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SOME EFFECTS OF 3-CHLOROISOXAZOLE-5-CARBOXYLIC ACID ON LIPID AND CARBOHYDRATE METABOLISM

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Abstract—The effect of 3-chloroisoxazole-5-carboxylic acid (CIC) on lipid and carbohydrate metabolism has been investigated. CIC lowered plasma NEFA levels in fasted glucose-primed rats and was significantly more active than 5-methylpyrazole-3-carboxylic acid (MPC). Both CIC and MPC had hypoglycaemic activity. With both CIC and MPC the fall in plasma NEFA always preceded the fall in blood sugar levels. CIC and MPC reduced the blood sugar and plasma NEFA in alloxan diabetic rats. CIC and MPC inhibited the basal and adrenaline and growth hormone stimulated NEFA release from *in vitro* rat epididymal fat pads. This CIC inhibition of growth hormone stimulated NEFA release was confirmed in intact rabbits. It seems that both MPC and CIC inhibit triglyceride breakdown in adipose tissue and that the effect upon blood glucose levels is probably secondary to the effect on fat metabolism.

THE HYPOGLYCAEMIC and non-esterified fatty acid (NEFA) lowering effects of 3,5-dimethylpyrazole (DMP)[1, 2] and 5-methylpyrazole-3-carboxylic acid (MPC)[3, 4] and the corresponding isoxazoles[5, 6] have recently been described. Similar activity has also been found with 3-chloroisoxazole-5-carboxylic acid (CIC). We have also studied the effects of all these compounds on the release of NEFA from unstimulated and adrenaline and growth hormone stimulated rat epididymal fat pads *in vitro* and have further investigated the effect of CIC on growth hormone stimulated increases in circulating NEFA in rabbits.

EXPERIMENTAL

Animals. Rats of a Sprague–Dawley (Charles River C.U.B.J.) strain of from 150 to 250 g, but within a 20 g range for any one experiment, and male New Zealand White rabbits of 1·5–2·5 kg were used.

Materials. These were 3,5-dimethylpyrazole, 5-methylpyrazole-3-carboxylic acid and 3-chloroisoxazole-5-carboxylic acid (prepared by Drs. K. Bowden and W. J. Ross); l-adrenaline hydrochloride (B.D.H.); growth hormone (porcine) (Sigma), alloxan (B.D.H.); insulin B.P. (Burroughs Wellcome), and bovine serum albumin (Fraction V) (Armour).

In vivo *NEFA lowering and hypoglycaemic activity*

In groups of eight female rats, food was withdrawn 18 hr before the subcutaneous injection of 1 g/kg of glucose and this was followed immediately by oral administration of drug or tap water. The compounds were dissolved in tap water and dose vo

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were adjusted to 0·1 ml/100 g body wt. Two hours later blood samples were taken by cardiac puncture under CO_2 anaesthesia. Blood sugar levels of all samples were determined by the ferricyanide method using the Autoanalyser (Technicon), and plasma NEFA levels were estimated on 4 samples from each group by the method of Trout, Estes and Friedberg.[7] Diabetic rats were used in one experiment. Rats were fasted for 24 hr and an experimental diabetes was produced by the subcutaneous injection of 1/4 U/kg insulin followed ½ hr later by 75 mg/kg of alloxan given subcutaneously. Approximately 75 per cent of the animals became diabetic and those having blood sugar greater than 350 mg % were used 4 weeks later.

In vitro *rat hemidiaphragm experiments*

The method used was that of Vallance-Owen and Hurlock.[8]

In vitro *rat epididymal fat pad experiments*

Whole fat pads from rats fasted for 18 hr were used in these experiments.

Initial results indicated that epididymal fat pads from one animal released similar amounts of NEFA, but that there was a wide variation between animals.

In experiments on unstimulated pads, one pad from each of 5 animals was incubated in Krebs–Henseleit bicarbonate buffer containing 2% bovine serum albumin (Fraction V) and 100 mg/100 ml glucose for 2 hr at 37·5° in an atmosphere of 95 per cent O_2/5 per cent CO_2 with constant shaking. The other pad from each animal was incubated as above but with varying concentrations of drug added to the medium.

