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THE EPOXIDATION OF ALDRIN BY HOUSEFLY MICROSOMES AND ITS INHIBITION BY CARBON MONOXIDE

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Abstract—Housefly (*Musca vicina*) microsomes contain a system which converts aldrin to dieldrin and which requires the presence of NADPH and O_2. The pH optimum is 8·2 and the reaction is stimulated by cyanide and inhibited by EDTA, sesamex and carbon monoxide. The inhibition by carbon monoxide is partially reversed by light. Carbon monoxide combines with the reduced microsomes to give a difference spectrum with a peak at 450 mμ which shifts to 420 mμ on addition of deoxycholate. The possibility of the presence of two terminal oxidases is discussed briefly.

COMPARATIVE STUDIES of the detoxication mechanisms in insects and mammals is of considerable importance in the development of selective insecticides. The few comparative studies made to date have indicated little difference between the microsomal systems of insects and mammals. A microsomal system has been reported in *Blattella germanica* which hydroxylated DDT to give a kelthane-like derivative[1] and the co-factors, NADPH and O_2, required for this reaction were similar to those reported for hydroxylation in rabbit liver microsomes.[2] Housefly[3] and rat liver[4] microsomes both hydroxylate naphthalene to give the same two major products, 1-naphthol and 1,2-dihydro-1,2-dihydroxy naphthalene. The epoxidation of aldrin has been demonstrated in both mammals and insects using rabbit liver microsomes and housefly homogenates[5] and also in microsomal preparations from houseflies.[6] Mammalian microsomes have been shown to contain a cytochrome which, in its reduced form, binds carbon monoxide giving an absorbance spectrum with a peak at 450 mμ.[7, 8] Carbon monoxide has been shown to inhibit the hydroxylation of sterols,[9] and later it was demonstrated that the photo-chemical action spectrum corresponded to the spectrum of the P450/CO complex.[10] The presence of P450 has been reported in a number of vertebrates[11] but the question of its existence in invertebrates has received little attention. Trace amounts of P450 have been found in lobster gill, but not in any other lobster organ,[11] and its presence has been reported in three species of insects.[12]

MATERIALS AND METHODS

The houseflies were from a dieldrin-resistant strain which are no longer reared under dieldrin pressure. Flies, collected in cages as they emerged from puparia, were fed on sugar and water only and used for the preparation of microsomes when 3–4 days old.

Preparation of microsomes

All apparatus was pre-cooled to 0° and subsequent operations were carried out as

near to 0° as possible. Unsexed adult flies (130–200 g) were immobilized at approx. −15°, and homogenized for 30 sec in 600 ml 1·15% KCl in a Waring blender. The homogenate was strained through a single layer of mutton cloth, centrifuged at 6500 *g* for 10 min and the supernatant filtered through a pad of glass wool, and re-centrifuged at 12,000 *g* for a further 10 min. The 12,000-*g* supernatant was again filtered through glass wool and the filtrate centrifuged for 30 min at 105,000 *g*. The final pellet was reddish-orange in colour and appeared to be made up of a heavier darker zone and a lighter pale zone. The yield was of the order of 300 mg of microsomal protein.

Suspension of the microsomes

Initially the microsomes were suspended using an Ultraturex homogenizer operated with a reduced input voltage, in order to decrease violence of homogenization. This method was later replaced by one in which the microsomes were suspended in the centrifuge tubes using a hand-driven PTFE pestle. Different suspension media were tried as indicated in Table 1. For spectrophotometry the microsomes were either sus-

TABLE 1. THE EFFECT OF SUSPENDING MICROSOMES IN DIFFERENT MEDIA

Suspension medium	Dieldrin formation (moles × 10^{-8})
1	2·3
2	2·9
3	2·4
4	3·0

Incubation time 10 min at 30° pH 8·3, volume 5 ml containing 12·25 mg microsomal protein. Composition of suspension media: (1) 1·15% KCl; (2) 1·15% KCl + 2% w/v bovine plasma albumen fraction V; (3) 0·25 M sucrose 0·01 M tris phosphate buffer pH 8·3, 5×10^{-4} M NADP, 10^{-2} M nicotinamide, 10^{-2} M G6P; (4) 0·25 M sucrose, 0·01 M tris phosphate buffer pH 8·3, 5×10^{-4} M NADP, 10^{-2} nicotinamide, 10^{-2} M G6P, 2% w/v bovine plasma albumen fraction V, aldrin $5{\cdot}6 \times 10^{-5}$ M.

pended in (4) (see Table 1) or, more usually, in sucrose 0·25 M 10^{-2} M Tris phosphate buffer pH 8·3.

