Biochemical Pnarmacology, Vol. 18, pp. 749–756. Pergsmon Press. 1969. Printed in Great Britain

EFFECTS OF DEOXYFRENOLICIN ON ISOLATED ADIPOSE CELLS—I

GLUCOSE AND FRUCTOSE UTILIZATION

J. F. KUO,* I. K. DILL and C. E. HOLMLUND†

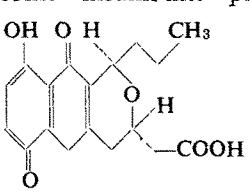
Lederle Laboratories, American Cyanamid Company,
Pearl River, N.Y. 10965, U.S.A.

(*Received* 18 *July* 1968; *accepted* 25 *September* 1968)

Abstract—Deoxyfrenolicin, at an optimal concentration of about 15 to 20 μg/ml, stimulated to a greater extent than did insulin the oxidation of glucose and fructose, presumably via the pentose cycle, by isolated adipose cells. Stimulation of glucose uptake and lipogenesis from glucose and fructose were lower than by insulin, however. At a higher concentration (60 μg/ml) of deoxyfrenolicin, the stimulatory effects were reduced or abolished. Deoxyfrenolicin, at a suboptimal concentration (10·5 μg/ml), stimulated still further the maximally stimulated lipogenesis from glucose and fructose in response to insulin or several "insulin-like" proteases.

It is suggested that deoxyfrenolicin stimulated sugar utilization in isolated adipose cells, at least in part, through a mechanism different from the actions of insulin or proteases.

RECENTLY, numerous substances other than peptide hormones have been reported to mimic insulin effects in adipose cells or adipose tissue. These include inorganic sulfhydryl agents such as $NaAsO_2$[1, 2] and $CdCl_2$;[1] organic compounds such as *p*-chloromercuribenzoate,[1] ouabain[3, 4] and polyene antibiotics;[5] and hydrolytic enzymes such as phospholipases,[6–9] trypsin and chymotrypsin,[10, 11] and microbial proteases.[11–13] Deoxyfrenolicin, an analog of frenolicin, an antibiotic produced by *Streptomyces fradiae* with a novel naphthoquinone epoxide structure,[14, 15] was found to mimic insulin effects on isolated adipose cells. This report consists of the effects of deoxyfrenolicin, as compared with some "insulin-like" proteases, on sugar utilization. An



Deoxyfrenolicin

accompanying report[16] deals with its effects on the hormone-induced lipolysis and the adenosine 3′,5′-monophosphate levels in adipose cells.

MATERIALS AND METHODS

Collagenase was purchased from Worthington; norepinephrine and α-chymotrypsin (crystalline) from Calbiochem; insulin (24 i.u./mg), corticotropin (121 units/mg),

* Present address: Department of Pharmacology, Yale University School of Medicine, New Haven, Conn. 06510, U.S.A.

† Present address: Department of Chemistry, University of Maryland, College Park, Md. 20740, U.S.A.

S. griseus protease, *B. subtilis* protease (crystalline) and caffeine from Sigma; glucose-U-^{14}C (0·5 mc/m-mole) and fructose-U-^{14}C (0·5 mc/0·5 mg) from New England Nuclear; bovine serum albumin (Fraction V) from Armour; and theophylline, from Mann. Avenaciolide was a kind gift of Dr. F. H. Stodola of Northern Utilization Research and Development Division, U.S. Department of Agriculture.