In experiments on stimulated fat pads, incubations were carried out as described above but with the addition of the stimulant of NEFA release (adrenaline or growth hormone) to the incubation medium for one pad of each of 5 animals, and the addition of stimulant plus drug to the medium for the other pad. For each of these experiments controls were set up to compare NEFA release from unstimulated pads with those stimulated with adrenaline or growth hormone. This served to check the activity of the stimulant from day to day.

In vivo *growth hormone experiments*

Rabbits were fasted for 18 hr. Blood samples were taken from one marginal ear vein and intravenous injections were given into the marginal vein of the other ear. Drug or saline was given 30 min before the injection of growth hormone or saline, and blood samples were taken immediately prior to each of these injections and also 30 min after the second injections. Blood sugar and plasma NEFA concentrations were measured as described previously.

Toxicity tests

Oral LD_{50}'s were obtained in male mice (18–20 g) and the results calculated by the method of Litchfield and Wilcoxon.[9] Drugs were made up in 0·25% tragacanth and were adjusted to pH 7·0 with sodium bicarbonate before use. Dose volumes were adjusted to 0·2 ml/20 g.

RESULTS

Plasma NEFA and blood sugar levels in vivo

The effects of CIC and MPC on plasma NEFA in fasted, glucose-primed rats are shown in Fig. 1. The lowest doses of CIC and MPC which produced statistically significant falls in circulating NEFA were 25 μg/kg and 200 μg/kg, respectively.

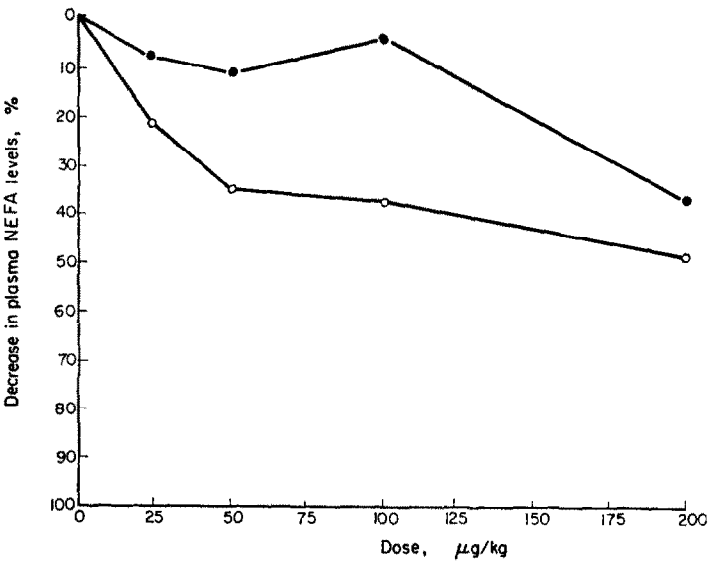

FIG. 1. Dose–response curves showing the effect of CIC (-○-) and MPC (-●-) on plasma NEFA levels.

The hypoglycaemic response to these substances was inconsistent but was probably influenced by the environmental temperature of the rats during and for 18 hr before the experiment. Table 1 gives a comparison of the hypoglycaemic effects of CIC and MPC at a room temperature of 56°F. There was no significant difference between the

TABLE 1. HYPOGLYCAEMIC EFFECTS OF CIC AND MPC IN FASTED, GLUCOSE-PRIMED RATS

Treatment	Dose (mg/kg)	Blood sugar (mg%) ± S.E.	Inhibition (%)
Control	—	83·1 ± 2·3	—
CIC	1·0	65·3 ± 5·0	21·4
	0·5	68·3 ± 2·9	16·8
	0·25	74·3 ± 2·6	10·6
	0·125	82·0 ± 4·1	1·3
MPC	1·0	61·4 ± 3·3	26·1
	0·5	75·1 ± 3·5	9·6
	0·25	74.8 ± 3·2	9·9
	0·125	64·3 ± 1·4	22·6

