Some Metabolic Effects of Phenformin in Rat Adipose Tissue

J. N. Pereira, Ph.D., N. O. Jangaard, Ph.D., and E. R. Pinson, Ph.D., Groton, Conn.

SUMMARY

In rat adipose tissue, phenformin (5×10^{-4}M) inhibited the oxidation of C_1 and C_6 of glucose to CO_2, blocked the stimulatory effect of insulin (32 μU./ml.) on C_1 oxidation, inhibited lipogenesis from C_1 and C_6 of glucose, and abolished the stimulatory effect of acetate on glucose oxidation and lipogenesis. These effects were accompanied by lactate accumulation. Such inhibitory effects suggest that phenformin interrupts the pentose shunt and lipogenesis, both of which are pyridine nucleotide-linked processes. Phenformin was shown, by inhibition of oxidation of pyruvate C_1, to interfere with the sequence at the point of pyruvate decarboxylation, since acetate incorporation was not affected. Both pyruvate decarboxylation and oxidation of acetate in the tricarboxylic acid cycle were blocked, presumably by interference with electron transport. This effect of phenformin on pyruvate decarboxylation is sufficient to explain the observed reduction in pentose shunt activity and lipogenesis as well as the increased lactate accumulation. DIABETES *16*:869-72, December, 1967.

The hypoglycemic agent, phenformin, has been shown to cause increased glucose uptake in the isolated rat diaphragm[1-3] and perfused rat heart.[4] A major end product of glucose uptake and utilization in the presence of phenformin is lactic acid. The finding that phenformin inhibits NAD-linked Krebs cycle oxidations as well as the respiratory chain cytochrome oxidase reaction[3,5] suggested that this agent might cause an increased rate of glycolysis; and therefore hypoglycemia, by a mechanism similar to the Pasteur effect.[6] In the light of our current understanding of the mechanism of the Pasteur effect,[7] phenformin would be expected to reduce the levels of ATP and increase the levels of AMP and inorganic phosphate (P_i) and in this way relieve the inhibition of the rate-controlling glycolytic enzyme, phosphofructokinase. Such a reduction in tissue ATP levels by phenformin has been reported by Williamson et al.[4] and Patrick.[8]

From the Pharmacology Research Department, Medical Research Laboratories, Chas. Pfizer & Co., Groton, Connecticut.

In adipose tissue, on the other hand, the effects of phenformin are less clear. Ditschuneit et al.[1] have reported a pronounced decrease in the oxidation of C-1 of glucose accompanied by a very modest increase in glucose uptake caused by phenformin. More recently, Waterbury and Jaffe[9] showed that phenformin depressed glucose uptake by adipose tissue. The depressed glucose uptake was associated with decreased incorporation of uniformly labeled glucose into CO_2 and lipid. On the other hand, several workers have reported slight to moderate increases in C-1 oxidation by adipose tissue in the presence of biguanides.[10,11] These and other observations have led at least one investigator[12] to the conclusion that phenformin acts primarily in muscle and not in adipose tissue.

The meager and conflicting information concerning the effects of phenformin on adipose tissue metabolism and the need for elucidation of the metabolic effects of phenformin in this tissue have stimulated the investigations described in this report.

MATERIALS AND METHODS

Male Sprague-Dawley rats (150-200 gm.) were used throughout these studies. Nonfasted rats were killed by stunning and exsanguination. Epididymal adipose tissue was excised, placed in Krebs-Ringer bicarbonate buffer and minced into pieces of approximately 10 mg. each (Steelman et al., 1960).[13] Each experimental flask contained 150 mg. of tissue in a final volume of 2 ml. The incubation procedures were those described by Renold et al.,[14] (1960) except that the incubation period was extended to five hours.

The total radioactivity in each flask was 0.4 microcuries regardless of substrate. The final concentrations of substrates were as follows: glucose, 300 mg. per 100 ml.; pyruvate and acetate, 10^{-2}M. The insulin and phenformin concentrations were as described in table legends.

At the end of the incubation period 0.3 ml. 6N H_2SO_4 was injected into the incubation medium and 0.2 ml. hyamine hydroxide 10X injected into a glass well suspended from a rubber stopper. After a one-hour

equilibration period, the tissue lipids were recovered by the procedure of Winegrad and Renold.[15] Radioactivity was determined in a Packard model 314EX liquid scintillation counter. Lipid and carbon dioxide radioactivity was counted in a scintillation fluid composed of 0.4 per cent 2,5-diphenyloxazole and 0.05 per cent p-bis (phenyloxazolyl)-benzene in 30 per cent ethanol-70 per cent benzene.

