the case: in the presence of NAD(P)H and/or vitamin K_3 or cytochrome *c*, the ratio was hardly affected, indicating that the effects on the observed I_{45} of light absorption due to these substances were practically negligible.

NADH (or NADPH)-induced light-scattering changes and shrinkage of microsomal vesicles

As discussed in ref. 1, if NADH (or NADPH)-induced changes in light scattering are really caused by shrinkage of the microsomal vesicles, the angular light-scattering pattern of microsomes suspended in a hypertonic sucrose solution of a certain osmolarity would be nearly identical with that in 0.25 M sucrose solution containing NAD(P)H and cytochrome *c*, or NADPH and vitamin K_3. Indeed, as shown in Fig. 4A, the angular variation curves of a microsomal suspension in 0.42 M

sucrose with and without cytochrome *c* and in 0.25 M sucrose containing both NAD(P)H and cytochrome *c* were found to overlap almost completely for the angle 45–135°. Applying Eqn. 8 presented in the previous report[1], therefore, it might be said that microsomal vesicles shrink to about 90 % of their original volume in the presence of NAD(P)H, and cytochrome *c* alone causes practically no shrinkage. Similarly, the overlapping of the angular scanning patterns of the NADPH–vitamin K_3 system also showed that microsomal shrinkage was about 15 %.

Alternatively, shrinkage of vesicles could be evaluated by using the dissymmetry coefficient $[Z]$, as reported earlier[1]. Indeed, upon the addition of NAD(P)H, the dissymmetry coefficient decreased significantly as I_{45} declined in the presence of cytochrome *c* (Fig. 5) but hardly changed in the absence of the cytochrome *c*. Applying the value of $[Z]$ thus obtained to Eqn. 7 of our previous report[1], the shrinkage induced by NADH or NADPH in the presence of cytochrome *c* was expected to be about 10 %.

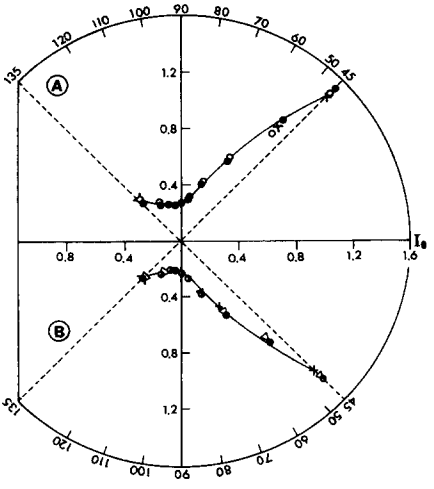

Fig. 4. Angular variation curves of light scattering at 578 mμ, from a brain microsomal suspension (173 μg protein per ml). (A) ×—×, in 0.42 M sucrose solution; ○—○, in 0.42 M sucrose solution containing cytochrome *c* (10^{-6} M); ●—●, in 0.25 M sucrose solution containing cytochrome *c* (10^{-6} M) and NADH (10^{-4} M). (B) ×—×, in 0.47 M sucrose solution; △—△, in 0.47 M sucrose solution containing vitamin K_3 (10^{-6} M); ◐—◐, in 0.25 M sucrose solution containing vitamin K_3 (10^{-6} M) and NADPH (10^{-4} M).

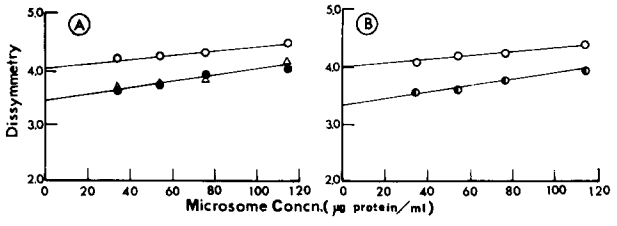

Fig. 5. The dissymmetry coefficient $[Z]$ at a wavelength of 578 mμ of a microsomal suspension. (A) ○—○, in 0.25 M sucrose solution containing cytochrome *c* (10^{-6} M); △—△, in 0.25 M sucrose solution containing cytochrome *c* (10^{-6} M) and NADH (10^{-4} M); ●—●, in 0.25 M sucrose solution containing cytochrome *c* (10^{-6} M) and NADPH (10^{-4} M). (B) ○—○, in 0.25 M sucrose solution containing vitamin K_3 (10^{-6} M); ◐—◐, in 0.25 M sucrose solution containing vitamin K_3 and NADPH (10^{-4} M).

The measurement of [Z] also suggested that the NADPH–vitamin K_3 system caused 10 % shrinkage in the microsomal volume. Thus the NAD(P)H-induced changes in microsomal volume estimated by both methods showed fairly good agreement. When the amount of added NADH was increased at a fixed concentration of cytochrome *c*, the microsomal shrinkage estimated by the overlapping method increased slightly, the maximum shrinkage being about 20 %.