Rats fasted overnight (18 hr) were dosed orally with drug or tap water immediately after a subcutaneous injection of 1 g/kg glucose. Blood was taken by cardiac puncture under CO_2 anaesthesia 2 hr later.

activities of the two compounds. At an environmental temperature below 60°F, and using a suitable dose of drug, a significant hypoglycaemia was usually obtained, but at higher temperatures the effect was very variable. Unfortunately, we could not control the room temperature over a sufficiently wide range to reach a definite conclusion on the effect of temperature on the hypoglycaemic response of rats to these compounds. We should point out, however, that other factors must also be involved, since in experiments performed at "high" temperatures some rats responded to drug treatment and conversely in "low" temperature experiments occasionally some rats did not become hypoglycaemic.

With each compound depression of plasma NEFA always preceded a fall in blood sugar and this is illustrated for MPC in Fig. 2. This suggests that the primary action of these compounds is on fat metabolism. Whether the two effects are related or independent has not been established.

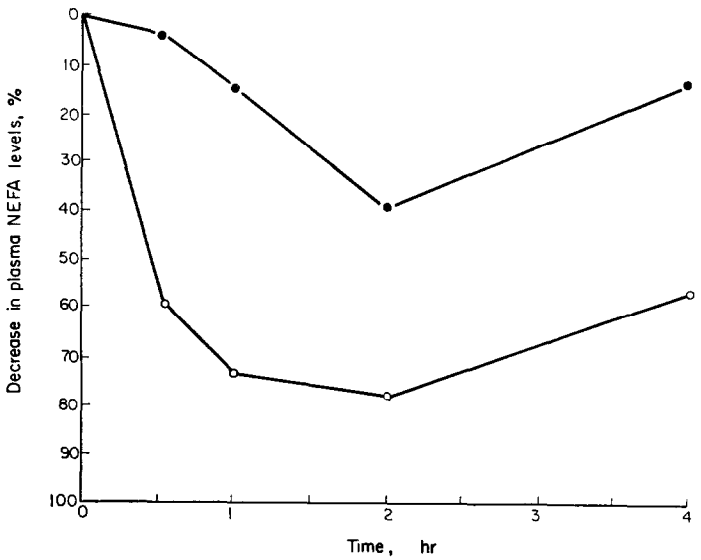

FIG. 2. Blood sugar (-●-) and plasma NEFA levels (-○-) at varying times after an oral dose of 10 mg/kg MPC.

In diabetic animals CIC and MPC effectively reduced the hyperglycaemia, and MPC was significantly more active than CIC ($P < 0.05$). Plasma NEFA levels were also lowered (Table 2).

TABLE 2. EFFECT OF CIC AND MPC ON BLOOD SUGAR AND PLASMA NEFA LEVELS OF ALLOXAN DIABETIC RATS

Treatment	Blood sugar (mg %) ± S.E. Before	Blood sugar (mg %) ± S.E. After	Plasma NEFA (μmoles/l) ± S.E. Before	Plasma NEFA (μmoles/l) ± S.E. After
Control	406 ± 50	357 ± 37 (8)	343 ± 79	343 ± 66 (4)
CIC (10 mg/kg)	561 ± 37	372 ± 19* (11)	322 ± 78	48 ± 9† (6)
MPC (10 mg/kg)	543 ± 28	294 ± 27*‡ (9)	242 ± 84	48 ± 14† (5)

* $P < 0.01$ compared with controls.
† $P < 0.001$ compared with controls.
‡ $P < 0.05$ compared with CIC.

The rats were made diabetic by alloxan injection 4 weeks previously. Blood samples were taken 18 hr before and 3 hr after oral dosing with drug or tap water. Blood sugar was determined on all samples and plasma NEFA estimated in approximately half the samples. Figures in parentheses indicate the number of samples used. The rats were fasted after dosing.