The epididymal fat pads used in the preparation of isolated adipose cells for this study were obtained from male Sprague–Dawley rats (120–150 g) that had been fed Purina chow. The procedures for preparing and incubating isolated adipose cells, for determining the incorporation of ^{14}C into CO_2, total lipid and fatty acid were essentially the same as described by Rodbell,[17] with some modifications.[11, 13] ^{14}C-labeled glycogen was determined by the method of Gutman *et al.*[18] Glucose in the medium was measured, without deproteinization, by the glucose oxidase procedure.[19] Unless otherwise specified, the incubation mixture consisted of 1 ml of Krebs–Ringer bicarbonate buffer, pH 7·4, containing 4% dialyzed bovine serum albumin (hereinafter referred to as the albumin–bicarbonate medium), free adipocytes ranging from 40 to 55 mg, and a specified amount of radioactive substrate, as indicated. Each treatment was incubated in triplicate or quadruplicate and each experiment was performed two to four times to insure its reproducibility. The data from one representative experiment are reported. The gas phase was 95% O_2–5% CO_2 (v/v).

RESULTS

As shown in Fig. 1, deoxyfrenolicin at an optimum concentration (about 20 μg/ml) stimulated CO_2 production from glucose-U-^{14}C (panel A) and fructose-U-^{14}C (panel B) to a greater degree than did insulin (1000 microunits/ml); 820 and 280 per cent of the basal were observed from glucose and fructose respectively, compared with 690 per cent and 260 per cent, respectively, with insulin. Lipogenesis from glucose-U-^{14}C (panel C) and fructose-U-^{14}C (panel D), however, was stimulated more by insulin (620 per cent and 240 per cent respectively) than by deoxyfrenolicin (400 per cent and 160 per cent respectively). As reported for several proteases that mimic insulin effects *in vitro*,[10–13] a sharp reduction in stimulation was observed with a high concentration of deoxyfrenolicin (60 μg/ml).

The effects of deoxyfrenolicin on glucose uptake were also studied (Table 1). Like insulin, deoxyfrenolicin stimulated glucose uptake. The optimal concentration of deoxyfrenolicin in eliciting stimulation coincided with the value illustrated in Fig. 1.

The effects of 3-*o*-methylglucose, an inhibitor of sugar transport,[8] and L-glucose on the utilization of glucose-U-^{14}C, stimulated by deoxyfrenolicin, are presented in Table 2. In contrast to 3-*o*-methylglucose, which inhibited both the basal and the deoxyfrenolicin-stimulated processes, L-glucose exerted no effect on either process. It is concluded, therefore, that deoxyfrenolicin stimulated glucose utilization at least in part via an enhanced stereospecific transport process. The same conclusion has been reported for the stimulatory effects of insulin,[8] phospholipase C,[8] and *B. subtilis* protease.[12]

It was desirable to compare the effects of deoxyfrenolicin with insulin and protease on the fate of isotope from glucose-1-^{14}C and glucose-U-^{14}C. It was found that about 75 per cent of the radioactive CO_2 (panel A, Fig. 2) produced by incubated adipocytes in the presence and absence of insulin or *B. subtilis* protease originated from C_1 of glucose. However, the radioactive CO_2 produced in response to deoxyfrenolicin

