BBA 45873

STUDIES ON THE MECHANISM OF INHIBITION OF THE MITOCHONDRIAL ELECTRON TRANSPORT BY ANTIMYCIN

III. BINDING OF ANTIMYCIN TO SUB-MITOCHONDRIAL PARTICLES AND TO COMPLEX III

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(Received June 13th, 1969)

SUMMARY

1. Extraction by ether removes only about one-half of antimycin added to sub-mitochondrial particles, independently of the amount of antimycin added up to that necessary for 100% inhibition.

2. The amount of antimycin extractable with ether remains the same even when the antimycin is redistributed between an antimycin-inhibited preparation and an untreated. The antimycin remaining after ether extraction is redistributed between ether-accessible and ether-inaccessible sites when the preparation is incubated on its own or with an untreated preparation.

3. Low concentrations of cholate increase the extractability of the antimycin by ether.

4. Complex III binds antimycin more firmly than sub-mitochondrial particles. However, antimycin is readily extracted by ether, leading to restoration of enzymic activity and cleavage of the complex by bile salts.

5. The results are consistent with an explanation of the sigmoidal inhibition curve with antimycin and preparations of the intact respiratory chain in terms of an allosteric model.

INTRODUCTION

In the first paper of this series[1] (see also ref. 2) it was shown that inhibition by antimycin of the respiratory chain in sub-mitochondrial preparations can be reversed by extraction with ether. However, not all the antimycin bound to the preparation can be removed even by repeated extractions with ether. The residual antimycin may be released by a subsequent extraction with ethanol–ether. Consequently, an ether-extracted antimycin-treated preparation is more sensitive to a second treatment with antimycin than a preparation not treated with antimycin. It was suggested that ether removes antimycin bound to a site involved in the enzymic

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activity, whereas some inhibitor remains tightly bound at another non-catalytic site.

This conclusion has not, however, been supported by further studies. In particular, it was shown that ether extraction removes only about one-half of the antimycin, independently of the degree of inhibition before extraction[3,4]. Moreover, THORN[5] has pointed out that the sigmoidal curves obtained on plotting activity against enzyme concentration, in the presence of a fixed amount of antimycin, do not support the view that sub-inhibitory concentrations of antimycin are bound more firmly than inhibitory.

In the preceding paper[6], evidence is presented favouring the view that the sigmoidal inhibition curve of antimycin may be described by the allosteric model of MONOD *et al.*[7]. According to this model the intrinsic binding constants of an inhibitor to the different conformational states are independent of the concentration of the inhibitor. A second requirement of this model is that antimycin should be bound more firmly to the dissociated monomer than the oligomer (or polymer).

Because of the very high binding constant of antimycin, the concentration of unbound inhibitor is only a small fraction of the bound until maximal inhibition is reached. This makes it experimentally impossible to determine binding constants by the conventional methods of enzyme kinetics. Relative binding constants may, however, be determined by the method introduced by THORN[5] (see also refs. 3, 4), in which an antimycin-treated preparation is mixed with an antimycin-free preparation. This method has been used to test the requirements of the allosteric model, and to investigate some related phenomena.

EXPERIMENTS

Extraction by ether of antimycin from antimycin-treated sub-mitochondrial particles

Table I shows that extraction by ether releases only about one-half of the antimycin added to beef-heart sub-mitochondrial particles (the Keilin and Hartree heart-muscle preparation), independent of the amount of antimycin added up to that necessary for 100% inhibition. This suggests that amounts of antimycin in-

TABLE I

RELEASE OF ANTIMYCIN BOUND TO HEART-MUSCLE PREPARATION BY EXTRACTION WITH ETHER OR ETHER–ETHANOL

Expt. No.	*Heart-muscle preparation (mg)*	*Added antimycin (nmoles)*	*Inhibition of succinate oxidase (%)*	*Antimicin extracted by*			
				Ether		*Ether–ethanol*	
				nmoles	%	*nmoles*	%
1	73.5	15.3	0	6.5	42	13.7	90
		31.1	30	15.0	48	28.6	92
		32.7	98	13.8	42	31.2	96
2	51.6	8.8	0	5.1	58	8.4	96
		17.5	10	9.3	53	16.8	96
		23.8	100	12.0	50	23.8	100
3	50.5	3.1	0	1.5	49	3.0	97
		8.2	10	4.2	51	7.5	91
		14.1	90	7.7	54	13.2	94

sufficient to inhibit the respiratory chain are not bound more firmly than larger amounts.

