Biochemical Pharmacology, Vol. 18, pp. 1093–1101. Pergamon Press. 1969. Printed in Great Britain

EFFECTS OF 5-METHOXYINDOLE-2-CARBOXYLIC ACID ON CARBOHYDRATE METABOLISM

N. BAUMAN and B. S. PEASE

Biochemistry Research Department, Lederle Laboratories,
Pearl River, N.Y., U.S.A.

(*Received* 16 *August* 1968; *accepted* 1 *November* 1968)

Abstract—The hypoglycemic effect of 5-methoxyindole-2-carboxylic acid (MICA) has been investigated. MICA promotes neither the uptake of glucose or galactose by peripheral tissues nor the oxidation of glucose, and therefore is not insulin-like. Because it does not produce insulin-like effects and because it is active in the alloxanized mouse, it is not an insulin releaser. MICA blocks gluconeogenesis both *in vivo* and in the perfused liver; it is proposed that MICA causes hypoglycemia by interfering with gluconeogenesis. MICA depletes liver glycogen even in the face of exogenous hyperglycemia.

THE HYPOGLYCEMIC effect of the indole-2-carboxylic acids was discovered in the course of systematic screening for such activity in the rat.[1] This paper reports studies on 5-methoxyindole-2-carboxylic acid (MICA), a representative member of this group of compounds, demonstrating that the hypoglycemia is caused by inhibition of hepatic gluconeogenesis. These conclusions agree with those of Hanson *et al.*[2]

MATERIALS AND METHODS

MICA was purchased from the Aldrich Chemical Co. and recrystallized from water. It was dissolved in 0·15 M sodium bicarbonate for use. Crystalline zinc insulin (glucagon-free) was a gift from Dr. O. Behrens of the Eli Lilly Co. Glucose oxidase and galactose oxidase, each with peroxidase, were purchased from the Boehringer Mannheim Corp. and the Worthington Biochemical Corp. U-^{14}C-D-glucose (5·76 μc/mg) was from Nutritional Biochemicals; L-alanine-U-^{14}C (0·75 mc/m-mole) was from Volk Radiochemicals, Inc. AG1X8 (acetate) and AG50X4(H) ion-exchange resins were purchased from BioRad Laboratories. BSP (sulfobromophthalein) was from Hynson, Wescott & Dunning, Inc.

Adult male rats of the CFE strain (Carworth Farms) and mice of the MF-1 strain (Manor Farms) were fed Purina chow *ad libitum*, except as otherwise stated.

Glucose was determined by the alkaline ferricyanide method[3] as modified by the Technicon Corp. for the autoanalyzer. Radioglucose was separated from radioactive ionic materials of deproteinized blood by passage through a column 5 mm in diameter, consisting of consecutive 2-cm beds of AG1X8 (acetate) and AG50X4(H) ion-exchange resins, separated by 2 mm sand. Separate experiments showed that alanine, lactate, pyruvate and bicarbonate are retained on such columns and that the radioactivity in the effluent was convertible to retained material by glucose oxidase. Liver glycogen was determined by digestion of the tissue in 30% KOH at 100°, precipitation with 1·2 vol. ethanol, extraction into 10% trichloroacetic acid, hydrolysis

in 1·6 N HCl at 100° for 30 min, and determination of glucose on a suitably diluted and neutralized aliquot as above. Glycogen is reported as milligrams of glucose equivalent.

Galactose was determined by the same procedure as that used for glucose after digestion of the sample with glucose oxidase, and was read against a standard curve prepared from D-galactose. This method was occasionally checked with a galactose–oxidase–peroxidase reagent.