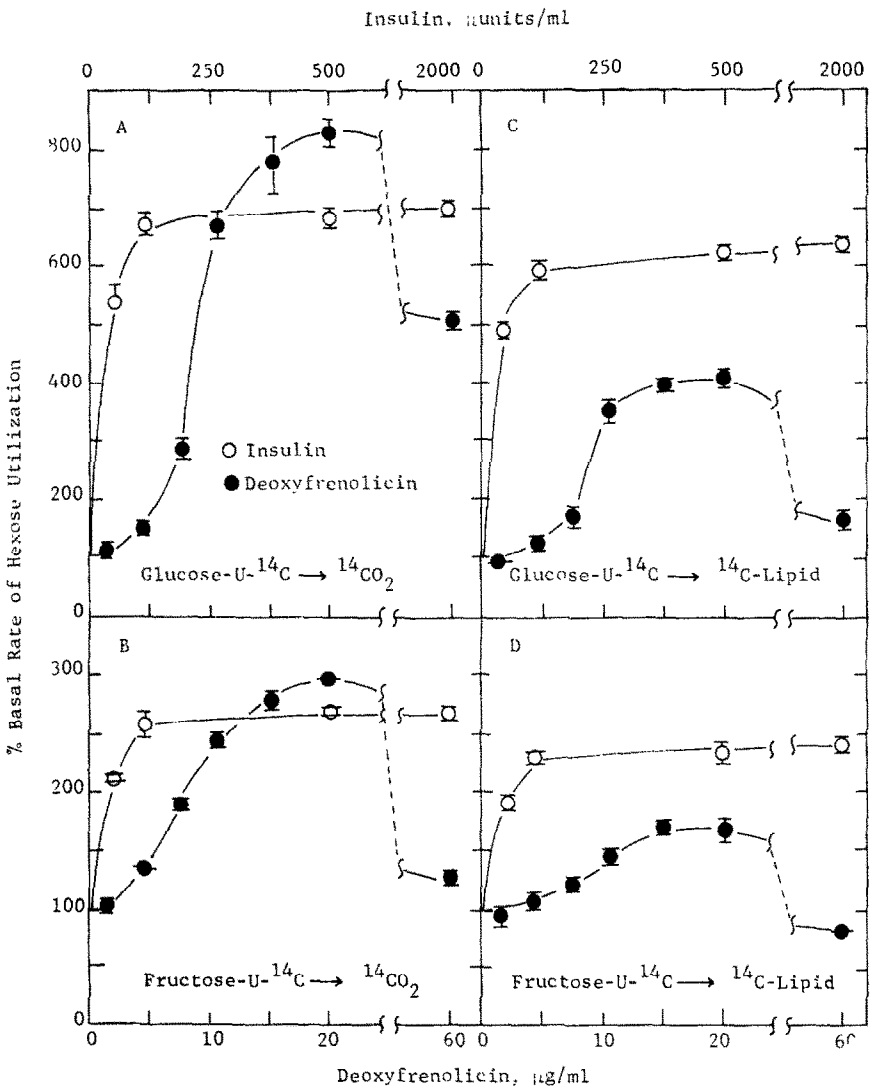


FIG. 1. Comparison of the effects of deoxyfrenolicin and insulin on the utilization of glucose-U-^{14}C and fructose-U-^{14}C by isolated adipose cells. Free adipocytes were incubated for 2 hr in 1 ml of albumin-bicarbonate medium with either 0·1 μc (1 μmole) glucose-U-^{14}C or 0·5 μc (20 μmoles) fructose-U-^{14}C and varying concentrations of insulin and deoxyfrenolicin, as indicated. The basal rate of conversion of glucose to CO_2 (panel A) and total lipid (panel C), expressed as μmoles per g cells per 2 hr, were 0·37 and 0·58, respectively, and conversion of fructose to CO_2 (B panel) and total lipid (panel D) were 0·83 and 1·41 respectively. Each treatment was performed in quadruplicate and the means (± S. E., expressed as vertical bars) are presented.

was found to originate exclusively from C_1, with a rate exceeding 36 per cent of that of glucose-1-^{14}C oxidation stimulated by insulin. Compared with insulin and *B. subtilis* protease, the degree of stimulation of fatty synthesis (panel B, Fig. 2) from glucose-1-^{14}C by deoxyfrenolicin was considerably lower than from glucose-U-^{14}C.

The effects of deoxyfrenolicin on the stimulatory actions of insulin and several proteases with regard to the utilization of glucose-U-^{14}C were examined. As shown in Fig. 3, the data indicate that deoxyfrenolicin at 10·5 μg/ml, a concentration that gave about 4·6-fold stimulation of lipogenesis from glucose-U-^{14}C, augmented by 8, 34, 64 and 59 per cent the already maximally elevated lipogenesis in response to insulin, *B. subtilis* protease, *S. griseus* protease and α-chymotrypsin respectively.