In vitro *rat hemidiaphragm experiments*

Neither CIC nor MPC, at concentrations up to 2 mg/ml, affected the uptake of glucose into rat hemidiaphragm; insulin increased it at 100 μU/ml (Table 3).

TABLE 3. EFFECTS OF CIC, MPC AND INSULIN ON UPTAKE OF GLUCOSE BY RAT HEMIDIAPHRAGMS *IN VITRO*

No. of estimations	Treatment	Final concentration/ml	Glucose uptake mg%/ 10 mg/dry tissue ± S.E.
12	Control	—	15·9 ± 1·1
6	CIC	2 mg	15·1 ± 1·0
5	MPC	2 mg	16·4 ± 1·4
4	Insulin	100 μU	20·4 ± 1·0*

* $P < 0{\cdot}01$.

Rat hemidiaphragms were incubated Gey and Gey's medium for 90 min at 37·5°.

In vitro *rat epididymal fat pad experiments*

We investigated the effects of DMP, CIC, MPC and insulin on basal NEFA release and on adrenaline and growth hormone stimulated NEFA release from rat epididymal fat pads. The results are given in Tables 4, 5 and 6. DMP caused a slight increase in

TABLE 4. INHIBITION OF UNSTIMULATED NEFA RELEASE FROM RAT EPIDIDYMAL FAT PAD

Drug	Final concentration	NEFA release (μ mole/g/hr) Unstimulated control	Unstimulated + drug	Change (%)	*P*
CIC	1 μg/ml	3·09	2·09	−33	< 0·001
	20 μg/ml	3·99	0·61	−85	< 0·001
MPC	1 μg/ml	3·64	0·89	−76	< 0·001
	20 μg/ml	2·83	0·73	−74	< 0·01
DMP	1 μg/ml	2·88	3·43	+19	> 0·05
	20 μg/ml	2·91	3·29	+13	> 0·05
Insulin	3·3 mU/ml	2·99	0·41	−86	< 0·001

Rat epididymal fat pads were incubated in 3 ml Krebs–Henseleit bicarbonate buffer containing 2% bovine serum albumin (Fraction V) and 100 mg% glucose, for 2 hr at 37·5°. Fat pads from five animals were used for each experiment, one pad from each animal serving as a control.

TABLE 5. INHIBITION OF ADRENALINE STIMULATED NEFA RELEASE FROM RAT EPIDIDYMAL FAT PAD

Drug	Final concentration/ml	NEFA release (μmole/g/hr) Adrenaline alone	Adrenaline + drug	Stimulation or inhibition (%)	*P*
CIC	50 μg	7·84	4·26	−46	<0·01
MPC	50 μg	5·95	2·64	−56	<0·01
DMP	50 μg	7·19	8·10	+12	>0·05
Insulin	3·3 mU	10·09	3·40	−66	<0·001

Rat epididymal fat pads were incubated in 3 ml Krebs–Henseleit bicarbonate buffer containing 2% bovine serum albumin (Fraction V) and 100 mg % glucose for 2 hr at 37·5°. Fat pads from five animals were used for each experiment, one pad from each animal serving as the adrenaline-stimulated control. Concentration of adrenaline was 0·05 μg/ml. The average increase in NEFA release in fat pads incubated with 0·05 μg/ml adrenaline was 170 per cent compared with release from unstimulated tissues and controls were set up for each experiment to check the activity of the adrenaline.

TABLE 6. INHIBITION OF GROWTH HORMONE STIMULATED NEFA RELEASE FROM RAT EPIDIDYMAL FAT PAD

Drug	Final concentration/ml	NEFA release (μmoles/g/hr) Growth hormone alone	Growth hormone + drug	Stimulation or inhibition (%)	P
CIC	50 μg	4·39	2·36	−46	<0·01
MPC	50 μg	6·61	2·24	−66	<0·01
DMP	50 μg	5·63	6·37	+13	<0·01
Insulin	3·3 mU	6·47	3·53	−46	<0·01

Rat epididymal fat pads were incubated in 3 ml Krebs–Henseleit bicarbonate buffer containing 2% bovine serum albumin (Fraction V) and 100 mg % glucose, for 2 hr at 37·5°. Fat pads from five animals were used for each experiment, one pad from each animal serving as the growth hormone stimulated control. Concentration of growth hormone was 100 μg/ml. The average increase in NEFA release was 144 per cent when compared with release from unstimulated tissue and controls were set up for each experiment to check the activity of the growth hormone.

