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INTERACTION OF METYRAPONE WITH ADRENAL MICROSOMAL CYTOCHROME P450 IN THE GUINEA PIG

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Abstract—Studies were carried out to compare the actions of metyrapone on adrenal mitochondrial and microsomal cytochrome P450-containing enzymes in the guinea pig and rat. As expected, addition of metyrapone to adrenal mitochondria inhibited 11β-hydroxylation in both species. The shape of the type II difference spectrum produced by metyrapone in mitochondria differed somewhat in rat ($\Delta O.D._{425-405nm}$) and guinea pig ($\Delta O.D._{425-390nm}$) and the magnitude of the spectrum was far greater in rat adrenal mitochondria, paralleling species differences in cytochrome P450 concentration (rat > guinea pig). In rat adrenal microsomes, metyrapone produced a small "reverse type I" spectral change ($\Delta O.D._{420-385nm}$) but did not affect either 21-hydroxylation or the interaction of progesterone with cytochrome P450 (as determined spectrally). In guinea pig adrenal microsomes, in contrast, metyrapone produced a large type II spectral change ($\Delta O.D._{423-408nm}$) and inhibited both 21-hydroxylation and ethylmorphine demethylation, cytochrome P450-dependent reactions. The magnitudes of type I spectra produced by 17α-hydroxyprogesterone and ethylmorphine in guinea pig adrenal microsomes were significantly diminished by prior addition of metyrapone. The results indicate that metyrapone interacts with both microsomal and mitochondrial cytochrome P450 in the guinea pig and that its adrenal sites of action, therefore, are species dependent.

The actions of metyrapone on adrenocortical steroidogenesis have been extensively investigated (see Refs. 1–3). Although originally proposed to be a relatively specific 11β-hydroxylase antagonist, metyrapone has been found to inhibit various other steroidogenic reactions, including 18- and 19-hydroxylation and cholesterol side chain cleavage [3–11]. Each of these enzymes is located in the mitochondrion of the adrenal cell and contains the hemeprotein, cytochrome P450, as its terminal oxidase. Hepatic microsomal drug-metabolizing enzymes which contain cytochrome P450 are similarly inhibited by metyrapone [12, 13]. The mechanism of metyrapone inhibition appears to involve interaction of the drug with cytochrome P450, blocking the binding of steroid or drug substrates [6,11,14]. Upon binding to cytochrome P450, metyrapone induces a characteristic spectral perturbation [6,14–16] and inhibits the spectral changes produced by substrate interactions with the cytochrome.

Despite the relatively non-specific effects of metyrapone on adrenal mitochondrial and hepatic microsomal mixed function oxidases, the results of several studies indicate that adrenal microsomal enzymes are not affected by the drug. Neither 21-hydroxylase [5,17] nor 17α-hydroxylase [18] activity was inhibited by metyrapone in bovine or rat adrenals, although cytochrome P450 is required for both reactions. In addition, Satre and Vignais [19] demonstrated high affinity binding of a metyrapone metabolite to bovine adrenal mitochondria but no binding was demonstrable in microsomes. These observations suggested that metyrapone did not interact with adrenal microsomal cytochrome P450. However, we recently found [20] that addition of metyrapone to guinea pig adrenal microsomes produced a large spectral change similar to that seen in adrenal mitochondria [6,14–16]. Therefore, the following studies were carried out to determine the effects, if any, of metyrapone on adrenal microsomal enzymes in the guinea pig. Effects were compared with those in the rat, a species in which metyrapone does not affect adrenal microsomal metabolism.

MATERIALS AND METHODS

Adult, Sprague–Dawley rats (200–225 g) and English Smooth Hair guinea pigs (650–800 g) were obtained from Zivic–Miller Laboratories, Pittsburgh, PA, and Hilltop Farms, Scottdale, PA, respectively. Animals were maintained on a diet of Purina Laboratory Chow and water *ad lib.* under standardized conditions of light (6:00 a.m.–6:00 p.m.) and temperature (22°).