Lactate was determined by the Barker–Summerson method.[4] BSP was determined spectrophotometrically on a diluted alkalinized sample. Glucose uptake of rat hemidiaphragm was measured by a modification of the method of Owen.*

Evisceration and nephrectomy were performed by the two-stage method of Ingle and Griffith[5] or by a modification in which the entire procedure was performed in one stage, functional evisceration (removal of the gastrointestinal tract with the exception of the liver, cutting off the blood supply of the liver) according to Russell.[6] Diethyl ether was used as anesthetic after premedication with atropine sulfate, 150 mg/kg, subcutaneously. Atropine neither augmented nor decreased MICA-induced hypoglycemia.

Galactose space was calculated from the level of serum galactose 1 hr after i.v. injection of 700 mg/per kg of D-galactose: galactose space (per cent of body weight) = [70 mg/100 ml ÷ serum galactose (mg/100 ml)] × 100. (The weight after evisceration was used; the injection was made into the exposed jugular vein[7] so that one could be certain that the complete dose was injected.) Sugars were injected as 10% solutions; all other compounds were injected in 1 ml/100 g body weight.

Respiratory $^{14}CO_2$ was collected by entraining a stream of air through the rat container and thence through a trap containing 2-amino ethanol and 2-methoxy ethanol (1:2). Radioactivity was determined in the Packard liquid scintillation spectrometer, using "DAM 611."[8]

Liver perfusion was performed by the method of Green and Miller.[9]

RESULTS

As is well known, a decrease in blood glucose concentration must be caused by an increase in consumption (including excretion), a decrease in production, or both.† Results are therefore divided into those concerned with consumption of glucose and with production of glucose.

Lack of effect of MICA on peripheral consumption of glucose

Because MICA was hypoglycemic in alloxanized mice,[10] the possibility of its having a peripheral insulin-like action was investigated.

Glucose uptake by isolated rat diaphragm. In a system in which insulin (1 milliunit/ml) consistently increased the uptake of glucose, MICA (10^{-3} M) failed to produce an increase in glucose uptake or a potentiation of insulin. Treatment of the donor rat with MICA failed to increase the glucose uptake of the isolated diaphragm, while insulin did so. Representative data are in Table 1.

Galactose distribution in the eviscerated nephrectomized rat. Levine *et al.*[10] showed

* H. Levin, unpublished method.

† Mere dilution is untenable because of the great changes in fluid volume which would be required to account for the large changes in blood glucose this drug creates in minutes.

TABLE 1. GLUCOSE UPTAKE BY RAT HEMIDIAPHRAGMS*

Donor treatment	Blood sugar (mg/100 ml)	Treatment *in vitro*	
		None	MICA (10^{-3} M)
Expt. 4125C-64			
None	64	5·3	4·9
MICA (200 mg/kg, i.p.)	32	3·1	3·3
Insulin (1 U/rat, i.p.)	33	9·6	9·4

* Uptake is in micrograms glucose/milligrams tissue/90 min. Fasted male rats were treated as indicated; 1 hr later they were decapitated. Blood was taken for glucose determination and the hemidiaphragms were used for the incubation. Hemidiaphragms were incubated 90 min at 37° under 95 per cent O_2–5 per cent CO_2 in Krebs–Ringer bicarbonate buffer fortified with 2·5 mg/ml of bovine serum albumin at a glucose concentration of 2·0 mg/ml and MICA as indicated. Tissue weight was taken as the mean of the preincubation and postincubation wet weight. Glucose uptake was measured by subtracting the glucose in the medium after incubation from the mean of incubated controls in which the tissue was omitted. Each value is a mean of 4 hemidiaphragms; the pooled standard error of the mean is ± 0·15. Analysis of variance showed that the differences among donor treatments were significant while those between treatments *in vitro* were not.

that in the eviscerated nephrectomized animal galactose is an unmetabolizable analog of glucose and that its volume of distribution is increased by insulin. Accordingly, we sought an effect of MICA in eviscerated nephrectomized rats. The volume of distribution proved to be remarkably consistent within each group of rats. MICA was without effect on this volume of distribution, whether injected before or after evisceration, while insulin consistently increased it. Representative data are in Table 2.

