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Short Communications

Specific Binding of [^{14}C]Piericidin A in the Reduced Nicotinamide–Adenine Dinucleotide Dehydrogenase Segment of the Mitochondrial Respiratory Chain

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Rotenone inhibits the respiration of mammalian mitochondria by blocking the NADH dehydrogenase segment of the respiratory chain. Contrary to expectations, [^{14}C]rotenone binds, not only to the 'specific site' responsible for its inhibitory effects on NADH oxidation, but also at 'unspecific sites' unrelated to enzyme activity (Horgan & Singer, 1967; Horgan, Singer & Casida, 1968). Rotenone binding at the latter sites is relatively weak, and repeated washing with bovine serum albumin removes most of the unspecifically bound rotenone. Binding at the specific site is more tenacious and therefore washing with bovine serum albumin removes relatively little rotenone from this locus. These facts, coupled with the demonstration that rotenoids, barbiturates and piericidin A are bound at and compete for the same specific site (Horgan & Singer, 1967; Horgan *et al.* 1968; Palmer, Horgan, Tisdale, Singer & Beinert, 1968), permit the calculation of the amount of rotenone bound in the NADH dehydrogenase region of the respiratory chain. Unfortunately, in these studies it was not possible to define the experimental conditions in which rotenone binds exclusively at the specific site because, on repeated washing with bovine serum albumin, the bound rotenone is gradually removed also from this specific site, with consequent regeneration of enzyme activity.

Piericidin A inhibits mitochondrial NADH oxidation in the same manner as rotenone (Hall *et al.* 1966), but, unlike inhibition by rotenone, the inhibition caused by piericidin A is not reversed on washing the particles with bovine serum albumin (Horgan *et al.* 1968). This suggests that piericidin A is more specific than rotenone as a reagent for blocking electron transport from NADH dehydrogenase to the cytochrome system. In view of the previous work with [^{14}C]rotenone, it seemed desirable to utilize [^{14}C]piericidin A in ascertaining their comparative specificities.

Piericidin labelled with ^{14}C was prepared biosynthetically from [^{14}C]acetate by Professor S. Tamura and Professor N. Takahashi, University of Tokyo, Japan. Other reagents and methods were as in previous work (Horgan *et al.* 1968).

Fig. 1(*a*) shows that, on titration of ETP* with [^{14}C]piericidin in sucrose–phosphate buffer, a linear binding curve is obtained, just as with [^{14}C]rotenone (cf. Fig. 1*a* in Horgan & Singer, 1967), which bears no apparent relation to the curve for inhibition of NADH oxidase activity. However, on washing the inhibited particles twice with 2% (w/v) bovine serum albumin, the amount of [^{14}C]piericidin bound is drastically decreased without a significant effect on the inhibition (curve *A* in Fig. 1*b*). Further washing with bovine serum albumin does not remove additional [^{14}C]piericidin. Comparison of Fig. 1(*b*) with the corresponding Figure for [^{14}C]-rotenone (Horgan & Singer, 1967) clearly shows that piericidin binding is considerably more specific under these conditions than that of rotenone, since the point at which a plateau is reached in the binding curve of [^{14}C]piericidin coincides with the point for maximal inhibition of NADH oxidation. The unspecific binding that occurs beyond this point is very slight with [^{14}C]piericidin (curve *A* in Fig. 1*b*). As expected, when the particles are first treated with just sufficient unlabelled piericidin to give 99% inhibition of NADH oxidase, followed by titration with [^{14}C]piericidin and bovine serum albumin washes, the initial rapidly rising portion of

* Abbreviation: ETP, electron transport particles.