Animals were sacrificed by decapitation between 9:00 and 10:00 a.m. and adrenals were quickly removed and placed in cold 0.25 M sucrose. Adrenals were trimmed free of adhering tissue, weighed, and homogenized in 0.25 M sucrose containing 0.05 M Tris-HCl, pH 7.4. Homogenates were centrifuged at 900 *g* for 10 min in a Sorvall RC-5 refrigerated centrifuge to remove red blood cells, nuclei and unbroken cells. The supernatant fraction from rat adrenals was centrifuged at 9500 *g* for 15 min to obtain the mitochondrial pellet which was resuspended in sucrose–Tris buffer and recentrifuged at

9500 *g* for 15 min. Guinea pig adrenal mitochondria were isolated by centrifugation of the 900 *g* supernatant fraction at 6000 *g* for 20 min followed by resuspension and recentrifugation at 6000 *g* for 20 min. The "washed" mitochondria were used for 11β-hydroxylase assays and spectral studies. Aliquots of the first 9500 *g* or 6000 *g* supernatant fractions were taken for 21-hydroxylase and ethylmorphine (EM) demethylase assays and the remainder was centrifuged at 105,000 *g* for 60 min in a Beckman refrigerated ultracentrifuge to obtain the microsomes. Adrenal microsomes were resuspended in 1.15% KCl containing 0.05 M Tris–HCl, pH 7.4. Microsomal and mitochondrial preparations were relatively free of cross-contamination, as indicated by the absence of detectable 11β-hydroxylase activity in microsomes and of detectable 21-hydroxylase activity in mitochondria.

11β-Hydroxylase activity was determined by incubating aliquots of adrenal mitochondria equivalent to 5 mg (rat) or 20 mg (guinea pig) tissue in a reaction mixture containing 5 mM KCl, 5 mM $MgCl_2$, 80 mM NaCl, 100 mM sucrose, 50 mM Tris–HCl (pH 7.4), 2.5 mg sodium isocitrate and 150 nmoles of 11-deoxycortisol (guinea pig) or 11-deoxycorticosterone (rat) in a total volume of 1.0 ml. Differing amounts of rat and guinea pig adrenal mitochondrial protein were incubated to compensate for species differences in sp. enzyme act. (rat > guinea pig). Incubations were carried out in a Dubnoff metabolic incubator at 37° for 10 min under air. The reaction was stopped by the addition of 0.5 ml of 0.5% mercuric chloride to each incubation tube. Steroids were extracted with freshly distilled chloroform, and cortisol or corticosterone was determined fluorometrically [21]. Standards and blanks were carried through the entire procedure.

21-Hydroxylase activity was measured as the rate of conversion of 11β-hydroxyprogesterone to corticosterone by the adrenal 9500 *g* supernatant fraction. Homogeneity of the product (corticosterone) has been established using thin-layer and paper chromatography [22]. The supernatant fraction from 10 mg (guinea pig) or 5 mg (rat) adrenal was incubated with 3 mM glucose 6-phosphate, 0.3 mM NADP, 0.15 M NaCl, 5 mM KCl, 4 mM $CaCl_2$, 2 mM $MgSO_4$, 0.05 M Tris–HCl (pH 7.4) and 150 nmoles 11β-hydroxyprogesterone at 37° for 8 min under air. The reaction was stopped by adding 0.5 ml of 0.5% $HgCl_2$ to each tube. Steroids were extracted with chloroform, and corticosterone was measured fluorometrically [22].

Reaction mixtures for adrenal ethylmorphine metabolism contained 10 μmoles ethylmorphine, 9 mM glucose 6-phosphate, 24 μmoles $MgSO_4$, 2 μmoles NADP, 0.02 M Tris–HCl (pH 7.4) and adrenal 9500 *g* supernatant fraction equivalent to 50 mg tissue [20]. Incubations were carried out in a Dubnoff metabolic incubator at 37° for 15 min. Semicarbizide HCl (25 μmoles) served as a trapping agent for formaldehyde produced from ethylmorphine. Formaldehyde production was determined using the method of Nash [23]. All samples were read against appropriate tissue blanks and standards.

Substrate and inhibitor-induced spectral changes in mitochondria and microsomes were obtained using a Cary model 17 recording spectrophotometer at room temperature. Spectral dissociation constants (K_s) were calculated by the method of Schenkman *et al.* [24]. Cytochrome P450 was measured as the sodium dithionite reduced: CO complex as described by Omura and Sato [25]. Adrenal mitochondrial and microsomal protein content was determined by the method of Lowry *et al.* [26] using bovine serum albumin as the standard.