TABLE 1. STIMULATION OF GLUCOSE UPTAKE BY INSULIN AND DEOXYFRENOLICIN IN ISOLATED ADIPOSE CELLS*

Additives	Glucose uptake (μmoles/g cells/2 hr)
None (basal)	2·51 ± 0·08
Insulin (2000 μunits/ml)	7·96 ± 0·08
Deoxyfrenolicin (10 μg/ml)	4·10 ± 0·29
(20 μg/ml)	5·62 ± 0·05
(60 μg/ml)	3·95 ± 0·08

* Free adipocytes were incubated for 2 hr in 1 ml of albumin–bicarbonate medium containing 1 μmole of unlabeled glucose. Each treatment was performed in triplicate and the means (± S. E.) are presented.

TABLE 2. EFFECTS OF 3-*O*-METHYLGLUCOSE AND L-GLUCOSE ON THE DEOXYFRENOLICIN-STIMULATED UTILIZATION OF GLUCOSE BY ISOLATED ADIPOSE CELLS*

Additives		Conversion of glucose-U-^{14}C (μmoles/g cells/2 hr)		
		Control	3-*O*-methylglucose (15 mM	L-glucose (15 mM)
None	CO_2	0·47 ± 0·06	0·20 ± 0·02	0·47 ± 0·04
	Lipid	0·70 ± 0·02	0·31 ± 0·02	0·70 ± 0·04
Deoxyfrenolicin (15 μg/ml)	CO_2	2·27 ± 0·08	1·40 ± 0·03	2·22 ± 0·04
	Lipid	2·00 ± 0·08	0·99 ± 0·07	1·96 ± 0·03

* Free adipocytes were incubated in the usual manner with 0·1 μc (1 μmole) glucose-U-^{14}C per ml and the agents, as indicated. Each treatment was performed in triplicate and the means (± S. E.) are presented.

Deoxyfrenolicin at 30 μg/ml, a concentration that reduced by 23 per cent the enhanced sugar utilization achieved by it at 10·5 μg/ml and still exhibited a 3·6-fold stimulation, inhibited by 33, 13, 6 and 10 per cent respectively, the elevated lipogenesis in response to insulin and the aforementioned proteases. It was also found that the effects of deoxyfrenolicin on the basal and the insulin- or protease-stimulated lipogenesis from fructose-U-^{14}C were qualitatively similar to those observed with glucose, as shown above.

It was reported previously that avenaciolide, and antifungal lactone, inhibited the insulin- or protease-stimulated utilization of glucose[20] and fructose[21] without affecting the basal process. In the present study, it was also found that avenaciolide inhibited the deoxyfrenolicin-stimulated oxidation of glucose and fructose (Table 3).

Since deoxyfrenolicin exceeded insulin in its effects on stimulating glucose oxidation (Figs. 1, 2 and Table 3), it was desirable to know whether it also elicits a higher oxidation of ^{14}C-labeled cellular components in incubated adipocytes. The data

presented in Table 4 show that this is the case. Insulin was unable to stimulate oxidation. In contrast to insulin, which blocked puromycin- or norepinephrine-induced glycogen breakdown without significantly affecting the basal rate, deoxyfrenolicin not only accelerated the basal rate of glycogenolysis but also enhanced still further the elevated glycogenolysis in response to puromycin and norepinephrine. Ouabain