NEFA release in each experiment but marked inhibition occurred with CIC and MPC. Approximately 75 per cent inhibition of basal NEFA release was obtained with a dose as low as 1 μg/ml of MPC in the incubation medium and 50 μg/ml of this compound caused approximately 60 per cent inhibition of both adrenaline and growth hormone stimulated NEFA release; MPC was more potent than CIC in these *in vitro* tests.

As expected, insulin also inhibited NEFA release *in vitro*.

In vivo *inhibition of growth hormone stimulated NEFA release*

Inhibition by CIC of growth hormone stimulated NEFA release is shown in Fig. 3. Rabbits were used in these experiments as no effect of growth hormone on plasma NEFA could be shown in rats. In rabbits, however, growth hormone (0·25 mg/kg) produced a 4-fold increase in circulating NEFA, but in animals pretreated with 200 mg/kg CIC growth hormone had little effect and the plasma NEFA levels were maintained at about control values. This reduction in the response of plasma NEFA to growth hormone, in animals pretreated with CIC, was related to the dose of inhibitor; the per centage reduction in the response of growth hormone on plasma NEFA levels being 30, 72, and 81 with doses of 50, 100 and 200 mg/kg CIC, respectively.

Blood sugars were also estimated in these experiments but the results were meaningless as elevated levels were seen in all animals, including controls, following the repeated injections and bleedings necessary during the test. These figures have therefore not been included.

Toxicity tests

In mice MPC showed no toxic effects in doses up to 9 g/kg. The LD_{50} for CIC was 1·86 g/kg with fiducial limits at 95 per cent probability of 1·71–2·03 g/kg.

DISCUSSION

Dulin and Gerritsen[5] first described the hypoglycaemic activity of 3,5-dimethylisoxazole and this was followed by further papers on the hypoglycaemic effects of DMP and related compounds (e.g.[1]). Subsequent studies on the metabolism of DMP showed that it was metabolised to MPC,[3] and that this metabolite was the active

compound.[4] It became apparent that the primary action of this series of compounds was probably to depress plasma NEFA levels by inhibiting their release from triglycerides in adipose tissue.[4, 10] With this source of energy removed, increased glucose utilisation would occur, leading to a decrease in blood sugar.

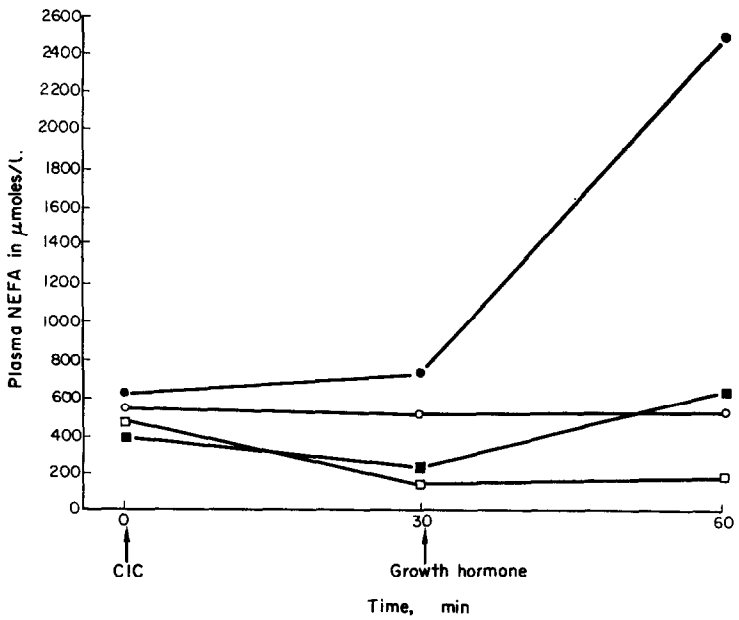

FIG. 3. Effect of CIC on the growth hormone induced increase in plasma NEFA levels in rabbits. CIC or saline given at time 0 and growth hormone or saline at 30 min. Blood samples were taken immediately before the injections of CIC, growth hormone or saline and also at 60 min.