This conclusion is shown more directly by the redistribution experiment shown in Table II. The amount of antimycin extractable with ether remains the same even when the antimycin is redistributed between an antimycin-inhibited and an untreated preparation, resulting in the inhibition declining from 90 % to 22 %.

Moreover, the antimycin remaining after ether extraction is redistributed when an ether-extracted antimycin-treated preparation is preincubated with an untreated preparation. About one-half of the antimycin is now extractable with ether (Table III). Even more significantly, allowing a suspension of the ether-ex-

TABLE II

EXTRACTION OF ANTIMYCIN AFTER PREINCUBATION OF HEART-MUSCLE PREPARATION TREATED WITH VARIOUS AMOUNTS OF ANTIMYCIN WITH UNTREATED PREPARATION

Heart-muscle preparation (2.5 ml containing 50.6 mg protein) was incubated with the indicated amounts of antimycin and then either extracted with ether or preincubated with 2.5 ml of the untreated preparation (P) for 5 min and then extracted with ether. A_0, A_{10} and A_{90} refer to preparations inhibited by 0, 10 and 90%, respectively.

Preparation	*Antimycin added (nmoles)*	*Inhibition of succinate oxidase (%)*	*Antimycin extracted*	
			nmoles	(%)
A_0	3.1	0	1.5	49
A_{10}	8.2	10	4.2	51
A_{90}	14.1	90	7.7	54
$A_0 + P$	3.1	0	1.4	45
$A_{10} + P$	8.2	0	3.5	42
$A_{90} + P$	14.1	22	7.3	51

TABLE III

RE-DISTRIBUTION OF ANTIMYCIN BETWEEN ETHER-EXTRACTED ANTIMYCIN-TREATED PREPARATION AND UNINHIBITED PREPARATION

Heart-muscle preparation (71 mg in Expt. 1 and 158 mg in Expt. 2) treated with 0.24 nmole antimycin per mg protein, was extracted with ether (preparation E). The amounts of antimycin remaining in E were determined by extraction with ether–ethanol (see ref. 1). The extractability with ether was determined after 5 min preincubation of E with an untreated preparation. 71 mg and 157 mg protein of preparations E in Expts. 1 and 2, respectively, were mixed with 131 mg and 158 mg protein of control preparations (P) in Expts. 1 and 2, respectively. The extractability with ether of E was determined either without (Expt. 2a) or after re-suspension in phosphate buffer and standing for 30 min at 0° before the repeated extraction with ether (Expts. 1 and 2b).

Expt. No.	*Antimycin extracted with*	*Amounts of antimyin extracted from*			
		E		*E + P*	
		nmoles	%	*nmoles*	%
1	Ether–ethanol	14.3	100	14.3	100
	Ether	8.4	58	7.4	51
2a	Ether–ethanol	18.8	100		
	Ether	2.4	13		
2b	Ether–ethanol	16.7	100	17.2	100
	Ether	9.5	56	9.5	55

tracted antimycin-treated preparation to stand resulted in it becoming possible, in time, to extract about 50% of the antimycin remaining after the first extraction (Table III, Expts. 1 and 2). Presumably, the antimycin is redistributed between the ether-accessible and ether-inaccessible sites during this preincubation.

The effect of previous treatment with antimycin followed by extraction with ether, which is to shift the inhibition curve to the left[3], is now largely understandable, since during the measurement of the enzymic activity, antimycin will be redistributed within the particles. The inhibition curves of ether-extracted antimycin-treated preparations should then be plotted with on the abcissa the total amount of antimycin present (both that retained after ether extraction and that subsequently added), rather than the added antimycin as was done previously. In Fig. 1, the previously published data (Fig. 1 of ref. 3) have been re-plotted in this way. The effect of treatment with antimycin, followed by ether extraction, although still present, is much less marked than previously appeared to be the case. The much lower antimycin sensitivity of the rat-liver mitochondria (Fig. 1B) than that of either heart-muscle preparation (Fig. 1A) or beef-heart mitochondria (unpublished results) is presumably due to the lower concentration of antimycin-binding sites in these mitochondria.