The C-14-labeled substrates were obtained from the New England Nuclear Corporation, Boston, Massachusetts.

Glucose was determined by the Hofmann ferricyanide reduction method[16] modified for use in the Auto-Analyzer. The Barker and Summerson technic[17] was used for lactate determinations.

RESULTS

Adipose tissue incubated with specifically labeled glucose oxidized both C-1 and C-6 to CO_2. The oxidation of C-1 to carbon dioxide was significantly more complete than the corresponding oxidation of C-6 (table 1). Under these conditions, phenformin decreased the oxidation of C-1 and C-6 to CO_2. An approximately 75 per cent reduction in the oxidation of these glucose carbons was observed with a phenformin concentration of 5×10^{-4}M.

Sodium acetate, 10^{-2}M, stimulated C-1 oxidation significantly ($p < 0.05$) and caused a two-fold increase in the oxidation of glucose C-6. When phenformin was added to the acetate-containing medium, it completely blocked the stimulatory effect of acetate on C-1 oxidation. In a similar manner, phenformin blocked the effect of acetate on C-6 oxidation. Although acetate did not significantly increase C-1 oxidation in the presence of phenformin, the oxidation of C-6 was stimulated by acetate above the levels observed with phenformin alone.

Insulin stimulated the oxidation of glucose C-1 but was without effect on the oxidation of C-6. Phenformin blocked the stimulatory effect of insulin and reduced the C-1 and C-6 oxidation almost to the levels observed in the presence of phenformin alone.

An examination of the incorporation of specifically labeled glucose into total lipids and fatty acids by adipose tissue revealed a disparity in the degree of incorporation of C-1 and C-6 (table 2). Under the conditions described, C-6 was incorporated approximately two and one-half times as efficiently as was C-1.

Phenformin blocked the conversion of both C-1 and

TABLE 1

Effect of phenformin, sodium acetate and insulin on the conversion of specifically labeled glucose to CO_2 by adipose tissue in vitro

	Glucose-1-C-14		Glucose-6-C-14	
	Thousands of DPM*	Per cent	Thousands of DPM*	Per cent
Control	25.6 ± 6.2	3.1	11.3 ± 1.3	1.2
Phenformin, 5×10^{-4}M	6.1 ± 1.1	0.7	2.5 ± 0.4	0.2
Na acetate, 10^{-2}M	35.6 ± 3.0	4.3	22.3 ± 0.8	2.3
Phenformin + Na acetate	8.1 ± 2.5	1.0	4.3 ± 0.2	0.4
Insulin, 32 μU./ml.	59.1 ± 6.3	7.1	12.2 ± 1.8	1.2
Phenformin + insulin	8.7 ± 1.7	1.0	3.3 ± 0.6	0.3

*Values represent the means ± standard deviations of six experimental flasks.

TABLE 2

Effect of phenformin, sodium acetate and insulin on the conversion of specifically labeled glucose to total lipid and fatty acids by adipose tissue in vitro

	Glucose-1-C-14				Glucose-6-C-14			
	Total lipid		Fatty acids		Total lipid		Fatty acids	
	Thousands of DPM*	Per cent	Thousands of DPM	Per cent	Thousands of DPM*	Per cent	Thousands of DPM	Per cent
Control	26.3±5.0	3.2	18.5±3.0	2.3	71.5±17.0	7.2	45.4± 6.0	5.8
Phenformin, 5×10^{-4}M	6.8±0.8	0.8	4.2±0.8	0.4	34.6± 6.5	3.5	22.3± 3.5	2.8
Na acetate, 10^{-2}M	31.1±4.1	3.7	20.4±3.4	2.5	199.1±28.0	20.2	126.8±16.0	15.8
Phenformin + Na acetate	6.5±1.3	0.8	3.9±1.0	0.4	29.1± 1.7	2.9	19.1± 2.3	2.4
Insulin, 32 μU./ml.	53.5±3.0	6.4	39.8±5.0	5.0	114.2±18.7	11.6	74.9±11.4	9.4
Phenformin + insulin	11.9±1.4	1.4	6.6±1.1	0.8	31.6± 6.7	3.2	18.8± 3.4	2.3

*Values represent the means ± standard deviations of six experimental flasks.