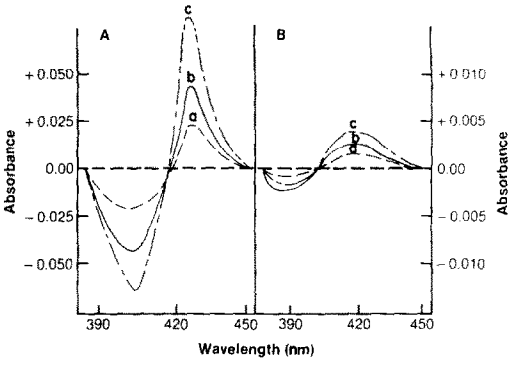

Fig. 1. Metyrapone-induced difference spectra in (A) rat adrenal mitochondria and (B) microsomes. Metyrapone was added to the sample cuvette in final concentrations of (a) 1.8×10^{-7} M; (b) 1.3×10^{-6} M; and (c) 1.2×10^{-5} M. Cuvettes contained 1.3 mg of mitochondrial protein/ml with 0.9 nmole cytochrome P450/mg of protein and 1.1 mg of microsomal protein/ml with 0.4 nmole cytochrome P450/mg of protein, respectively. Equal volumes of ethanol were added to the reference cuvette with each addition of metyrapone.

RESULTS

Interaction of metyrapone with rat adrenal mitochondria and microsomes. Addition of metyrapone to adrenal mitochondria from adult male or female rats produced a typical type II difference spectrum (Fig. 1, part A). The magnitude of the metyrapone-induced spectrum was similar to that of the type I spectral change produced by 11-deoxycorticosterone (DOC), the normal substrate for 11β-hydroxylation (Table 1). The affinity of each compound for cytochrome P450, as determined spectrally (spectral dissociation constant; K_s), was also similar (Table 1). As expected, metyrapone inhibited the conversion of DOC to corticosterone by adrenal mitochondria in a dose-dependent manner (Table 2). Prior addition of metyrapone to mitochondria also diminished the magnitude of the DOC-induced spectral change.

In rat adrenal microsomes, metyrapone produced a small "reverse type I" difference spectrum (Fig. 1, part B) suggesting, perhaps, displacement of endogenous substrates. However, metyrapone had no effect on 21-hydroxylase activity (Table 2) or on the type I spectral changes produced by progesterone and 11β-hydroxyprogesterone, substrates for 21-hydroxylation.

Interaction of metyrapone with guina pig adrenal mitochondria and microsomes. Addition of metyrapone to adrenal mitochondria from male or female guinea pigs produced a spectral change

Table 1. Metyrapone- and substrate-induced spectral changes in rat adrenal mitochondria and microsomes*

Compound	Type spectrum (max–min)	Spectral dissociation constant (M)	Maximum spectral change (ΔA/mg protein $\times 10^{-2}$)
Mitochondria†			
11-Deoxycorticosterone	(385–420)	1.6×10^{-6} (1.2–1.8)	12.9 (11.9–14.0)
Metyrapone	(425–405)	2.5×10^{-6} (2.0–3.0)	10.1 (8.9–12.6)
Microsomes‡			
Progesterone	(385–420)	5.2×10^{-7} (4.8–5.6)	1.7 (1.1–2.2)
Metyrapone	(420–385)	2.4×10^{-5} (1.6–3.2)	1.9 (1.3–2.5)

* Values represent means of triplicate determinations for each compound. Range of values is indicated in parentheses.

† Cytochrome P450 content of 1.0 ± 0.1 nmoles/mg of protein.

‡ Cytochrome P450 content of 0.5 ± 0.1 nmole/mg of protein.

Table 2. Effects of metyrapone *in vitro* on 11β- and 21-hydroxylase activities in rat adrenals*

Metyrapone concn. (M)	Enzyme activity (% of control)	
	11β-Hydroxylase	21-Hydroxylase
0	100†	100‡
5×10^{-5}	84 ± 4	103 ± 5
1×10^{-4}	66 ± 5	100 ± 4
5×10^{-4}	35 ± 2	100 ± 3
1×10^{-3}	22 ± 3	96 ± 8

* Values are expressed as means ± S. E. of three to four determinations for each concentration of metyrapone.

† Equivalent to 205.3 nmoles/min/g of tissue.