Control	-○-	(5)
Growth Hormone (0·25 mg/kg)	-●-	(5)
CIC (200 mg/kg)	-⊡-	(4)
CIC (200 mg/kg) + Growth Hormone (0·25 mg/kg)	-■-	(4)

No. of animals given in parentheses.

CIC also depresses plasma NEFA and has potent hypoglycaemic activity. It is about as active as MPC, although differences in their relative potencies can be seen in some tests. For example, MPC is more active than CIC in reducing blood sugar levels in diabetic rats but not in normal animals. On the other hand, CIC is more active than MPC in lowering plasma NEFA levels *in vivo*, but the reverse is probably true *in vitro*.

Under our experimental conditions the hypoglycaemic activities of CIC and MPC were very variable and appeared to depend to some extent on the environmental temperature. This contrasts with the effect of these compounds on plasma NEFA, which was unaffected by changes in temperature. An explanation for the inconsistent hypoglycaemic effects at higher temperatures may be that under these conditions the basal metabolic rate of the animals is low, and less energy is expended in maintaining normal body temperature, whereas, at lower environmental temperatures more energy is expended for this purpose with a resultant decrease in blood sugar.

In alloxan diabetic rats the elevated blood sugars were significantly reduced by both CIC and MPC, and the latter compound was significantly more active than the former. However, in normal animals both compounds caused marked depression in circulating NEFA.

In vitro basal NEFA release from epididymal fat pad was unaffected by DMP. MPC and CIC, on the other hand, were extremely potent inhibitors of this *in vitro* release, whether basal, or adrenaline or growth hormone stimulated, MPC being somewhat more active than CIC. These *in vitro* results suggest that DMP acts *in vivo* either indirectly through some unknown mechanism or after its metabolic conversion to an active compound. As mentioned previously, we know that DMP is metabolised to MPC and that any *in vivo* NEFA lowering effects are due to the action of the latter metabolite.

It is probable also that the *in vivo* activity of MPC and CIC is due to decreased lipolysis although some other mechanisms may be involved; for example, increased re-esterification of NEFA may occur *in vivo* under the action of these drugs and further work is needed to clarify this point. In fact Dulin and Gerritsen[11] using palmitate-1-^{14}C produced data which was interpreted to mean that the decrease in plasma NEFA level after 3,5-dimethylisoxazole administration was a result of decreased release from the depots and not of increased re-esterifications.

Many other substances are known to depress the release of NEFA from adipose tissue e.g. salicylates[12] and nicotinic acid[13] but they are far less potent than CIC or MPC.

The *in vitro* inhibition, with MPC and CIC, of growth hormone induced release of NEFA from epididymal fat pad, led us to investigate the activity of CIC against growth hormone *in vivo*. We were unable to show an effect of growth hormone (porcine or bovine) on plasma NEFA in acute experiments in fasted rats, or in chronic experiments on fed rats using intravenous doses of up to 10 mg/day (unpublished results). In rabbits, however, an intravenous dose of 0·25 mg/kg of growth hormone (porcine) caused an approximately 4-fold increase in plasma NEFA. It can be seen from Fig. 3 that pretreatment of rabbits with CIC significantly reduced the growth hormone induced rise in circulating NEFA, although the doses of drug needed to achieve this effect were higher than might have been expected from the other *in vivo* and *in vitro* experiments reported in this paper. 200 mg/kg CIC prevented the rise in plasma NEFA, following administration of growth hormone, exceeding the normal levels for untreated controls.