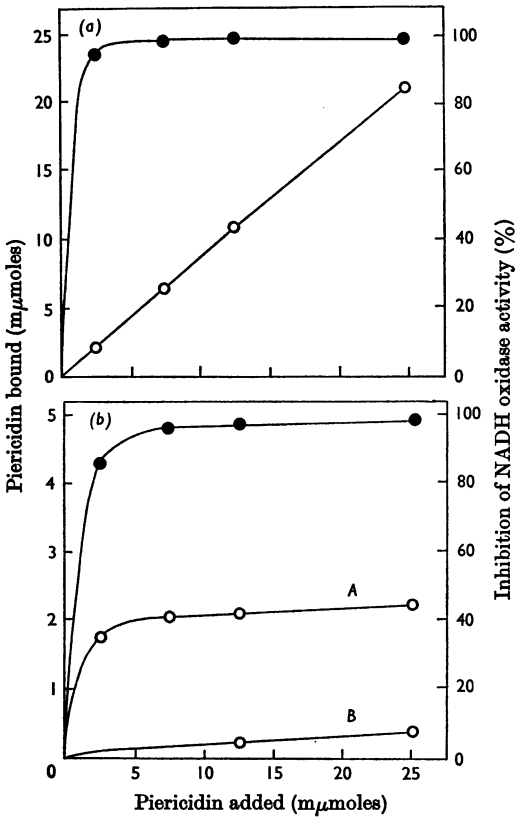

Fig. 1. (*a*) Titration of 25mg. portions of ETP with [^{14}C]-piericidin A in 0·25M-sucrose–25mM-phosphate buffer, pH7·4. The inhibition of NADH oxidase activity was measured spectrophotometrically. The [^{14}C]piericidin sample contained 70% of piericidin A and 30% of a mixture of two slightly less polar compounds, as shown by their thin-layer chromatographic behaviour. Though these impurities have not been identified, they are probably closely related to piericidin A because of the purification procedure involved after biosynthesis and because they bind to ETP in the same manner as piericidin A itself. The amount of piericidin added and bound to the particles refers to the total ^{14}C radioactivity of the material described. (*b*) Titration of ETP with [^{14}C]piericidin as above followed by two washes with sucrose–phosphate buffer containing bovine serum albumin (2%, w/v). Curve *A*, [^{14}C]piericidin added without pretreatment of the particles; curve *B*, particles first inhibited (99%) with unlabelled piericidin A before addition of [^{14}C]piericidin. ●, Inhibition of NADH oxidase activity; ○, [^{14}C]piericidin bound.

the binding curve is abolished and the small amount of unspecific binding remains unaffected (curve *B* in Fig. 1*b*). Thus there is good evidence that piericidin is more specific than rotenone in this system, because it is more tightly bound at the specific site than rotenone and is less tightly bound at the unspecific sites. This conclusion is supported by the findings that unlabelled piericidin displaces [^{14}C]rotenone from the specific site, but that unlabelled rotenone does not release [^{14}C]piericidin from this site.

There are four other findings relevant to the use of piericidin as a selective inhibitor of NADH oxidase. First, inhibition of NADH oxidase by piericidin A shows the same type of lag period as that shown by rotenone. Secondly, on comparing the amount of [^{14}C]piericidin bound on incubation individually with various submitochondrial particles of increasing simplicity (ETP, NADH–cytochrome *c* reductase particle, complex I), the binding per mg. of protein increases with purification of the particle in the expected manner. Thirdly, the amount of [^{14}C]piericidin specifically bound by a given type of particle is in good agreement with the amount of [^{14}C]rotenone specifically bound. Fourthly, [^{14}C]piericidin bound at the specific site of ETP is not removed by denaturation with 5% (w/v) trichloroacetic acid, but is quantitatively extracted with acetone from freeze-dried particles.

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Hall, C., Wu, M., Crane, F. L., Takahashi, N., Tamura, S. & Folkers, K. (1966). *Biochem. biophys. Res. Commun.* **25**, 373.

Horgan, D. J. & Singer, T. P. (1967). *Biochem. J.* **104**, 50c.

Horgan, D. J., Singer, T. P. & Casida, J. E. (1968). *J. biol. Chem.* **243**, 834.

Palmer, G., Horgan, D. J., Tisdale, H., Singer, T. P. & Beinert, H. (1968). *J. biol. Chem.* **243**, 844.