Reaction mixture for epoxidation

Total volume of the reaction mixture was 5 ml and contained Tris phosphate pH 8·3 0·05 M; G6P 5×10^{-3} M; nicotinamide 5×10^{-3} M; NADP 10^{-4} M; KCl $12{\cdot}5 \times 10^{-3}$ M; glucose 6-phosphate dehydrogenase 1·4 units. When the effect of pH was examined, a potassium dihydrogen phosphate sodium hydroxide buffer 0·05 M was used in place of the tris-phosphate. The aldrin was added in 100 μl of alcohol.

Spectra

The difference spectra were measured on a Unicam SP 800. Carbon monoxide was generated by reacting sulphuric and formic acids, and washed by bubbling it through a dilute potassium hydroxide solution.

Extraction and measurement of dieldrin

The reaction mixture was shaken at 30° for 10 min in 25 ml conical flasks. The reaction was stopped by adding 3 ml of acetone and the mixture transferred to a 25ml

stoppered tube. The flask was rinsed with a further 1 ml of acetone and this, together with a further 3 ml of acetone, was added to the mixture. After addition of a few mg of anhydrous sodium sulphate, the mixture was extracted twice with two 4-ml aliquots of petroleum spirit (40–60° b.p. fraction). The extracts were combined and aliquots of this solution (1 or 3 μl) were assayed for aldrin and dieldrin on a Pye Panchromatograph employing a 100 mc tritium foil electron capture detector of the type first described by Lovelock.[13] The column was 6 in. long and consisted of a stationary phase of 2·5% Apiezon + 0·25% Epicote on celite (100–200 mesh). The carrier gas was nitrogen. Dieldrin was measured by comparing peak heights with those obtained using a standard curve.

Materials

The aldrin was recrystallized by Dr. G. T. Brooks. NADP, G6P and G6P dehydrogenase were purchased from Boehringer, London; bovine plasma albumen fraction V from Armour Pharmaceutical Co. Ltd., Eastbourne; and the crude lipase from Koch-Light Ltd., Colnbrook. All other chemicals were AR grade.

RESULTS

The effect of different suspension media

Table 1 shows the effect on epoxidase activity of suspending microsomes in different media and indicates that epoxidation was slightly greater in the presence of albumen. The activity of housefly microsome preparations is rapidly lost on storage. Storage for 16 hr at 0° in any of the four media used resulted in the loss of 50–60 per cent of the initial activity; however when microsomes were frozen and stored for 16 hr at −15° in suspension medium (4), they lost only 15 per cent of their initial activity. In the present work the microsomes were used within two hours of suspension of the microsomal pellet.

Stability of the microsomes when incubated with aldrin

Figure 1 shows that the rate of production of dieldrin from aldrin is almost linear up to 20 min under the conditions described. No product other than dieldrin was found on analysis by GLC although the possibility of other products present in small amounts cannot be eliminated.

pH *Optimum for aldrin epoxidation system in houseflies microsomes*

The effect of pH on the epoxidation activity is shown in Fig. 2. There is a very steep rise in activity over the pH range 6·7–7·5, the activity increasing 4½ times. The maximum enzyme activity was observed around pH 8·2 and thereafter declined only slightly as the pH was increased to 9·7. The activity in the sodium and potassium phosphate buffer was the same as that in the tris phosphate at pH 8·3.