C-6 to lipid, causing a more pronounced inhibitory effect on the C-1 transformation. Sodium acetate did not affect the incorporation of C-1 into lipid but caused a profound stimulation of lipogenesis from C-6. Phenformin completely blocked the acetate stimulation of lipogenesis.

Insulin caused increased lipogenesis from both C-1 and C-6 and this effect was markedly reduced by phenformin. Throughout these studies, 60-70 per cent of the radioactivity incorporated into lipid could be accounted for as fatty acids.

Insulin stimulated the uptake of glucose by adipose tissue in vitro but did not change the level of lactate secreted into the incubation medium (table 3). Phenformin had no significant effect on glucose uptake but caused a distinct increase in the level of medium lactate. When both phenformin and insulin were included in the incubation mixture, the stimulatory effect of insulin on glucose uptake was not influenced by phenformin. However, insulin and phenformin caused an increase in lactate levels over those observed with phenformin alone.

TABLE 3

Effects of phenformin, alone and in combination, on glucose uptake, and lactate production by adipose tissue in vitro*

	Glucose uptake (μmoles/ gm. tissue)	Lactate production (μmoles/ gm. tissue)	Per cent of theoretical lactate yield
Control	10.3 ± 0.8	12.0 ± 0.7	58
Insulin, 32 μU./ml.	14.9 ± 0.6	16.5 ± 1.2	55
Phenformin, 5×10^{-4}M	11.0 ± 0.8	24.7 ± 1.8	112
Phenformin + insulin	15.3 ± 1.2	32.3 ± 2.9	106

*Mean ± standard deviations of ten experimental values.

Adipose tissue efficiently converted C-1 of pyruvic acid to carbon dioxide. This conversion was markedly inhibited by phenformin at a concentration of 5×10^{-4}M (table 4). The conversion of pyruvate C-1 to lipid was very inefficient and was further reduced by the presence of phenformin. In a similar fashion, the oxidation of pyruvate C-2 to carbon dioxide as well as the incorporation of that carbon into lipid was reduced by phenformin.

The levels of acetate oxidation and incorporation into lipid were comparable. The oxidation of C-1 was very much reduced by phenformin. However, the incorporation of acetate into lipid was not influenced by phenformin.

Figure 1 demonstrates the inhibitory effects of a range

TABLE 4

Effect of phenformin on the conversion of specifically labeled pyruvate and acetate to CO_2 and lipid. Radioactivity expressed in thousands of DPM*

		Control	Phenformin (5×10^{-4}M)
Pyruvate-1-C-14	CO_2	288.1 ± 10.0	133.1 ± 11.1
	Lipid	1.6 ± 0.1	0.4 ± 0.1
Pyruvate-2-C-14	CO_2	63.6 ± 10.0	13.1 ± 2.9
	Lipid	55.4 ± 9.0	5.0 ± 0.6
Acetate-1-C-14	CO_2	30.2 ± 4.0	9.1 ± 1.8
	Lipid	36.3 ± 2.3	35.8 ± 3.5

*Values represent the means ± standard deviations of six experimental flasks.

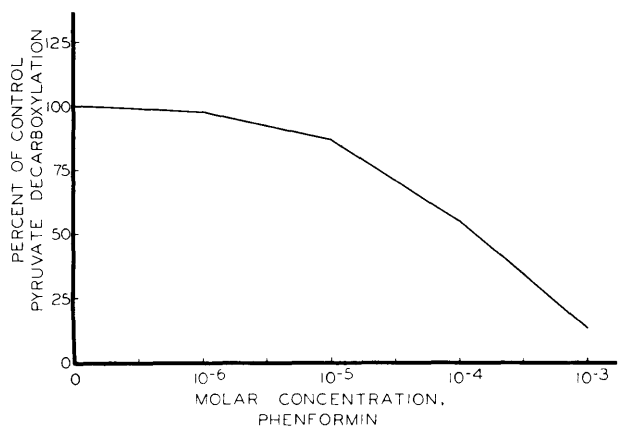

FIG. 1. The effect of various concentrations of phenformin on pyruvate decarboxylation in adipose tissue in vitro. Experimental points represent the means of six experimental values. Values at 10^{-5}, 10^{-4}, 10^{-3} M are significantly decreased from control values ($p < 0.05$).

of phenformin concentrations on pyruvate decarboxylation. The inhibition is first observed at a concentration of 10^{-5}M ($p < 0.05$) and increases with increasing concentrations up to 10^{-3}M at which point a 90 per cent inhibition is produced.