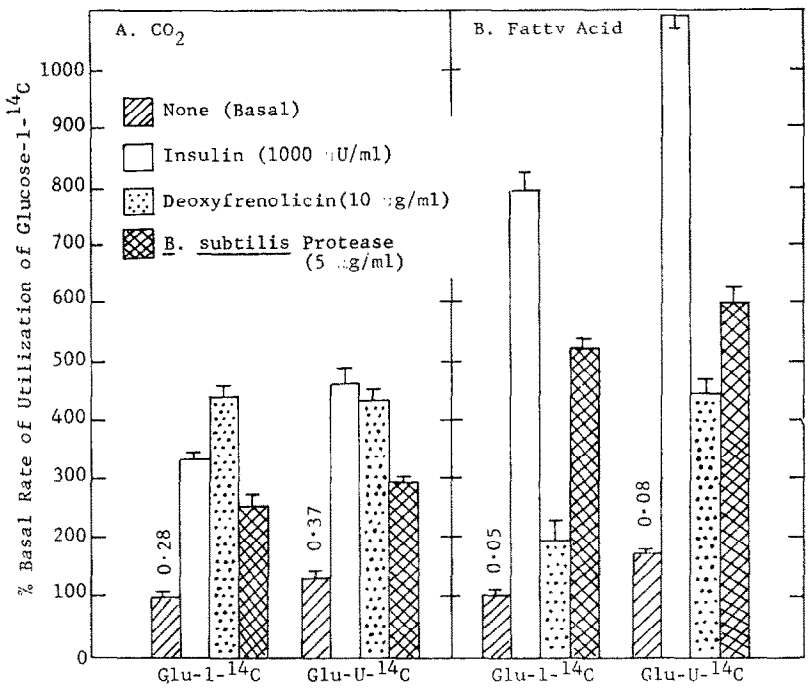


FIG. 2. Comparison of the effects of deoxyfrenolicin, insulin and *B. subtilis* protease on the utilization of glucose-U-^{14}C and glucose-1-^{14}C. Experimental conditions were essentially the same as for Fig. 1. The amount of radioactive glucose was, in each instance, 0·1 μc (1 μmole) per ml of incubation medium. The values indicated in the figure refer to the actual rate of conversion of glucose carbon to the indicated metabolites, expressed as μmoles per g cells per 2 hr. Each treatment was performed in triplicate and the means (± S. E., expressed as vertical bars) are presented.

has also been reported to exhibit a similar effects.[3] The stimulatory effects of puromycin[18] and norepinephrine[3] on glycogenolysis have been reported elsewhere.

DISCUSSION

The obvious differences between the effects of deoxyfrenolicin and the effects of insulin and proteases on isolated adipose cells include the following: (1) deoxyfrenolicin exceeded insulin and proteases in its effects on stimulating sugar oxidation via the pentose cycle but was less stimulatory to sugar uptake and lipogenesis therefrom; (2) deoxyfrenolicin stimulated oxidation of cellular materials whereas insulin did not; and (3) deoxyfrenolicin accelerated the basal as well as the puromycin- or norepinephrine-induced glycogenolysis, whereas insulin blocked the effects of puromycin and norepinephrine.

TABLE 3. EFFECTS OF AVENACIOLINE ON HEXOSE OXIDATION BY ISOLATED ADIPOSE CELLS INCUBATED WITH OR WITHOUT INSULIN AND DEOXYFRENOLICIN*

Additives	Avenaciolide (50 μg/ml)	Hexose-U-^{14}C → $^{14}CO_2$ (μmoles/g cells/2 hr)	
		Glucose-U-^{14}C	Fructose-U-^{14}C
None (basal)	−	0·23 ± 0·01	0·38 ± 0·01
	+	0·26 ± 0·03	0·42 ± 0·02
Insulin (300 μunits/ml)	−	0·86 ± 0·01	0·83 ± 0·01
	+	0·36 ± 0·01	0·46 ± 0·03
Deoxyfrenolicin (15 μg/ml)	−	1·22 ± 0·02	0·99 ± 0·04
	+	0·33 ± 0·02	0·40 ± 0·02

* Free adipocytes were incubated for 2 hr in 1 ml of medium with either 0·1 μc (1 μmole) glucose-U-^{14}C or 0·5 μc (20 μmoles) fructose-U-^{14}C in the presence or absence of insulin and deoxyfrenolicin, as indicated. Each treatment was performed in triplicate and the means (± S. E.) are presented.