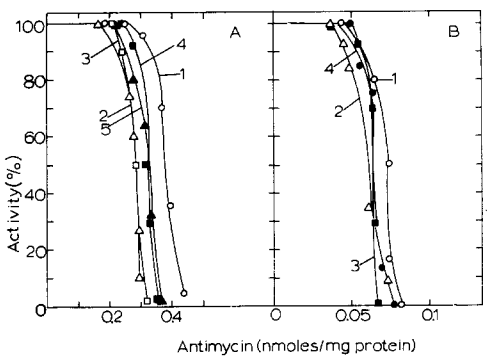

Fig. 1. Effect of antimycin on succinate oxidase activity of heart-muscle preparation (A) and rat-liver mitochondria (B) previously treated with various amounts of antimycin and then extracted with ether. A. Curve 1, untreated preparation; Curves 2, 3, 4 and 5, preparations previously treated with 0.13 nmole (no inhibition), 0.27 nmole (2% inhibition), 0.35 nmole (57% inhibition) and 0.44 nmole (97% inhibition) of antimycin per mg protein, respectively, and then extracted with ether. B. Curve 1, untreated mitochondria; Curves 2, 3, and 4, mitochondria previously treated with 0.04 nmole (no inhibition), 0.06 nmole (68% inhibition), and 0.10 nmole (100% inhibition) of antimycin per mg protein, respectively, and then extracted with ether. In calculating the amount of antimycin present, it was assumed that one-half of the antimycin initially added was not extracted by the ether.

The addition of cholate causes an increase in the amount of antimycin that can be extracted with ether to 80–95% (Tables IV and V). The amount of cholate giving this effect (1 mg/mg protein) is much smaller than that required to transform the sigmoidal inhibition curve into a linear (14 mg/mg protein)[5]. Also contrary to the effect on the inhibition curve, the increased extractability was largely retained after removal of much of the cholate by dialysis (Table V).

Binding of antimycin to Complex III

Table VI shows that untreated Complex III is able completely to reactivate antimycin-inhibited heart-muscle preparation, even in the presence of cholate. In-

TABLE IV

THE EFFECT OF CHOLATE ON THE RELEASE OF ANTIMYCIN BOUND TO THE HEART-MUSCLE PREPARATION BY EXTRACTION WITH ETHER

Cholate, where indicated, was added to concentrations of 13.3 and 13.9 mg/mg protein (46 and 53 mg/ml) in Expts. 1 and 2, respectively.

Expt. No.	*Heart-muscle preparation (mg)*	*Added antimycin (nmoles)*	*Inhibition of succinate–cytochrome c reductase (%)*	*Antimycin extracted*			
				No cholate		*With cholate*	
				nmoles	%	*nmoles*	%
1	113	40	93	17.8	45	36.4	81
		40	87	16.7	42	32.0	80
2	116	48	94	18.2	38	40.6	84

TABLE V

EXTRACTION OF ANTIMYCIN FROM CHOLATE-TREATED PREPARATIONS BEFORE AND AFTER DIALYSIS

Heart-muscle preparation, treated with 0.4 nmole antimycin per mg protein and various amounts of cholate, was extracted with ether, either before or after dialysis for 8 h.

Cholate		*Antimycin extracted*			
mg/mg protein	*mg/ml*	*Before dialysis*		*After dialysis*	
		nmoles	%	*nmoles*	%
0	0	2.7	44	—	—
1	6	5.1	88	—	—
2	12	4.9	79	—	—
4	22	5.5	89	—	—
7	35	5.9	95	—	—
14	61	5.9	95	5.1	83

activated Complex III is much less effective. Table VII shows that Complex III, on the other hand, does not donate its antimycin to heart-muscle preparation, even when the amount of antimycin bound to Complex III is twice as great as that necessary completely to inhibit the heart-muscle preparation. Even the cholate-treated preparation is unable to remove the antimycin from Complex III. Thus, the higher affinity of Complex III for antimycin is not solely due to the treatment with cholate during its preparation.

Heat-inactivated heart-muscle preparation also has a higher affinity for antimycin than a normal preparation (Table VIII).

Attempts by RIESKE *et al.*[8] to demonstrate reversal of antimycin inhibition of Complex III were unsuccessful and they concluded that antimycin is bound irreversibly to the complex. Although the antimycin could be removed by extraction with acetone, taurocholate or freezing in the presence of guanidine, these treatments caused complete loss of enzymic activity. Extraction with ether can be used to remove the antimycin firmly bound to Complex III without completely inactivating the

TABLE VI

REACTIVATION BY COMPLEX III OF ANTIMYCIN-INHIBITED HEART-MUSCLE PREPARATION

P, untreated heart-muscle preparation; P_A, heart-muscle preparation treated with 0.43 nmole antimycin per mg protein; C, complex III (containing 2.8 nmoles cytochrome c_1 per mg protein); C_I, Complex III inactivated by standing for 1 h at 40°. Before measurement of enzymic activity, 2.2 mg of P or P_A were mixed with 0.21 mg of C or C_I for 5 min at 0°. Samples containing 27 μg/ml P or P_A and/or 2.6 μg/ml C or C_I were used in the measurements. In Expt. B, P and P_A contained 13.6 mg cholate per mg protein.