DISCUSSION

The disparity between the oxidation of glucose C-1 and C-6 to CO_2 in adipose tissue has been observed by other investigators[18,19] and provides evidence for the contribution of a nonglycolytic pathway of glucose catabolism. This pathway has been shown to be the pentose cycle. The preferential incorporation of C-6 into lipid is another manifestation of the pentose cycle participation in adipose tissue. The observed stimulatory effects of insulin on the conversion of C-1 to CO_2 and lipid as well as the conversion of C-6 to lipid are in agree-

ment with the findings of earlier investigators.[18,19] In addition, the absence of a stimulatory effect on C-6 oxidation is consistent with the earlier findings.[19]

The stimulatory effect of acetate on the pentose cycle and lipogenesis from glucose has recently been described[20] and the results reported here confirm those findings. Of interest is the observation that C-6 oxidation to CO_2 is also stimulated by acetate. Such a finding, by itself, does not distinguish between the glycolytic pathway and the pentose cycle. However, the absence of a significant stimulatory effect on the incorporation of glucose C-1 into lipid suggests that only the pentose cycle is affected by acetate. Flatt and Ball [20] have calculated that the pentose cycle furnished 65 per cent of the reduced coenzymes required for lipogenesis in the presence of insulin. This figure was increased to 95 per cent when acetate, a substance which utilizes reduced coenzymes without producing them, was added to the incubation mixture. The acetate stimulation pattern is best understood as a direct substrate stimulation of lipogenesis and utilization of reduced coenzymes which results, indirectly, in a stimulation of the pentose cycle, an oxidized coenzyme-requiring process. It would be expected that pyruvate, a substance which yields reduced coenzymes in the course of its metabolism,[21] would have a much lessened stimulatory effect on the pentose cycle but retain the capacity to stimulate lipogenesis.

Phenformin inhibited all oxidation of glucose regardless of the labeling pattern and blocked lipogenesis from glucose. Furthermore, phenformin blocked the stimulatory effects of insulin and acetate on these processes. Several possibilities exist to explain these inhibitions. It would be expected that a direct inhibitory effect of phenformin on the pentose cycle would indirectly reduce lipogenesis by interfering with the major reduced pyridine nucleotide-generating system of adipose tissue. Conversely, a direct inhibitory effect on lipid synthesis would be expected to produce a concomitant reduction in the flow of glucose through the pentose cycle.

Of particular interest is the finding that phenformin blocked the incorporation of glucose C-1 into lipid. Since that process would proceed efficiently only by way of the glycolytic pathway, these findings leave open the possibility that phenformin blocks a step in glucose metabolism beyond the pentose cycle, thereby inhibiting lipogenesis and the pentose cycle. In order to permit the choice between these possibilities, studies with specifically labeled pyruvate and acetate were undertaken. The finding that phenformin blocked the otherwise efficient conversion of the carboxyl carbon of pyruvate to CO_2 provided strong evidence for an inhibitory effect of phenformin at the point of pyruvate decarboxylation. This conclusion was further strengthened by the observation that phenformin blocked the incorporation of pyruvate C-1 into lipid as well as the oxidation or incorporation into lipid of C-2 of pyruvate.

Pyruvate decarboxylation is accomplished by an enzyme complex catalyzing the following over-all reaction:

$$CH_3COCOOH + Co.ASH + DPN^+ \rightarrow Co.ASCOCH_3 + DPNH + H^+ + CO_2.$$

This reaction sequence is oxidative in nature and requires, in addition to NAD, coenzyme A, lipoic acid and thiamine pyrophosphate.[22]

The manner in which phenformin blocks pyruvate decarboxylation is not established, but it appears likely that the reported inhibitions by this compound and other biguanides and guanidines of the phosphorylation steps of the electron transport system[23-26] are adequate to explain the effect. In this respect, the effect of phenformin on pyruvate oxidation may be analogous to the inhibition of a variety of Krebs-cycle oxidations reported by earlier investigators.[3,5,27] Although this mechanism appears adequate to explain the effects of phenformin on pyruvate oxidation, another possibility exists. The finding by Hansen and Henning (1966)[28] that NADH, one of the products of the pyruvate oxidase reaction sequence, inhibits pyruvate oxidation competitively with NAD in *E. coli* provides the basis for another mechanism. Since lactate production was markedly increased by phenformin (table 3) high enough levels of the reduced nucleotide might have been achieved to cause this type of negative feedback inhibition if a comparable effect is seen in mammalian tissues. Studies of the mechanism of the inhibitory action of phenformin on pyruvate decarboxylation are continuing. The finding that phenformin is without effect on partially purified pyruvate dehydrogenase[29] indicates that the inhibitory effect is an indirect one.