‡ Equivalent to 185.4 nmoles/min/g of tissue.

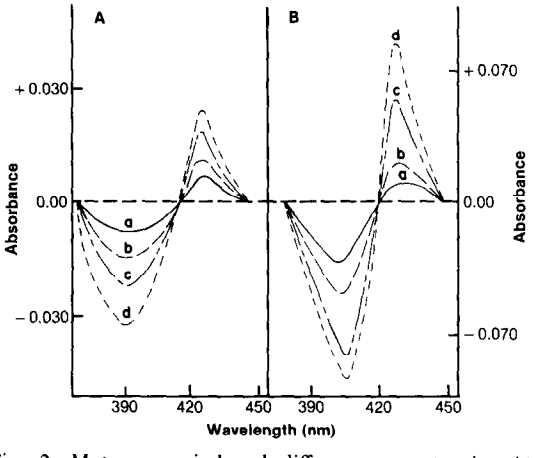

Fig. 2. Metyrapone-induced difference spectra in (A) guinea pig adrenal mitochondria and (B) microsomes. Metyrapone was added to the sample cuvette in final concentrations of (a) 1.8×10^{-7} M (b) 1.3×10^{-6} M; (c) 7.2×10^{-6} M; and (d) 1.0×10^{-5} M. Cuvettes contained 1.5 mg of mitochondrial protein/ml with 0.5 nmole cytochrome P450/mg of protein and 2.5 mg of microsomal protein/ml with 1.7 nmoles cytochrome P450/mg of protein respectively. Equal volumes of ethanol were added to the reference cuvette with each addition of metyrapone.

($\Delta O.D._{425-390nm}$; Fig. 2) differing somewhat from that seen in rat adrenals. The metyrapone-induced spectrum and the 11-deoxycortisol-induced type I spectrum were similar in magnitude (Table 3). However, 11-deoxycortisol, a normal substrate for 11β-hydroxylation in the guinea pig adrenal, had a far greater affinity than metyrapone for mitochondrial cytochrome P450. Both the rates of 11β-hydroxylation (Table 4) and the size of the 11-deoxycortisol-induced spectra were diminished by prior addition of metyrapone to guinea pig adrenal mitochondria.

In guinea pig adrenal microsomes, metyrapone produced a large type II spectral change (Fig. 2), similar to that seen in rat adrenal mitochondria (Fig. 1). The type I spectra produced by 17α-hydroxyprogesterone and 11β-hydroxyprogesterone, substrates for 21-hydroxylation, were larger than the metyrapone spectrum and both steroids had a higher affinity for microsomal cytochrome P450 than metyrapone. Ethylmorphine, a type I drug substrate rapidly metabolized by guinea pig adrenal microsomes [20], had a far lower affinity for cytochrome P450 than the other compounds studied (Table 3). Addition of metyrapone to adrenal microsomes or the 9500 *g* supernatant fraction produced a dose-dependent inhibition of 21-hy-

Table 3. Metyrapone- and substrate-induced spectral changes in guinea pig adrenal mitochondria and microsomes*

Compound	Type spectrum (max–min)	Spectral dissociation constant (M)	Maximum spectral change (ΔA/mg protein $\times 10^{-2}$)
Mitochondria†			
11-Deoxycortisol	(385–420)	$4.8 \pm 0.9 \times 10^{-7}$	5.2 ± 0.3
Metyrapone	(425–390)	$2.2 \pm 0.2 \times 10^{-5}$	6.1 ± 0.2
Microsomes‡			
17α-Hydroxyprogesterone	(385–420)	$6.6 \pm 1.2 \times 10^{-7}$	16.0 ± 0.5
11β-Hydroxyprogesterone	(385–420)	$9.2 \pm 1.3 \times 10^{-7}$	13.9 ± 0.8
Ethylmorphine	(385–420)	$2.1 \pm 0.3 \times 10^{-4}$	2.4 ± 0.3
Metyrapone	(423–408)	$7.4 \pm 1.0 \times 10^{-6}$	6.9 ± 0.7

* Values are expressed as means ± S. E. of four to five determinations for each compound.
† Cytochrome P450 content of 0.5 ± 0.1 nmole/mg of protein.
‡ Cytochrome P450 content of 1.9 ± 0.2 nmoles/mg of protein.