TABLE 2. GALACTOSE SPACE IN EVISCERATED NEPHRECTOMIZED RATS*

Treatment	No. of rats	Galactose space (% of body wt.)
Expt. 8445B-160		
Control	3	24 ± 1†
MICA (75 mg/kg)	3	24 ± 1
Insulin (1 U/kg)	2	35 ± 3
Expt. 8445B-163		
Control	6	32 ± 2
MICA (75 mg/kg)	6	29 ± 2
Insulin (1 U/kg)	4	44 ± 3

* In experiment 8445B-160 the rats were totally eviscerated and nephrectomized, while in 8445B-163 functional evisceration was used. In both experiments galactose was injected i.v. at 700 mg/kg and heart blood was sampled for galactose at 1 hr.
† Mean ± standard error of the mean.

Blood sugar in the eviscerated nephrectomized rat. MICA (100 mg/kg, s.c.) was also examined for its effect on blood sugar in glucose-primed (500 mg/kg, i.v.) eviscerated nephrectomized rats. No significant lowering was seen in 1 hr, e.g. the control group's mean blood sugar at 1 hr was 176 ± 10 mg per 100 ml, while that of the MICA group as 179 ± 10 mg per 100 ml (six rats/group; mean ± standard error of the mean).

Oxidation of glucose-U^{14}C to $^{14}CO_2$. In these experiments the eviscerated

nephrectomized rat was used, since in this preparation the specific activity of a radioglucose load is unchanged for at least an hour.* This makes it possible to interpret CO_2 collection directly as glucose oxidized. Table 3 shows an experiment of this sort, in which it can be seen that MICA has no effect, while insulin increases glucose oxidation.

TABLE 3. GLUCOSE OXIDATION BY EVISCERATED NEPHRECTOMIZED RATS

Treatment	No. of rats	Glucose converted to CO_2 (mg/hr)
Control	11	7·5 ± 0·6*
MICA (100 mg/kg, s.c.)	6	7·6 ± 0·8
Insulin (1 U/kg, s.c.)	4	11·2 ± 0·9†

* Mean ± standard error of the mean.
† $P < 0·01$ by analysis of variance. Eviscerated nephrectomized rats were injected i.v. with 700 mg/kg of U-^{14}C-D-glucose (0·01 μc/mg) and respiratory $^{14}CO_2$ was collected. At the end of the experiment, heart blood was drawn for determination of glucose specific activity (see Methods). Glucose oxidized was estimated as the quotient of cpm in $^{14}CO_2$ and glucose specific activity (cpm/mg).

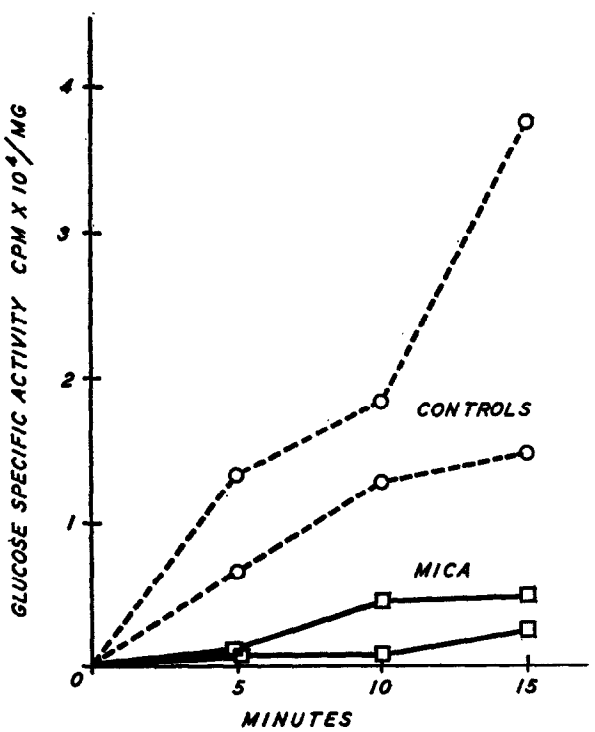

FIG. 1. Effect of MICA on incorporation of ^{14}C-alanine into glucose in the mouse. Two experimental and two control mice were each injected i.p. with 5 μc U-^{14}C-alanine at time zero. In addition, the experimental pair received MICA (75 mg/kg, i.p.). Blood samples were withdrawn from the orbital sinus at intervals and assayed for glucose and radioglucose as in Methods. Experimentals are significantly lower than controls by *t*-test on slopes ($P < 0·05$).