Factors affecting the microsomal epoxidation of aldrin

It can be seen from Table 2 that insect microsomes, in common with mammalian microsomes, require the presence of both oxygen and NADPH for the conversion of aldrin to dieldrin. There is sufficient glucose 6-phosphate dehydrogenase present in the microsomes to maintain the maximum rate of dieldrin formation. It can also be seen in Table 2 that EDTA inhibits epoxidation and cyanide stimulates it. Replication in

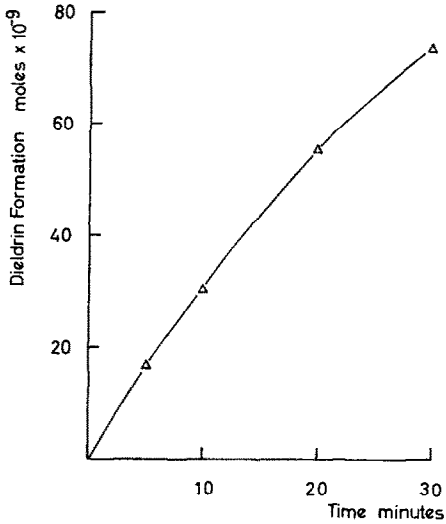

FIG. 1. The rate of formation of dieldrin. Incubation at 30°. Reaction mixture; tris phosphate buffer 0·05 M pH 8·3; nicotinamide 5×10^{-3} M; NADP 10^{-4} M; KCl $12{\cdot}5 \times 10^{-3}$ M; aldrin $5{\cdot}6 \times 10^{-5}$ M; glucose 6-phosphate dehydrogenase 1·4 units; microsomal protein 12·14 mg; total volume 5 ml.

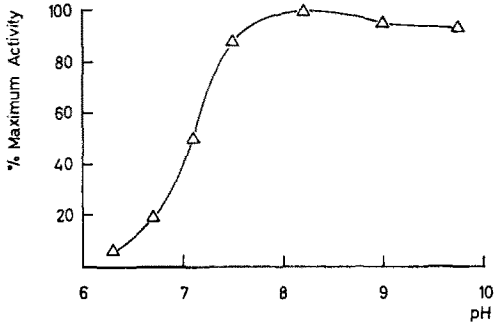

FIG. 2. The pH optimum for dieldrin formation. Incubations at 30° for 10 min. Reaction mixture: potassium dihydrogen phosphate buffer 0·05 M; nicotinamide 5×10^{-3} M; NADP 10^{-4} M; KCl $12{\cdot}5 \times 10^{-3}$ M; glucose 6-phosphate dehydrogenase 1·4 units; aldrin $5{\cdot}6 \times 10^{-5}$ M; microsomal protein 15 mg; total volume 5 ml.

TABLE 2. EFFECT OF THE ADDITION AND OMISSION OF VARIOUS SUBSTANCES ON THE DIELDRIN FORMED IN 10 min BY HOUSEFLY MICROSOMES

Complete reaction mixture	100%
No NADPH	trace
No O_2	trace
No G6P dehydrogenase	100%
EDTA 10^{-4} M	88%
KCN 2×10^{-4} M	114%

Reaction mixture unless otherwise indicated: tris phosphate buffer 0·05 M pH 8·3; nicotinamide 5×10^{-3} M; NADP 10^{-4} M; KCl $12{\cdot}5 \times 10^{-3}$ M; aldrin $5{\cdot}6 \times 10^{-5}$ M; glucose 6 phosphate dehydrogenase 1·4 units volume 5 ml.

the controls was better than 5 per cent and the EDTA and cyanide results are the means of three determinations.

Determination of K_m of aldrin epoxidation and effect of sesamex

Figure 3 shows the effect of increasing concentrations of aldrin on the rate of epoxidation of aldrin plotted by the Lineweaver–Burk[14] method. It is surprising that

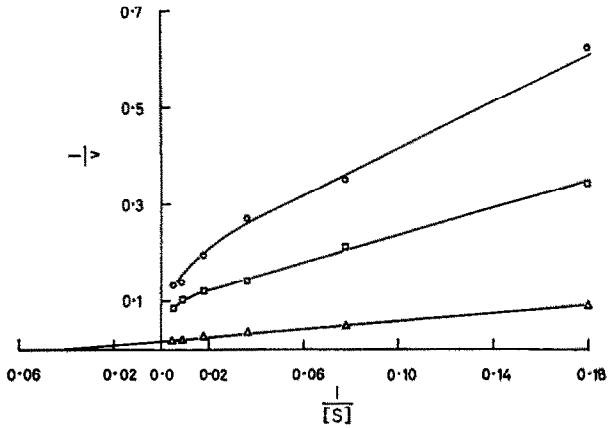

FIG. 3. Reciprocal plot of dieldrin formation against aldrin concentration and effect of sesamex Incubations at 30° for 10 min. Reaction mixture: tris phosphate buffer pH 8·3, 0·05 M; nicotinamide 5×10^{-3} M; NADP 10^{-4} M; KCl $12{\cdot}5 \times 10^{-3}$ M; glucose 6-phosphate dehydrogenase 1·4 units; microsomal protein 12·1 mg; total volume 5 ml. [s] = 10^{-6} M aldrin v = dieldrin formed 10^{-9} moles/10 min.
△ = 10 μl alcohol; ○ = 10 μl alcohol 5×10^{-5} sesamex; □ = 10 μl alcohol 10^{-4} sesamex.