Table 4. Effects of metyrapone *in vitro* on the activities of adrenal mitochondrial and microsomal enzymes in guinea pigs*

Metyrapone conc. (M)	Enzyme activity (% of control)		
	11β-Hydroxylase	21-Hydroxylase	Ethylmorphine demethylase
0	100†	100‡	100§
5×10^{-5}	81 ± 5	84 ± 7	62 ± 7
1×10^{-4}	67 ± 4	73 ± 3	48 ± 3
5×10^{-4}	42 ± 6	52 ± 3	32 ± 2
1×10^{-3}	29 ± 4	28 ± 2	24 ± 4

* Values expressed as means ± S. E. of four to six determinations for each concentration of metyrapone.
† Equivalent to 45.2 nmoles/min/g of tissue.
‡ Equivalent to 172.3 nmoles/min/g of tissue.
§ Equivalent to 255.7 nmoles/min/g of tissue.

droxylase and ethylmorphine demethylase activities (Table 4). Metyrapone also diminished the magnitude of the 17α-hydroxyprogesterone, 11β-hydroxyprogesterone and ethylmorphine-induced type I spectra in guinea pig adrenal microsomes. Lineweaver–Burk plots of 17α-hydroxyprogesterone or ethylmorphine concentrations v. the magnitude of the resulting spectral changes, in the presence and absence of metyrapone, indicated that the inhibition of binding was competitive.

DISCUSSION

The inhibitory actions of metyrapone on a variety of hepatic microsomal and adrenal mitochondrial cytochrome P450-catalyzed reactions are well known [1–3,12,13]. In contrast, effects on cytochrome P450-containing enzymes in adrenal microsomes, such as 17α- and 21-hydroxylation, were not demonstrable in rat or bovine tissue [5,17,18]. These observations indicated a subcellular specificity of metyrapone action in the adrenal cortex. Consequently, most investigators using metyrapone have assumed that, in the adrenal, its effects were limited to mitochondrial mixed function oxidases. However, Leblanc *et al.* [27] have reported that metyrapone inhibits 21-hydroxylation in duck adrenal microsomes and we recently found that metyrapone produced a large type II difference spectrum in guinea pig adrenal microsomes [20], suggesting that the adrenal sites of action of metyrapone are species dependent. The data presented here lend further support to this hypothesis.

Although the shape of the metyrapone-induced spectral change in adrenal mitochondria differed somewhat in rat and guinea pig, effects of the drug on substrate binding to mitochondrial cytochrome P450 and on 11β-hydroxylase activity were similar in both species. However, interaction of metyrapone with adrenal microsomes differed considerably in the rat and guinea pig. Even at high concentrations, metyrapone had no effect on 21-hydroxylase activity in rat adrenals. Similary, the interaction of steroid substrates with microsomal cytochrome P450 in the rat was unaffected by prior addition of metyrapone to microsomal preparations. The small "reverse type I" spectral change produced by metyrapone in rat adrenal microsomes is similar to that seen by Estabrook *et al.* [28] in bovine adrenal microsomes and may represent displacement of endogenous (steroid) substrates from cytochrome P450.

In the guinea pig, by contrast to the rat, metyrapone was a potent inhibitor of adrenal microsomal cytochrome P450-dependent metabolism. The guinea pig adrenal is of interest to us, in part, because of its capacity to oxidatively metabolize

drugs as well as steroid substrates [20,22], resembling, therefore, the human fetal adrenal [29,30]. Accordingly, we were able to evaluate the effects of metyrapone on ethylmorphine demethylation as well as on 21-hydroxylation. Both reactions were inhibited by metyrapone. The mechanism of inhibition appears similar to that in adrenal mitochondria and hepatic microsomes. That is, metyrapone, upon addition to guinea pig adrenal microsomes, produced a large type II spectral change indicating an interaction with cytochrome P450, and prevented the subsequent binding of steroid and drug substrates to the cytochrome. Analysis of the spectral data indicated that metyrapone inhibition of substrate binding to adrenal microsomal cytochrome P450 was competitive in nature.

These observations clearly establish species differences in metyrapone effects on adrenal microsomal metabolism. The differences may reflect a multiplicity of cytochrome P450 moieties with varying substrate specificities in adrenal microsomes from species to species. Multiple forms of cytochrome P450 have been demonstrated in adrenal mitochondria [31,32] and hepatic microsomes [33, 34] and probably exist in adrenal microsomes as well [20]. Therefore, the specificity of metyrapone action on the adrenal cortex must be established for each species studied and cannot be extrapolated from one animal model to another.