* N. Bauman and C. Hill, unpublished data.

Urinary excretion of glucose. Four mice treated with MICA (200 mg/kg, i.p.) failed to develop glycosuria, as measured with a glucose oxidase tape (available from Eli Lilly Co. as Tes-Tape). From these experiments we conclude that MICA has no insulin-like activity and is unable to promote the utilization of glucose by muscle. It does not deplete glucose via excessive urinary excretion. The effects of MICA on gluconeogenesis were therefore examined.

Effect of MICA on gluconeogenesis in vivo

Mice were injected with a tracer dose of U-^{14}C alanine, i.p., and the incorporation of radioactivity into glucose was followed in serial blood samples (see Methods). Figure 1 shows the results of one such experiment; incorporation of radioalanine into radioglucose is reduced about 80 per cent by MICA.

Effect of MICA on perfused rat liver

Gluconeogenesis was studied in the perfused rat liver. Fig. 2, A and B, shows the results of a typical experiment consisting of two individual liver perfusions, one treated with MICA and one control. After 30–40 min, during which starting values of glucose and lactate were measured, 10 mg MICA in 1 ml of 0·15 M $NaHCO_3$ was administered to one perfusion, and vehicle alone to the other (this defined zero time). At 5 min, 5 μc DL-sodium lactate-U-^{14}C was administered. In the control perfusion, glucose rose and lactate fell, while in the presence of MICA, the opposite was seen (Fig. 2A) In the control there was rapid oxidation of lactate to CO_2 and rapid incorporation of lactate into glucose, while in the presence of MICA lactate oxidation was depressed and gluconeogenesis from lactate was almost nil (Fig. 2B).

In all, fourteen such perfusions were performed, some as described above and some with a substrate amount (100 mg) of alanine-U-^{14}C replacing the lactate tracer. In all cases the qualitative results were the same; i.e. the control liver was able to keep up with the glycolysis of the red cells (in this system the mass of the red cells is about three times that of the liver) and reconvert the lactate to glucose. The glucose would rise to 50–100 mg per 100 ml and the lactate would fall to 10–20 mg per 100 ml; incorporation of radioactivity into glucose showed that this was the true gluconeogenesis. The MICA-treated liver showed deficient gluconeogenesis; the glucose levels would fall while the lactate rose, sometimes up to 100 mg per 100 ml, and incorporation of radioactivity into glucose was minimal. $^{14}CO_2$ production proceeded at about $\frac{1}{6}$ the rate seen in controls. In some of the liver perfusion experiments, in addition to the parameters mentioned, the excretion of BSP into bile was also assessed. This was done by injecting 5 mg BSP into the perfusion fluid and collecting fractions of bile for spectrophotometric determination of BSP after appropriate dilution and alkalinization. From time to time during the course of the perfusion, further BSP was added. The amount of BSP available at any time was calculated as the amount injected less the amount excreted in the bile; thus, throughout the procedure, an estimate could be made of the ability of the liver to excrete BSP. Under all circumstances, although there was considerable variability, BSP was excreted at a rate of 2–13 per cent per 15 min, unaffected by MICA. Bile flow was also continuous and unaffected by MICA.