Randle, Garland, Hales and Newsholme[14] proposed that the impaired sensitivity to insulin which occurs in diabetes may be due to the higher rate of release of NEFA and ketone bodies in these subjects. They suggest the existence of a glucose-fatty acid cycle in which the metabolism of glucose by the tissue inhibits the release of NEFA and conversely the release of NEFA inhibits the metabolism of glucose. For example, phosphorylation of glucose by the perfused rat heart was reduced by addition of fatty acids and ketone bodies to the perfusion fluid. Similar effects were seen in hearts from diabetic or starved animals.[15] In 1940, Young[16] suggested that hypersecretion of growth hormone could be an important factor in the pathogenesis of diabetes, and one mechanism for this effect may be the opposite actions of insulin and growth hormone on plasma NEFA.[17] Certainly juvenile diabetes, in which insulin secretion is often eventually reduced to zero, is frequently excacerbated by spurts of growth or by other

crises such as infections.[18] Under these conditions growth hormone or adrenocorticoid secretion is presumably high and consequently plasma NEFA output would be expected to rise also.

In view of the work of Young and of Randle and his associates it would be interesting to know whether CIC could prevent the development of a growth hormone induced diabetes in experimental animals by inhibiting the rise in plasma NEFA.

However, it must be noted that Froesch *et al.*[19] have reported that escape mechanisms to the antilipolytic activity of MPC, developed in rats that had been pretreated with large doses of MPC. The authors also found evidence that under chronic administration of MPC, both the synthesis of glycerides from glucose as well as the hydrolysis of freshly synthesised glycerides were activated to a marked degree.

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REFERENCES

1. J. B. Wright, W. E. Dulin and J. A. Markillie, *J. mednl Chem.* **7**, 102 (1964).
2. G. C. Gerritsen and W. E. Dulin, *Diabetes* **14**, 507 (1965*a*).
3. D. L. Smith, A. A. Forist and W. E. Dulin, *J. mednl Chem.* **8**, 350 (1965).
4. G. C. Gerritsen and W. E. Dulin, *J. Pharmac exp. Ther.* **150**, 491 (1965*b*).
5. W. E. Dulin and G. C. Gerritsen, *Proc. Soc. exp. Biol. Med.* **113**, 683 (1963).
6. W. E. Dulin and G. C. Gerritsen, *Proc. Soc. exp. Biol. Med.* **121**, 777 (1966).
7. D. L. Trout, E. H. Estes and S. J. Friedberg, *J. Lipid Res.* **1**, 199 (1960).
8. J. Vallance-Owen and B. Hurlock, *Lancet* **i**, 68 (1954).
9. J. T. Litchfield and F. Wilcoxon, *J. Pharmac. exp. Ther.* **96**, 99 (1949).
10. W. E. Dulin, G. H. Lund and G. C. Gerritsen, *Proc. Soc. exp. Biol. Med.* **118**, 499 (1965).
11. W. E. Dulin and G. C. Gerritsen, *Fedn. Proc.* **23**, 502 (1964).
12. A. Bizzi, A. M. Godegoni and S. Garattini, *Nature, Lond.* **204**, 1205 (1964).
13. A. M. Barrett, *Br. J. Pharmac. Chemother.* **26**, 363 (1966).
14. P. J. Randle, P. B. Garland, C. N. Hales and E. A. Newsholme, *Lancet* **i**, 785 (1963).
15. P. J. Randle, E. A. Newsholme and P. B. Garland, *Biochem. J.* **93**, 652 (1964).
16. F. G. Young, *Endocrinology* **26**, 345 (1940).
17. F. G. Young, *Endocrinology* **73**, 654 (1963).
18. P. White, *Diabetes* (Ed. R. H. Williams) p. 381. P. A. Hoeber (1960).
19. E. R. Froesch, M. Waldvogel, U. A. Meter, A. Jakob and A. Labhart, *Molec. Pharmac.* **3** 442 (1967).