the line is straight because even the lowest concentration of aldrin exceeds its solubility in water. The K_m for this reaction lies in the region of $2\text{–}2{\cdot}5 \times 10^{-5}$ M. The same figure also shows the effect of two concentrations of sesamex, a pyrethrum synergist, on the epoxidation of aldrin.

Effect of CO *on system*

The gaseous mixtures all contained 20 per cent oxygen, the appropriate amount of CO and the difference between this and 100 per cent was made up with nitrogen. The incubation medium was equilibrated with the appropriate gas mixture for 5 min before addition of the microsomes and substrate. The inhibition of dieldrin formation by a 1:1 carbon monoxide:oxygen mixture was of the order of 80 per cent, although the actual inhibition varied slightly from one microsome preparation to another. With a 2:1 carbon monoxide:oxygen ratio the level of inhibition rose to approx. 90 per cent. With either gas mixture it was possible to reverse partially the inhibition brought about by carbon monoxide by illuminating the reaction vessel. Light, from a 1000 W tungsten–iodine lamp was passed through a 6 in. condenser and a copper sulphate solution as a heat filter, only reduced the level of inhibition by 8–12 per cent.

The presence of a carbon monoxide binding pigment in housefly microsomes

When carbon monoxide is passed through a suspension of housefly microsomes in buffer saturated with N_2 and reduced either by NADPH or dithionite a difference

spectrum is obtained (Fig. 4). There is a sharp peak at 450 mμ and a shallow trough at about 520 mμ. The height of the peak at 450 mμ is greatly reduced in the presence of oxygen when NADPH is used as the reducing agent and under these conditions addition of dithionite caused an increase in absorption at 450 mμ. When the microsomes are reduced with a NADPH generating system in the presence of carbon

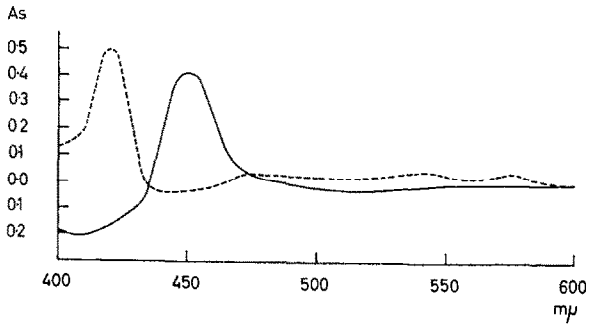

FIG. 4. The two forms of the CO binding pigment in housefly microsomes. Path length 10 mm. Microsomes in 0·25 M sucrose 0·01 M tris phosphate buffer reduced with approx. 1 mg sodium dithionite, protein concentration 8·6 mg/ml.
Microsomes reduced by dithionite and saturated with CO minus dithionite-reduced microsomes.
As solid line but deoxycholate added to both.

monoxide and the absence of oxygen, the peak at 450 mμ shows a gradual drift in absorption to 452 mμ. There is also a slight production of a peak at 420 mμ, with the consequent decrease in height around 450–452 mμ.

Addition of deoxycholate to reduced microsomes saturated with CO leads to the disappearance of the peak at 450 mμ and the formation of a new peak at 420 mμ. This absorption spectrum is shown in Fig. 4, not only is there a peak at 420 but two smaller peaks at 545 mμ and 575 mμ. Using the extinction coefficient of 91 cm^{-1} mM^{-1} for the P450 in rabbit liver microsomes,[15] the housefly microsomes have been calculated to contain 0·20–0·41 mμ moles P450/mg of microsomal protein.