Thus, these experiments demonstrated inhibition of oxidation of lactic acid to CO_2 and a block in the conversion of lactic acid to glucose. The isolated liver perfusion reproduces the major phenomenon we see in the whole rat on administration of

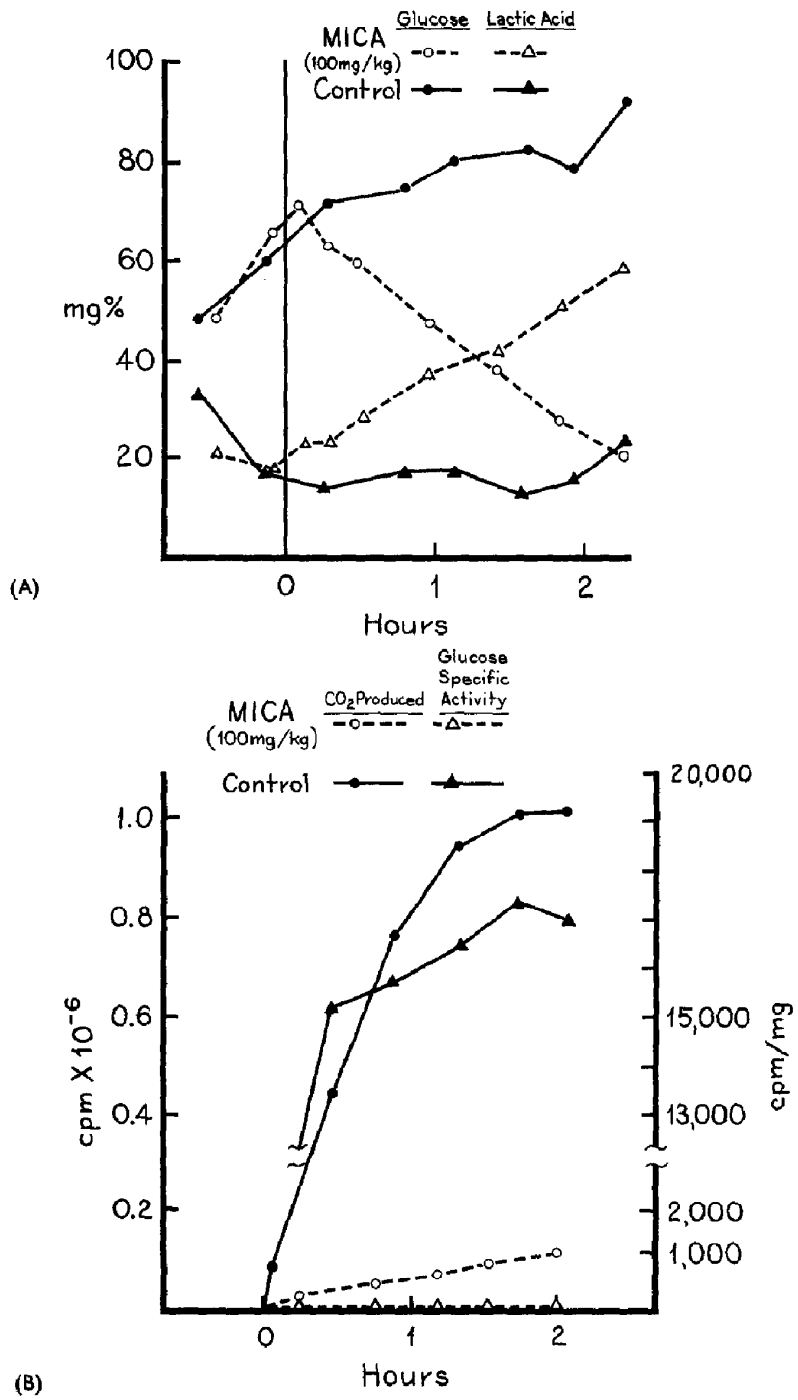

FIG. 2. Rat liver perfusion.

(A) shows the concentrations of glucose and lactate in the perfusate; (B) shows the specific activity of the perfusate glucose and the cumulative production of $^{14}CO_2$. For details, see text.