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REFERENCES

1. D. B. Gower, *J. Steroid Biochem.* **5**, 501 (1974).
2. T. E. Temple and G. W. Liddle, *A. Rev. Pharmac.* **10**, 199 (1970).
3. R. Gaunt, B. G. Steinetz and J. J. Chart, *Clin Pharmac. Ther.* **9**, 657 (1968).
4. J. J. Chart, H. Sheppard, M. J. Allen, W. L. Bencze and R. Gaunt. *Experientia* **14**, 151 (1958).
5. O. V. Dominguez and L. T. Samuels, *Endocrinology* **73**, 304 (1963).
6. D. G. Williamson and V. J. O'Donnell, *Can. J. Biochem.* **45**, 153 (1967).
7. N. P. Sanzari and F. G. Peron, *Steroids* **8**, 929 (1966).
8. F. W. Kahnt and R. Neher, *Experientia* **27**, 959 (1971).
9. K. Griffiths, *J. Endoc.* **26**, 445 (1963).
10. I. Kraulis and M. K. Birmingham, *Can. J. Biochem.* **43**, 1471 (1965).
11. S. C. Cheng, B. W. Harding and A. Carballeira, *Endocrinology* **94**, 1451 (1974).
12. K. C. Leibman, *Molec. Pharmac.* **5**, 1 (1969).
13. K. J. Netter, G. F. Kahl and M. P. Magnussen, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **265**, 205 (1969).
14. L. D. Wilson, S. B. Oldham and B. W. Harding, *Biochemistry* **8**, 2975 (1969).
15. M. L. Sweat, R. B. Young and M. J. Bryson, *Archs Biochem. Biophys.* **130**, 66 (1969).
16. H. D. Colby and A. C. Brownie, *Archs Biochem. Biophys.* **138**, 632 (1970).
17. M. L. Sweat, J. S. Dutcher, R. B. Young and M. J. Bryson, *Biochemistry* **8**, 4956 (1969).
18. H. Levy, C. H. Cha and J. J. Carolo, *Steroids* **5**, 469 (1965).
19. M. Satre and P. V. Vignais, *Biochemistry* **13**, 2201 (1974).
20. J. W. Greiner, R. E. Kramer, D. A. Robinson, W. J. Canady and H. D. Colby, *Biochem. Pharmac.* **25**, 951 (1976).
21. L. E. Mejer and R. C. Blanchard, *Clin. Chem.* **19**, 718 (1973).
22. J. W. Greiner, R. E. Kramer, R. C. Rumbaugh and H. D. Colby, *Life Sci.* **20**, 1017 (1977).
23. T. Nash, *Biochem. J.* **55**, 416 (1953).
24. J. B. Schenkman, H. Remmer and R. W. Estabrook *Molec. Pharmac.* **3**, 113 (1967).
25. T. Omura and R. Sato *J. biol. Chem.* **239**, 2370 (1964).
26. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
27. H. Leblanc, J.-G. Lehoux and T. Sandor, *J. Steroid Biochem.* **3**, 683 (1972).
28. R. W. Estabrook, J. I. Mason, J. Baron, D. Lambeth and M. Waterman, *Ann. N.Y. Acad. Sci.* **212**, 27 (1973).
29. M. R. Juchau and M. G. Pedersen, *Life Sci.* **12**, 193 (1973).
30. M. R. Juchau, M. G. Pedersen and K. G. Symms, *Biochem. Pharmac.* **21**, 2269 (1972).
31. C. R. Jefcoate, R. Hume and G. S. Boyd, *Fedn Eur. Biochem. Soc. Lett.* **9**, 41 (1970).
32. S. Isaka and P. R. Hall, *Biochem. biophys. Res. Commun.* **43**, 747 (1971).
33. D. A. Haugen, T. A. van der Hoeven and M. J. Coon, *J. biol. Chem.* **250**, 3567 (1975).
34. P. E. Thomas, A. Y. H. Lu, D. Ryan, S. B. West, J. Kawalek and W. Levin, *J. biol. Chem.* **251**, 1385 (1976).