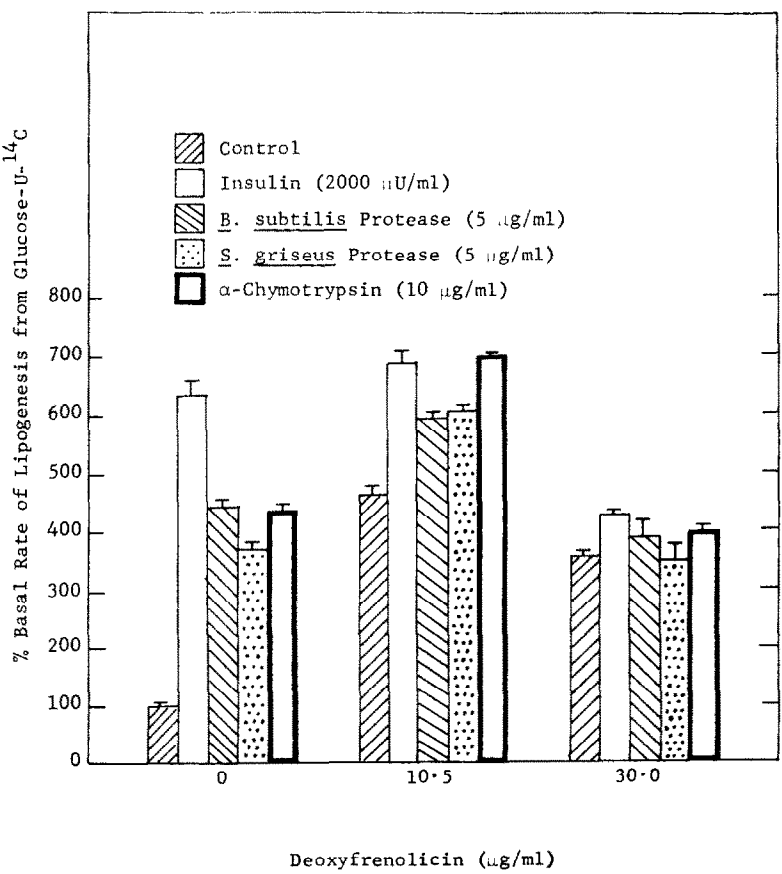


FIG. 3. Effects of deoxyfrenolicin on lipogenesis from glucose-U-^{14}C by isolated adipose cells incubated in the presence and absence of insulin and several proteases. Free adipocytes were incubated in 1 ml of medium for 2 hr in the presence of additives, as indicated. The basal (control without additives) rate of lipogenesis from 0·1 μc (1 μmole) glucose-U-^{14}C was 0·54 μmole/g cells per 2 hr. Each treatment was performed in triplicate and the means (± S. E., expressed as vertical bars) are presented.

It is also of interest to note that deoxyfrenolicin, at a suboptimal concentration of 10·5 μg/ml, enhanced still further the lipogenesis from glucose and fructose which was already maximally stimulated by insulin or by some proteases respectively. The rather small elevation of the insulin-stimulated process by deoxyfrenolicin may be explained if one assumes that insulin has already saturated the transport systems or

TABLE 4. EFFECTS OF DEOXYFRENOLICIN AND INSULIN ON $^{14}CO_2$ PRODUCTION AND DISAPPEARANCE OF ^{14}C-GLYCOGEN IN ISOLATED ADIPOSE CELLS INCUBATED WITH OR WITHOUT PUROMYCIN AND NOREPINEPHRINE*

Additives	Radioactivity (cpm/52 mg cells/hr)					
	Carbon dioxide produced			Glycogen remained		
	Control	Deoxyfrenolicin	Insulin	Control	Deoxyfrenolicin	Insulin
None	1270 ± 104	2424 ± 109	1409 ± 5	479 ± 12	225 ± 25	523 ± 28
Puromycin (130 μg/ml)	1330 ± 81	2369 ± 39	1301 ± 33	317 ± 7	213 ± 21	420 ± 44
Norepinephrine (0·1 μg/ml)	1476 ± 34	2331 ± 16	1448 ± 61	265 ± 4	207 ± 15	366 ± 24