MICA, namely, hypoglycemia. In addition, the perfused liver system also demonstrates a phenomenon seen in the whole mouse, the drug-induced failure of incorporation of glucose precursors into blood glucose. Thus, an effect of MICA is the blocking of gluconeogenesis from smaller molecules, and this could account for its hypoglycemic effect *in vivo*.

In addition, a new effect was noted, namely, the inhibition of oxidation of lactate to CO_2. This has been the basis of a further investigation of the mechanism of action of MICA at the subcellular level[11] in which it was demonstrated that MICA blocks mitochondrial oxidation of pyruvate.

Liver glycogen

The administration of MICA to alloxanized or normal mice causes a striking depletion of liver glycogen to levels even below fasting levels. This could be illustrated in another way by fasting mice, then administering a carbon source and measuring the repletion of hepatic glycogen. Table 4 shows such an experiment. Under these

TABLE 4. EFFECT OF MICA ON HEPATIC GLYCOGEN SYNTHESIS IN MICE*

Carbon source	Control	MICA (75 mg/kg, i.p.)
D-Glucose	> 6†	0·35
L-Alanine	> 6†	0·28
None	3·7	—
D-Glucose	12·5	2·8
D-Fructose	19·7	2·8
None	3·1	—

* Entries represent glycogen in mg glucose/g liver (wet wt.). Groups of five mice, fasted overnight, were dosed with 1000 mg/kg of the carbon source, i.p., with or without MICA as indicated. Exactly 1 hr later, the mice were decapitated and the livers were removed for glycogen determination (see Methods). Each value is the median of a group of five mice.

† Off scale; exact value not determined.

circumstances we see that MICA prevents formation of glycogen from glucose. It should be noted that in this type of experiment the glucose load prevents hypoglycemia (see Fig. 3); the mouse is hyperglycemic throughout the course of the entire experiment, yet despite the elevated blood sugar levels, liver glycogen falls. This is not simply a mobilization of glycogen to meet the demands of hypoglycemia, an effect which can be produced even by excessive dosage of insulin; this is distinctly a mobilization of liver glycogen in the face of hyperglycemia. This interesting effect has not been pursued further.

DISCUSSION

MICA is a representative of a newly discovered class of hypoglycemic indoles, the indole-2-carboxylic acids. These compounds lower blood sugar of laboratory animals in 30 min or less and are active in alloxan diabetic mice. They are of relatively low acute toxicity, provided that fatal hypoglycemia is prevented by glucose administration.*

* S. Gordon and B. S. Pease, unpublished data.