Studies with labeled acetate demonstrated clearly that the path of acetate to lipid is not affected by phenformin although acetate oxidation is severely inhibited. This inhibition of acetate oxidation is probably a manifestation of the inhibition of Krebs cycle oxidations reported by several investigators[3,5,27] and is in agreement with the findings of Wick et al.[5] in adipose tissue.

The inhibition of the pentose cycle and lipogenesis by phenformin is readily understood in the light of the blockade of pyruvate decarboxylation. If one considers that the major processes, pentose cycle, glycolysis and

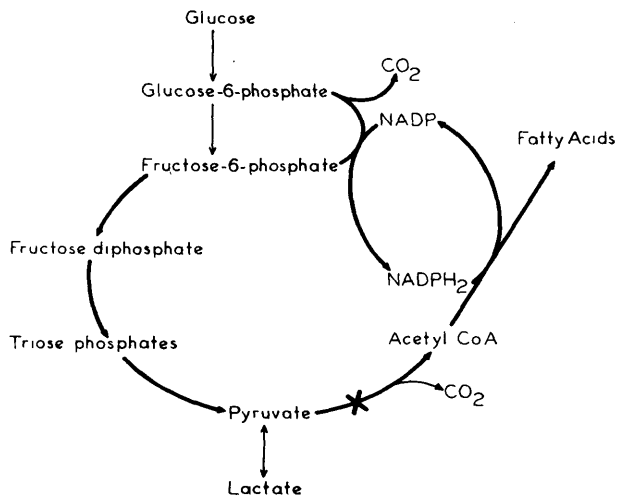

FIG. 2. Diagram representing the manner in which the pentose cycle, glycolysis and lipogenesis are coupled in adipose tissue. Heavy arrows indicate the closed loop pattern of the interactions. Cross indicates the point of interference by phenformin with the pyruvate decarboxylation step.

lipogenesis are coupled together in a closed loop described in figure 2, then the blockade of pyruvate decarboxylation effectively interrupts the loop. In so doing, the flow of carbon through the pentose cycle, the glycolytic pathway and lipogenesis would be disrupted.

The report of hypolipemic effects of phenformin in diabetic humans[30,31] may be a clinical manifestation of the effect at the pyruvate decarboxylation step. Other metabolic effects which may be readily explained on the basis of the inhibition of pyruvate metabolism are the accumulation of lactate in blood[32] and the reduced pyruvate tolerance reported by Fajans et al.[33] The possible relationship between the inhibition of pyruvate decarboxylation and the hypoglycemic effects of phenformin are currently under investigation.

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Copper Deficiency in Malnourished Infants

(Continued from page 851)

stration of copper. Prior to this, the dietary intake of copper had been calculated at 28 μg. per kg. body weight per day. An increase to 42 μg. was without effect, but larger doses of copper (up to 362 μg.) produced a striking response.

It should be mentioned that there was a partial response to ascorbic acid in two of the cases. The authors consider that the response to copper therapy—a response which included myeloid and erythroid elements in blood and marrow, and improvement in bone maturation and architecture—was sufficiently pronounced to warrant a diagnosis of copper deficiency.

This diagnosis is supported by the low levels of serum copper, 43 to 72 μg. per 100 ml., prior to therapy. These can be contrasted to the normal (adult) value of 109 ± 17 μg. per 100 ml. Following therapy, the levels rose to the range of 160 to 183 μg.

The authors suggest that these "cases represent striking examples of growth 'imbalance'—Cu deficiency resulting from growth acceleration produced by a high-calorie, low-Cu intake in infants whose stores were probably low." Hence the situation which they have described would not be apt to occur in everyday practice. The ordinary solid food supplements given to infants contain adequate amounts of copper.

As a result of their experiences, Cordano and coworkers estimate the daily copper requirement "of rapidly growing infants with poor stores" to be in the range of 42 to 135 μg. per kg. body weight. This amount is similar to that required by the growing pig (Nutrition Reviews 9:317, 1951).

Rehabilitation of the malnourished thus presents a special nutritional challenge. Not only must the requirements for protein and calories be met, but the diet must be sufficiently varied to provide for all the essential nutrients, and in amounts which will sustain rapid synthesis of new protoplasm.

From *Nutrition Reviews*, Vol. 23, No. 6, June 1965, pp. 164-66.