* About 4 g of fat pads was digested for 40 min with collagenase in 7 ml of medium containing 40 μc (20 μmoles) glucose-U-^{14}C. The dispersed adipocytes were washed with the glucose-free medium and were suspended in the same medium. The ^{14}C-labeled adipocytes were incubated for 1 hr in the same medium with various agents, as indicated. When presented, the amounts of deoxyfrenolicin and insulin were 10 μg and 1000 μunits/ml respectively. Each treatment was performed in triplicate and the means (± S. E.) are presented.

hexose kinase or both with sugar, and thence such a "supplementary" action of deoxyfrenolicin could no longer affect the process as whole. Nevertheless, the additive effect seems to indicate further that deoxyfrenolicin may stimulate sugar utilization in isolated adipose cells by a mechanism different from the actions of insulin and proteases.

There are no reports regarding either the mechanism of action of deoxyfrenolicin as an antifungal agent or the effects of the antibiotic on the normal metabolic pathway of animal tissues. However, it is clear from the data and the accompanying paper[17] that deoxyfrenolicin influenced adipocyte metabolism by acting at some specific point(s) within the cells rather than at the membrane.

The mechanism by which deoxyfrenolicin stimulated sugar utilization, inhibited lipolysis,[16] and unexpectedly elevated intracellular levels of adenosine 3′,5′-monophosphate in adipose cells[16] should be investigated.

Acknowledgement—The authors are grateful to Dr. G. A. Ellestad of these laboratories for the pure preparation of deoxyfrenolicin.

REFERENCES

1. E. G. BALL and R. L. JUNGAS, *Recent Prog. Horm. Res.* **20**, 183 (1964).
2. J. F. KUO, I. K. DILL and C. E. HOLMLUND, *Biochim. biophys. Acta* **148**, 683 (1967).
3. R. J. HO and B. JEANRENAUD, *Biochim. biophys. Acta* **144**, 61 (1967).
4. R. J. HO, B. JEANRENAUD, TH. POSTERNAK and A. E. RENOLD, *Biochim. biophys. Acta* **144**, 74 (1967).

5. J. F. KUO, *Archs Biochem. Biophys.*, **127**, 406 (1968).
6. M. BLECHER, *Biochem. biophys. Res. Commun.* **21**, 202 (1965).
7. M. BLECHER, *Biochem. biophys. Res. Commun.* **23**, 68 (1966).
8. M. RODBELL, *J. biol. Chem.* **241**, 130 (1966).
9. M. RODBELL and A. B. JONES, *J. biol. Chem.* **241**, 140 (1966).
10. J. F. KUO, C. E. HOLMLUND and I. K. DILL, *Life Sci.* **5**, 2257 (1966).
11. J. F. KUO, C. E. HOLMLUND, I. K. DILL and N. BOHONOS, *Archs Biochem. Biophys.* **117**, 269 (1966).
12. J. F. KUO, I. K. DILL and C. E. HOLMLUND, *J. biol. Chem.* **242**, 3659 (1967).
13. J. F. KUO *J. biol. Chem.* **243** 211 (1968).
14. J. C. VAN METER M. DANN and N. BOHONOS, in *Antrimicrobial Agents Annual*—1960 p. 77. Plenum, New York (1961).
15. G. A. ELLESTAD H. A. WHALEY and E. L. PATTERSON, *J. Am. chem. Soc.* **88**, 4109 (1966).
16. J. F. KUO, *Biochem. Pharmac.* **18**, 757 (1969).
17. M. RODBELL, *J. biol. Chem.* **239**, 375 (1964).
18. A. GUTMAN, A. P. AUTOR and W. S. LYNN, *Biochim. biophys. Acta* **130**, 19 (1966).
19. H. U. BERGMEYER and E. BERNT, in *Methods of Enzymatic Analysis*, (Ed. H. U. BERGMEYER), p. 123. Academic Press, New York (1963).
20. J. F. KUO, I. K. DILL, C. E. HOLMLUND and N. BOHONOS, *Biochem. Pharmac.* **17**, 345 (1968).
21. J. F. KUO, *Biochim. biophys. Acta* **165**, 208 (1968).