Cytochrome b_5 content of insect microsomes

Figure 5 shows the dithionite reduced minus oxidized difference spectrum of insect microsomes following digestion with 0·07% crude lipase ex pig pancreas at 0° for 16 hr followed by centrifugation at 105,000 *g* for 1 hr and resuspension in buffer. This treatment has been used to remove most of the flavoprotein in rabbit microsomes but only a small amount of b_5.[15] It can be seen that the absorbance bands are at 426, 528 and 559 mμ respectively. On solubilization of the b_5 there is a spectral shift of 2 mμ in the Soret band giving a peak at 424. The insect microsomes contain a pigment which comes from the head, probably the eyes, which has a reduced minus oxidized spectrum with a minimum at 450 mμ. This is a broad, shallow trough, and probably accounts for the minimum at 450 mμ on Fig. 5 and may account for the shift in absorption of the Soret band.

DISCUSSION

The insect microsomal epoxidation system, in common with that found in mammals, requires oxygen and NADPH for activity. The insect preparation tends to be variable in activity, the rate of aldrin epoxidation varying from 0·16 to 0·31 mμ moles dieldrin produced per min per mg of microsomal protein. This preparation is however

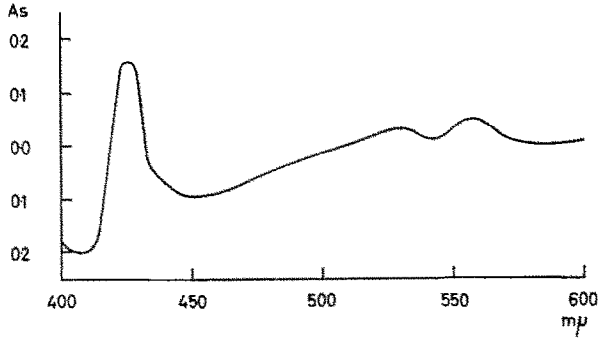

FIG. 5. Cytochrome b_5 in insect microsomes. Path length 10 mm. Microsomes digested for 16 hr at 0° with 0·07% crude pancreatic lipase ex pig, centrifuged 105,000 g for 1 hr and resuspended in 0·25 M sucrose 0·01 M tris phosphate buffer. Dithionite reduced minus oxidized.

4–7½ times more active than that produced from a strain of DDT-resistant flies.[6] Housefly microsomes have been stored (diluted in tris/HCl buffer pH 8·2) for up to 4 weeks without losing more than 20 per cent of their naphthalene hydroxylating ability, but the authors do not comment on the stability of the epoxidase system.[16]

The incorporation of bovine plasma albumen in the incubation medium for the C_{21} hydroxylation of sterols by bovine adrenocortical microsomes caused a two-fold increase in rate.[17] A similar but smaller effect has been found on the aldrin epoxidation by insect microsomes when albumen was incorporated in the suspension medium.

The pH optimum for the epoxidase is higher in housefly microsomes (pH 8·2) than for its counterpart in rabbit liver microsomes[5] (pH 7·1) and pig liver microsomes* (pH 7·3).

The epoxidase system of housefly microsomes is inhibited by EDTA and stimulated by cyanide. No explanation can be given for the former. Housefly microsomes contain tyrosinase activity† and it is thought that products from this system, e.g. benzoquinones, could possibly inhibit epoxidase activity. Both cyanide[18] and simply substituted methylenedioxyphenol compounds† inhibit tyrosinase activity, these latter compounds in low concentrations also stimulate the epoxidase system to the same extent as cyanide, although at higher concentrations‡ they inhibit epoxidation.

The insect microsomal epoxidation system is more sensitive to carbon monoxide than that found in pig liver microsomes and similarly the C_{21} hydroxylation of sterols by bovine adrenocortical microsomes is also less sensitive in this respect. This, combined with the differences in pH optima discussed above, suggest that real differences may exist between mammalian and insect microsomes at the enzymic level, although more comparative data is required in order to substantiate this hypothesis.

* S. E. Lewis, personal communication.
† C. F. Wilkinson, R. L. Metcalf and T. R. Fukuto, personal communication.
‡ J. W. Ray, unpublished data.