Effects of some inhibitors

Taking into account the scheme presented in Fig. 1, the results described above seem to suggest some participation of the electron transfer system in brain microsomes with NAD(P)H-induced light-scattering changes. These electron transfer pathways have been demonstrated to be insensitive to amylobarbitone, antimycin A and cyanide which were known as inhibitors of mitochondrial electron transport, but no specific inhibitor has been known. To exclude the possibility of some contribution by contaminated mitochondrial fragments, therefore, the effects of some inhibitors were examined. These included cyanide (10^{-5} M), rotenone (3 mM), antimycin A (1 μg), amylobarbitone (2 mM), ouabain (10^{-3} M), *p*-chloromercuribenzoate (PCMB, 10^{-5}, 10^{-6} M) and *N*-ethylmaleimide (10^{-5}, 10^{-6} M).

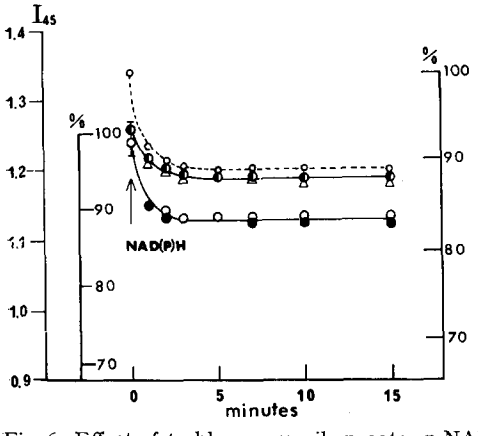

Fig. 6. Effect of *p*-chloromercuribenzoate on NAD(P)H-induced changes of light scattering from a brain microsomal suspension (156 μg protein per ml). O---- O, NADH (10^{-4} M) alone; ◐—◐, NADH (10^{-4} M) in the presence of PCMB (10^{-5} M); △—△, NADPH (10^{-4} M) in the presence of PCMB (10^{-5} M); ●—●, NADH (10^{-4} M) in the presence of PCMB (10^{-6} M); O—O, NADPH (10^{-4} M) in the presence of PCMB (10^{-6} M). Ordinates on the left: I_{45} at 578 mμ and % changes referred to I_{45} in the presence of PCMB (10^{-5} or 10^{-6} M). Ordinates on the right: % changes referred to I_{45} in the absence of PCMB, the difference in I_{45} between the levels of 100 % in the right and left scale indicating the effect of PCMB on the scattering.

The effects of NADH and NADPH were found to be practically unaffected by the presence of the inhibitors tested, the only exception being PCMB at a concentration of 10^{-5} M and *N*-ethylmaleimide (10^{-5} M). As already noted in our previous report[2], a scattering decrease (about 15 %) was caused by *p*-chloromercuribenzoate alone. As shown in Fig. 6, however, the percent decrease in I_{45} induced by NADH or NADPH in the presence of cytochrome *c* was depressed by 10^{-5} M PCMB to a level significantly lower than that with 10^{-6} M. The experiments with *N*-ethylmaleimide

provided results quite similar to those with PCMB. It might be said, therefore, that the effect of NADPH or NADPH *plus* cytochrome *c* is blocked by this inhibitor, a finding which suggests that the mechanism of SH enzymes might be involved. With the NADPH–vitamin K_3 system, PCMB and *N*-ethylmaleimide also showed inhibitory effects. Thus it is obvious that light-scattering changes induced by nicotinamide nucleotides of brain microsomes are insensitive to antimycin A, amylobarbitone, rotenone and cyanide, just as their electron transfer pathways relate to cytochrome b_5.

DISCUSSION

In spite of well-established high-amplitude turbidity changes of mitochondria, especially that of mitochondrial fragments[10], PACKER AND RAHMAN[3] failed to prove with liver microsomes that initiation of electron transfer from added NADH to cytochrome *c* through microsomal electron transfer systems induced a scattering change such as that observed with mitochondrial fragments. The results presented in this report suggest, however, that NAD(P)H–cytochrome *c* reductase activities in brain microsomes are closely related to decreases in scattering induced by some reducing agents such as ascorbate or nicotinamide nucleotides. The negative results reported by PACKER AND RAHMAN might be attributed to their employing only I_{90}, considerably weaker than the I_{45} used in the present study.

Indeed, NAD(P)H-induced decreases in light scattering of microsomal suspensions are markedly reinforced by the presence of cytochrome *c*. Moreover, such scattering changes are not affected by cyanide, antimycin A or amylobarbitone, thus excluding the possibility of the participation of oxidative activities of mitochondrial origin such as contaminated mitochondrial fragments. It is also worthy to note that NADPH-induced scattering changes are also remarkably enhanced by adding a catalytic amount of vitamin K_3. These effects on light scattering by nicotinamide nucleotides are inhibited by PCMB and *N*-ethylmaleimide.