Additions	*Succinate–cytochrome c reductase (μmoles cytochrome c per min)*	
	A. Without cholate	*B. With cholate*
P	0.228	0.204
P_A	0.021	0.009
C	0.021	0.021
P + C	0.303	0.212
P_A + C	0.273	0.159
C_I	0.003	0.003
P + C_I	0.249	0.192
P_A + C_I	0.090	0.096

TABLE VII

FAILURE OF ANTIMYCIN-INHIBITED COMPLEX III TO INHIBIT HEART-MUSCLE PREPARATION

P, heart-muscle preparation; C, Complex III (2.8 nmoles cytochrome c_1 per mg protein); C_A, Complex III treated with 2.8 nmoles antimycin per mg protein. Before measurement of enzymic activity, 2.2 mg of P (containing 0.9 nmole cytochrome c_1), or 1.1 mg were mixed with 0.31 mg of C or C_A (containing 0.9 nmole cytochrome c_1) for 5 min at 0°. Samples containing 27 μg/ml P and/or 3.8 μg/ml C or C_A were used in the measurements. In Expt. B, P contained 14 mg cholate per mg protein.

Additions	*Succinate–cytochrome c reductase (μmoles cytochrome c per min)*	
	A. Without cholate	*B. With cholate*
C	0.017	0.006
C_A	0.006	0.003
P	0.176	0.147
C + P (2.2 mg)	0.202	0.192
C_A + P (2.2 mg)	0.195	0.186
C + P (1.1 mg)	0.191	0.165
C_A + P (1.1 mg)	0.203	0.183

enzyme. Ether extraction inhibits the QH_2–cytochrome *c* reductase activity of Complex III by about one-half.

The slope of the inhibition curve was scarcely affected by extraction with ether (Fig. 2, Curve 2) or by treatment with antimycin followed by extraction with ether (Curve 3). Less antimycin was required after extraction with ether, presumably because the fraction of the Complex III inactivated by ether is unable to bind antimycin. Contrary to the experiments with heart-muscle preparations, previous treatment with antimycin followed by extraction with ether did not make the preparation more susceptible to the subsequent addition of antimycin.

TABLE VIII

TRANSLOCATION OF ANTIMYCIN FROM NORMAL PREPARATION TO HEAT-INACTIVATED

P, untreated heart-muscle preparation; P_A, preparation treated with 0.4 nmole antimycin per mg protein; P_I, preparation treated at 50° for 2 h; P_{AI}, antimycin-treated and heat-inactivated preparation; P_{IA}, heat-inactivated and antimycin-treated preparation.

Components	*Amounts of protein in the sample (mg)*	*Succinate oxidase activity*	
		μmoles succinate per min	%
P	0.8	252	100
P_A	0.8	38	15
P_I	0.8	56	22
P_{AI}	0.8	35	14
P_{IA}	0.8	46	18
P + P_A	0.4 + 0.4	230	92
P + P_I	0.8 + 0.8	271	108
P + P_{AI}	0.8 + 0.8	271	108
P + P_{IA}	0.8 + 0.8	271	108
P_I + P_A	0.8 + 0.8	136	54

TABLE IX

REVERSAL BY ETHER EXTRACTION OF INHIBITION BY ANTIMYCIN OF CLEAVAGE OF COMPLEX III

Complex III was extracted after 15-min preincubation with the indicated amounts of antimycin ("inhibited and extracted"). The control was neither treated with antimycin nor extracted with ether. "Inhibited" was treated with antimycin, without extraction with ether. "Extracted" was extracted with ether without treatment with antimycin. In Expt. 1, 12 mg Complex III, containing 35 nmoles cytochrome c_1, and 35 nmoles of antimycin were used; in Expt. 2, 16 mg Complex III (56 nmoles cytochrome c_1) and 54 nmoles antimycin; in Expt. 3, 9.4 mg Complex III (27.2 nmoles cytochrome c_1) and 26.5 nmoles antimycin. Cleavage was measured as described in METHODS on 0.1-ml samples.