The poor reversal of the carbon monoxide inhibition by light raises additional questions, either the light intensity is not sufficient or there are two carbon monoxide binding pigments acting as terminal oxidases only one of which is photodissociable. Evidence for the existence of two terminal oxidases each with a different K_m value for oxygen has been reported.[19] This has been supported by the discovery of two distinct Soret bands when P450 is treated with ethyl isocyanide,[20] each having a different affinity for molecular oxygen. If there are two terminal oxidases present in the microsomes it would be possible to explain the curved Lineweaver–Burke plots obtained in the presence of an inhibitor in the following way. The enzyme possessing the lower K_m appears to be inhibited non-competitively whereas the enzyme having the higher K_m is inhibited competitively. Similar results have been obtained for the epoxidation of aldrin by pig liver microsomes.*

The presence of cytochrome P450 in insects has been shown to occur in at least two other species, *Blattella germanica* and *Periplaneta americana*.[12] The P450 in housefly microsomes is not stable, and on standing in addition to the usual change to the P420 form, there is a gradual shift of the peak at 450 mμ to 452 mμ, a change which presumably involves some change in the binding of the cytochrome to the lipoprotein complex. The amount of P450 found in dieldrin-resistant housefly microsomes is approx. 1/10 that found in rabbit liver microsomes[13] and approx. 1/5 that found in pig liver microsomes† when expressed in terms of cytochrome per mg of microsoma protein.

So far there is no evidence to suggest that the microsomes from insects and mammals differ in any fundamental respect but there are minor differences for example, sensitivity to carbon monoxide and methylene-dioxyphenols and differences in pH optima. Work is now in progress to investigate the possibility of finding synergists which will exploit these differences.

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* S. E. Lewis, personal communication.
† J. W. Ray, unpublished data.

REFERENCES

1. M. AGOSIN, D. MICHAELI, R. MISKUS, S. NAGASAWA and W. H. HOSKINS, *J. econ. Ent.* **54**, 340 (1961).
2. C. H. MITOMA, H. S. POSNER, H. C. REITZ and S. UDENFRIEND, *Archs Biochem. Biophys.* **61**, 431 (1956).
3. R. O. ARIAS and L. C. TERRIERE, *J. econ. Ent.* **55**, 925 (1962).
4. J. BOOTH and E. BOYLAND, *Biochem. J.* **70**, 681 (1958).
5. T. NAKATSUGAWA, M. ISHIDA and P. A. DAHM, *Biochem. Pharmac.* **14**, 1853 (1965).
6. R. B. SCHONBROD, J. R. GILLETT and L. C. TERRIERE, *Bull. ent. Soc. Am.* **11**, 157 (1965).
7. M. KLINGENBURG, *Archs Biochem. Biophys.* **75**, 376 (1958).
8. D. GARFINKEL, *Archs Biochem. Biophys.* **77**, 493 (1958).
9. K. J. RYAN and L. L. ENGEL, *J. biol. Chem.* **225**, 103 (1957).
10. D. Y. COOPER, S. LEVIN, S. NARASIMHULA and O. ROSENTHAL, *Science, N.Y.* **147**, 400 (1965).
11. D. GARFINKEL, *Comp. Biochem. Physiol.* **8**, 367 (1963).
12. J. W. RAY, *Pest Infestation Research*, p. 59. H.M.S.O. (1965).
13. J. E. LOVELOCK and S. R. LIPSKY, *J. Am. chem. Soc.* **82**, 431 (1960).
14. H. LINEWEAVER and D. BURK, *J. Am. chem. Soc.* **56**, 658 (1934).

15. T. OMURA and R. SATO, *J. biol. Chem.* **239**, 2379 (1964).
16. R. B. SCHONBROD and L. C. TERRIERE, *Bull. ent. Soc. Am.* **11**, 158 (1965).
17. D. Y. COOPER and O. ROSENTHAL, *Archs Biochem. Biophys.* **96**, 331 (1962).
18. C. R. DAWSON and R. J. MAGEE, *Methods in Enzymology*, (Eds. S. P. COLOWICK and N. O. KAPLAN) vol. II, p. 817. Academic Press, New York (1955).
19. HJ. STAUDINGER, B. KEREKGARTO, V. ULLRICH and Z. ZUBRZYCKI, *Oxidases and Related Redox Systems*, (Eds. T. E. KING, H. S. MASON and M. MORISON) vol. 2, p. 815. Wiley, New York (1964).
20. Y. IMAI and R. SATO, *Biochem. Biophys. Res. Commun.* **23**, 5 (1966).