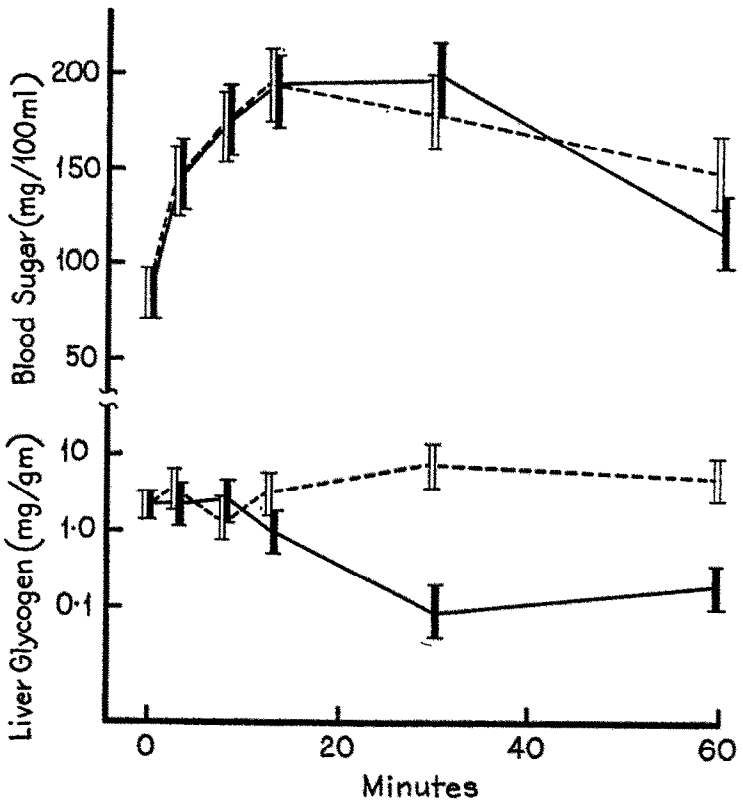

FIG. 3. Effect of MICA on blood sugar and liver glycogen in glucose-loaded mice. Fasted mice were injected i.p. at zero time with 1000 mg/kg of glucose with MICA, 75 mg/kg (solid lines and bars), or with glucose, 1000 mg/kg, without MICA (dotted lines and open bars). At intervals, groups of three mice were decapitated; blood and liver were taken for glucose and glycogen determinations. Each point is the mean of three mice; the bar represents ± one standard error, calculated from pooled data. (Glycogen data were handled with logarithmic conversion, as variation in liver glycogen was roughly proportional to its level.)

The present work has shown that MICA has no insulin-like effect on glucose uptake or oxidation, but that MICA can suppress hepatic gluconeogenesis. That this effect is relatively specific, rather than simply a poisoning of the liver, is shown by the continuance of bile formation and BSP secretion. We feel that suppression of gluconeogenesis is the mechanism of the hypoglycemic action of MICA.

In addition, MICA has two other effects: it inhibits oxidation of lactate and it depletes liver glycogen even in the face of hyperglycemia. The former effect has been explained by the demonstration that MICA blocks mitochondrial oxidation of pyruvate.[11] The latter has not been further explored.

MICA causes hypoglycemia in animals. Because no effect could be demonstrated on glucose uptake or oxidation or on the galactose space, it was concluded that the action is unlike that of insulin. Because MICA interferes with conversion of 3-carbon compounds to glucose both *in vivo* and in isolated perfused liver, it was concluded that MICA causes hypoglycemia through inhibition of gluconeogenesis. MICA also

causes hepatic glycogen depletion despite exogenous hyperglycemia; the mechanism of this action is not known.

Acknowledgement—The authors thank Dr. Paul H. Bell for his advice and encouragement.

REFERENCES

1. N. Bauman, S. Gordon and B. S. Pease, *Biochem. Pharmac.* **18**, 2298 (1969).
2. P. L. Hanson, P. D. Ray and H. A. Lardy, *154th National Meeting Am. Chem. Soc.*, Chicago Illinois (September 1967), Abstract 238C.
3. W. S. Hoffman, *J. biol. Chem.* **120**, 51 (1937).
4. S. B. Barker in *Methods in Enzymology* (Eds. S. P. Colowick and N. O. Kaplan), p. 241. Academic Press, New York (1957).
5. D. J. Ingle and J. Q. Griffith in *The Rat in Laboratory Investigation* (Eds. E. J. Farris and J. Q. Griffith), p. 450. Lipincott, Philadelphia (1949).
6. J. A. Russell, *Am. J. Physiol.* **136**, 95 (1942).
7. J. Q. Griffith and W. A. Jeffers in *The Rat in Laboratory Investigation* (Eds. E. J. Farris and J. Q. Griffith), p. 288. Lipincott, Philadelphia (1949).
8. J. D. Davidson and P. Feigelson, *Int. J. appl. Radiat. Isotopes* **2**, 1 (1957).
9. M. Green and L. L. Miller, *J. biol. Chem.* **235**, 3202 (1960).
10. R. Levine, M. S. Goldstein, B. Huddlestun and S. P. Klein, *Am. J. Physiol.* **163**, 70 (1950).
11. N. Bauman and C. J. Hill, *Biochemistry, N.Y.* **7**, 1322 (1968).