Expt. No.	*Complex III*	*Antimycin extracted (%)*	*Cytochrome b precipitated**	
			nmoles	%**
1	Control		64	97
	Inhibited		8	12
	Extracted		57	86
	Inhibited and extracted	87	52	79
2	Control		94	86
	Inhibited		14	12
	Extracted		84	75
	Inhibited and extracted	85	76	69
3	Control		120	88
	Inhibited		17	12
	Extracted		114	84
	Inhibited and extracted	64***	48	35

* Calculated for total amount of Complex III used.

** The amount of cytochrome *b* estimated by direct spectrophotometry (see METHODS) was assumed as a 100%.

*** Extracted only once with ether.

Extraction with ether has little effect on the cleavage by taurocholate of Complex III and reverses the inhibitory effect of antimycin on the cleavage (Table IX). Almost complete reversal was obtained when 85–87 % of the antimycin was extracted.

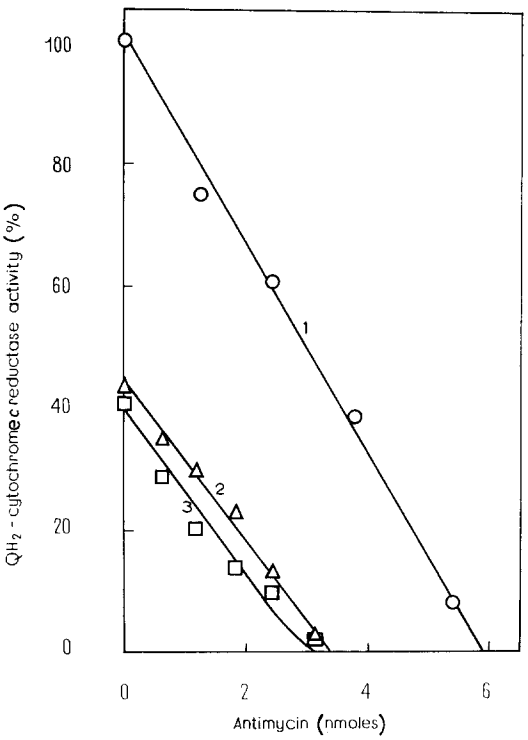

Fig. 2. The effect of antimycin on the QH_2–cytochrome *c* reductase activity of Complex III extracted in the absence of and after preincubation with antimycin. Curve 1, untreated Complex III (20.4 mg protein containing 56 nmoles cytochrome c_1); Curve 2, Complex III extracted in the absence of antimycin; Curve 3, Complex III treated with antimycin (0.9 mole/mole cytochrome c_1) and extracted with ether. Samples were treated with indicated amounts of antimycin (calculated for the total amount of Complex III used), incubated for 15 min at 0° and assayed for enzymic activity. The QH_2–cytochrome *c* reductase activity of the control was 24.2 nmoles of cytochrome *c* reduced per min per mg protein.

TABLE X

RELEASE OF ANTIMYCIN BOUND TO COMPLEX III BY EXTRACTION WITH ETHER OR ETHER–ETHANOL

6.2 mg (containing 18.8 nmoles cytochrome c_1 in 0.5 ml), 10.2 mg (containing 26.4 nmoles cytochrome c_1 in 0.7 ml), and 16.7 mg (containing 56 nmoles cytochrome c_1 in 1 ml) Complex III were extracted in Expts. 1, 2 and 3, respectively.

Expt. No.	*Added antimycin (nmoles)*	*Inhibition of cleavage (%)*	*Antimycin extracted by*			
			Ether		*Ether–ethanol*	
			nmoles	%	*nmoles*	%
1	17.5	90	14.0	80	15.4	88
2	24.5	87	21.4	87	21.6	88
3	51.4	88	43.2	84	47.4	92

but only slight reversal was obtained when 64 % was extracted. This is in agreement with RIESKE *et al.*[8] who found that removal of 70 % of the antimycin by acetone extraction did not restore the cleavage reaction.

Contrary to the experience with heart-muscle preparation, ether extraction removes nearly all the antimycin (Table X). In fact, ether extraction was as effective as a mixture of ethanol and ether which removes all the antimycin from the heart-muscle preparation[1].

DISCUSSION

In accordance with the predictions made on the basis of the allosteric model, low concentrations of antimycin are not bound more firmly than high, and antimycin is bound more firmly to Complex III, which may be considered the monomer, than to particulate preparations, even in the presence of cholate. On the other hand, ether is more effective in removing the antimycin from Complex III than from particulate preparations. Thus, the relative case of extraction by ether is not a satisfactory measure of the relative strength of binding.