Taking into consideration the scheme of electron transfer pathways in brain microsomes (Fig. 1), these findings could be easily explained by assuming that electron transfer through the oxidative chains presented causes some structural changes and/or shrinkage of microsomes. Both the angular scanning and dissymmetry measurements showed with high probability that observed structural changes such as decreases in light scattering are accompanied by shrinkage of the microsomal vesicles.

As INOUYE AND SHINAGAWA[4] showed, ascorbate and cysteine can reduce cytochrome b_5, though to a lesser extent than NAD(P)H. The effect of these substances as well as the nicotinamide nucleotides shown in Fig. 2 would be attributable to this cytochrome b_5 reductase activity utilizing oxygen as an acceptor. By acting as a powerful acceptor, cytochrome *c* enhances this activity, while the presence of a catalytic amount of vitamin K_3 evokes naphthoquinone-dependent NADPH oxidase activity. Thus, if the fp's in Fig. 1 are firmly bound to microsomal membranes as presumed from studies by STRITTMATTER[11] AND NISHIBAYASHI *et al.*[7], it is not so unexpected that these reductase activities couple with microsomal structural changes, which are naturally inhibited by PCMB and *N*-ethylmaleimide.

Since the scheme presented in Fig. 1 was only a tentative one to interpret the observed NAD(P)H oxidase activities in an analogy to the pathways elucidated in liver microsomes, it may afford some debate, especially as to the existence of an

NADPH–cytochrome b_5 pathway; its occurrence in liver microsomes is still in dispute. Recent studies by SATO *et al.*[12] and OMURA AND TAKEI[13], however, appear to lend further support to the occurrence in liver microsomes of this pathway. Even if the existence of a specific enzyme for the pathway, fp_2, is denied, our results are not controversial; an NADPH–cytochrome *c* pathway can be catalyzed, as shown by NISHBAYASHI and her co-workers[7], by a naphthoquinone-dependent NADPH oxidase, fp_3, its occurrence in brain microsomes being demonstrated by INOUYE AND SHINAGAWA[4,5].

In our laboratory, UYEDA and his co-workers[14] observed that the presence of NADH and cytochrome *c* in the incubation media caused a significant increase in the uptake of ^{22}Na by rabbit brain microsomes. But microsomal contraction by electron transfer through the microsomal oxidative chains is ouabain-insensitive, so that its coupling with the sodium and/or potassium pump does not seem so likely. Together with SIEKEVITZ[15], therefore, we have still no positive evidence for the possibility that the electron transport chain activates the microsomal sodium pump, resulting in vesicular contraction.

ACKNOWLEDGMENTS

The research reported in this communication was supported in part by a grant from the U.S. Army Research and Development Group (Far East), DA-CRD-AFE-S92544-67 CT69, and partly by a grant from the Ministry of Education, Japan.

REFERENCES

1 K. KAMINO AND A. INOUYE, *Biochim. Biophys. Acta*, 183 (1969) 36.
2 K. KAMINO, *Biochim. Biophys. Acta*, 183 (1969) 48.
3 L. PACKER AND M. M. RAHMAN, *Texas Rept. Biol. Med.*, 20 (1962) 414.
4 A. INOUYE, Y. SHINAGAWA AND Y. SHINAGAWA, *J. Neurochem.*, 12 (1965) 803.
5 A. INOUYE AND Y. SHINAGAWA, *J. Neurochem.*, 13 (1966) 385.
6 R. SATO, H. NISHIBAYASHI AND T. OMURA, *Biochim. Biophys. Acta*, 63 (1962) 550.
7 H. NISHIBAYASHI, T. OMURA AND R. SATO, *Biochim. Biophys. Acta*, 67 (1963) 550.
8 C. H. WILIAMS AND H. KAMIN, *J. Biol. Chem.*, 237 (1962) 587.
9 A. H. PHILIPS AND R. G. LANGDON, *J. Biol. Chem.*, 237 (1962) 2652.
10 L. PACKER AND A. L. TAPPEL, *J. Biol. Chem.*, 235 (1960) 525.
11 P. STRITTMATTER, in P. D. BOYER, H. LARDY AND K. MYRBÄCK, *The Enzymes*, Vol. 8, Academic Press, New York, 1962, p. 113.
12 R. SATO, H. NISHIBAYASHI AND A. ITO, in J. R. GILETTE, A. H. CONNEY, G. J. COSMIDES, R. W. ESTABROOK, J. R. FOUTS AND G. J. MANNERING, *Microsomes and Drug Oxidations*, Academic Press, New York, London, 1969, p. 111.
13 T. OMURA AND S. TAKEI, *J. Japan. Biochem. Soc.* (*Seikagaku*), 40 (1968) 608 (in Japanese).
14 A. INOUYE, Y. SHINAGAWA, M. UYEDA AND T. SHIMADZU, *J. Physiol. Soc. Jap.*, 28 (1966) 378 (in Japanese).
15 P. SIEKEVITZ, *Federation Proc.*, 24 (1956) 1153.