The reason for the incomplete extractability by ether of antimycin bound to sub-mitochondrial particles is not known. It appears to be a property of the highly aggregated state, since the addition of very low concentrations of cholate, insufficient to affect the sigmoidal titration curve, causes most of the antimycin to be extracted.

METHODS

Beef heart-muscle preparation was made by the method of KEILIN AND HARTREE[9] or by SLATER'S[10] modification. In all experiments in which the preparations were extracted with ether, the particles were suspended in 0.1 M phosphate buffer (pH 7.4). In some other experiments Complex III (QH_2–cytochrome *c* reductase) was prepared from bovine heart-muscle particles by the method of RIESKE *et al.*[11], except that 0.5 mg cholate instead of 0.25 mg per mg protein was used. The contents of cytochromes *b* and c_1 were determined spectrophotometrically after successive reductions by ascorbate and dithionite, as described by RIESKE[12]. Cleavage was carried out with taurocholate and ammonium sulphate as described by RIESKE *et al.*[13], except that it was done at 20° instead of 0°. The cytochrome *b* precipitated after cleavage was determined spectrophotometrically after conversion to the pyridine haemochrome as described by ZAUGG AND RIESKE[14].

Extraction of antimycin from particles with ethanol and ether–ethanol was carried out as described previously[1,3]. Diethyl ether of analytical or anaesthetic grade was used. The amount of antimycin extracted was measured by determining the amount required for 50 % inhibition of succinate–cytochrome *c* reductase in heart-muscle preparation, as described by BRYŁA AND KANIUGA[3].

Extraction of antimycin from Complex III was carried out at 0° once for 15 min and twice for 5 min with 50 vol. ether saturated with water.

Other methods are described in the previous paper[6].

ACKNOWLEDGEMENTS

We wish to thank Dr. D. E. Green and Merck, Sharp and Dohme for a generous gift of Q-2 and Mrs. A. E. Heggelund-Lem and Miss H. Ksiezak for assistance in some experiments.

This work was supported in part by grants (to J.B.) from the International Cell Research Organization and the European Molecular Biology Organization and (to E.C.S.) from the Life Insurance Medical Research Fund and from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.) under the auspices of the Netherlands Foundation for Chemical Research (S.O.N.).

REFERENCES

1 Z. KANIUGA, A. GARDAS AND J. BRYŁA, *Biochim. Biophys. Acta*, 153 (1968) 60.
2 Z. KANIUGA, J. BRYŁA, A. GARDAS AND I. CHMIELEWSKA, *Abstr. 3rd Meeting Federation European Biochem. Socs., Warsaw, 1966*, Academic Press and Polish Scientific Publishers, London and Warsaw, 1966, p. 140.
3 J. BRYŁA AND Z. KANIUGA, *Biochim. Biophys. Acta*, 153 (1968) 910.
4 Z. KANIUGA AND J. BRYŁA, *Abstr. 5th Meeting Federation European Biochem. Socs., Prague, 1968*, The Czechoslovak Biochemical Societies, Prague, 1968, p. 293.
5 M. B. THORN, *Biochem. J.*, 63 (1956) 420.
6 J. BRYŁA, Z. KANIUGA AND E. C. SLATER, *Biochim. Biophys. Acta*, 189 (1969) 317.
7 J. MONOD, J. WYMAN AND J. P. CHANGEUX, *J. Mol. Biol.*, 12 (1965) 88.
8 J. S. RIESKE, S. H. LIPTON, H. BAUM AND H. I. SILMAN, *J. Biol. Chem.*, 242 (1967) 4888.
9 D. KEILIN AND E. F. HARTREE, *Biochem. J.*, 41 (1947) 500.
10 E. C. SLATER, *Symp. Soc. Exptl. Biol.*, 10 (1957) 110.
11 J. S. RIESKE, W. S. ZAUGG AND R. E. HANSEN, *J. Biol. Chem.*, 239 (1964) 3023.
12 J. S. RIESKE, in R. W. ESTABROOK AND M. E. PULLMAN, *Methods in Enzymology*, Vol. 10, Academic Press, New York, 1967, p. 488.
13 J. S. RIESKE, H. BAUM, C. D. STONER AND S. H. LIPTON, *J. Biol. Chem.*, 242 (1967) 4854.
14 W. S. ZAUGG AND J. S. RIESKE, *Biochem. Biophys. Res. Commun.*, 9 (1962) 213.