Effects of 5-Methylpyrazole-3-Carboxylic Acid on Adipose Tissue

I. Inhibition of Lipolysis, Effects on Glucose, Fructose, and Glycogen Metabolism *in Vitro* and Comparison with Insulin

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SUMMARY

5-Methylpyrazole-3-carboxylic acid in concentrations between 10^{-8} and 10^{-6} M inhibited the glycerol release of adipose tissue of fasted-refed rats. Insulin and 5-methylpyrazole-3-carboxylic acid partially prevented the loss of glycogen even in the absence of any substrate in the medium. Maximal glycogen turnover *in vitro* in the presence of glucose, fructose, and insulin amounted to 5 μmoles of hexose per gram per hour. 5-Methylpyrazole-3-carboxylic acid stimulated the incorporation of fructose-U-^{14}C into glycogen out of proportion to its effects on overall fructose metabolism. This effect was counteracted by insulin but not by phlorizin. Insulin doubled fructose uptake of adipose tissue of fasted-refed rats but did not stimulate the incorporation of fructose-U-^{14}C into glycogen. It stimulated the incorporation of glucose-U-^{14}C into glycogen only at high rates of glucose uptake. These results support the concept that insulin and 5-methylpyrazole-3-carboxylic acid inhibit phosphorylase and increase glycogen synthetase activity. The difference between the activity of these two substances could best be explained by the existence of different pools of glucose 6-phosphate.

INTRODUCTION

Few substances mimic the action of insulin on adipose tissue metabolism. Purified nonsuppressible ILA (1) and phospholipase C (2, 3) and A (4) seem to increase glucose transport and to inhibit lipolysis similarly to insulin. We know of no substance which enhances the glucose transport similarly to insulin and which is devoid of the antilipolytic properties of insulin. These two types of effects seem to be expressions of one and the same action of insulin, presumably on the cell membrane. Inhibition of lipolysis does not depend on glucose transport since it also occurs in the complete absence of glucose or any other substrate in the medium (5–7). Whereas substances which act on glucose transport also seem to inhibit lipolysis, the reverse is not true. Nucleosides, nucleotides (8, 9), prostaglandins (10, 11), and nicotinic acid (12–14) are potent inhibitors of lipolysis with a definite but quantitatively unimportant effect on glucose uptake of adipose tissue. Such substances are of great interest as potential drugs in the treatment of diabetes mellitus. Whenever the concentration of plasma free fatty acids is decreased the muscle depends on glucose as substrate, and glucose consumption increases. These drugs would therefore have the advantage over insulin of indirectly increasing the glucose oxidation by muscle by keeping the level of the free fatty acids low without enhancing the uptake of glucose and its conversion to fat by adipose tissue.

This report deals with the mechanism of action on adipose tissue *in vitro* of 5-methylpyrazole-3-carboxylic acid which is many times as potent as nicotinic acid as an antilipolytic drug (G. C. Gerritsen, personal communication). The antilipolytic and blood sugar-lowering properties of this compound *in vivo* are the subject of a second communication (15).

MATERIALS AND METHODS

Glucose (16), glycerol (17), lactic acid (18), and pyruvic acid (19) were determined enzymically with the reagents of Biochimica Boehringer. $ZnSO_2$ and NaOH were used as precipitating agents. The extraction, isolation, and counting procedures for determination of incorporation of carbon-14 into total lipids, fatty acids of total lipids, and glycogen after incubation with ^{14}C-labeled substrates have been described previously (5, 20).

The incubation medium was a Krebs-Ringer bicarbonate buffer containing albumin. Other details regarding the incubation medium are listed in the legend to each experiment.

Male Osborne-Mendel rats weighing 125–180 g were used while fed with laboratory chow or in the fasted-refed state (5). After a fast of 120 hr duration these rats were refed with a high carbohydrate diet during 24 hours. The epididymal fat pads of 12 or more rats were cut into 12–16 pieces and pooled as described earlier (21).

5-Methylpyrazole-3-carboxylic acid was a gift of Drs. G. C. Gerritsen and W. E. Dulin of the Upjohn Company, Kalamazoo. Recrystallized glucagon-free insulin was used (courtesy Dr. K. Ege, Novo terapeutisk Laboratorium, Kopenhagen). Phlorizin dihydrate was purchased from Fluka AG, Buchs, Switzerland. Albumin fraction V, lot No. 65/08-10 was purchased from the Blutspendedienst of the Swiss Red Cross in Bern. It contained no insulin-like or insulin-inhibitory activity in concentrations up to 6 g/100 ml (21).

RESULTS

The smallest concentration of 5-methylpyrazole-3-carboxylic acid which inhibited glycerol release of adipose tissue of fasted-refed rats *in vitro* was 1×10^{-8} M (Table 1). Inhibition of lipolysis was almost complete at a 10^{-7} M concentration of 5-methylpyrazole-3-carboxylic acid. 5-Methylpyrazole-3-carboxylic acid stimulated the oxidation of glucose-U-^{14}C and its incorporation into total lipids, but not that into

TABLE 1

Effects of 5-methylpyrazole-3-carboxylic acid in various concentrations and of insulin on the glycerol-release and on glucose-U-^{14}C metabolism of adipose tissue of fasted-refed rats

Pooled epididymal adipose tissue of 120 hours fasted–24 hours refed rats was incubated during 4½ hr in a Krebs-Ringer bicarbonate buffer containing 200 mg of albumin and 100 mg of glucose-U-^{14}C per 100 ml. The means of the results of two flasks and their range are given.

Additions to medium	Release into medium (μmoles/g/hr)		Incorporation of uniformly labeled glucose-^{14}C (μmoles/g/hr) into			
	Glycerol	Lactic acid	CO_2	Total lipids	Glycogen	Total
5-Methylpyrazole-3-carboxylic acid						
None	6.91 ±0.22	3.68 ±0.07	0.70 ±0.03	0.97 ±0.02	0.04 ±0.001	1.71 ±0.05
1.0×10^{-8} M	3.31 ±0.12	3.58 ±0.05	0.77 ±0.04	1.23 ±0.07	0.03 ±0.001	2.03 ±0.11
3.1×10^{-8} M	1.82 ±0.16	3.51 ±0.11	0.87 ±0.02	1.18 ±0.01	0.03 ±0.005	2.09 ±0.02
1.0×10^{-7} M	1.37 ±0.05	3.45 ±0.12	0.81 ±0.02	1.28 ±0.07	0.03 ±0.001	2.12 ±0.08
1.0×10^{-5} M	1.05 ±0.01	2.96 ±0.31	1.17 ±0.02	1.74 ±0.01	0.03 ±0.002	2.95 ±0.01
1.0×10^{-3} M	1.00 ±0.01	3.65 ±0.02	1.00 ±0.03	1.42 ±0.05	0.04 ±0.003	2.43 ±0.08
Insulin, 1000 μU/ml	1.64 ±0.07	2.92 ±0.28	3.97 ±0.16	7.66 ±0.48	0.42 ±0.01	12.05 ±0.64

TABLE 2

Effects of 5-methylpyrazole-3-carboxylic acid and of insulin on the metabolism of fructose-U-^{14}C by adipose tissue of fed rats

In contrast to all other experiments pooled epididymal adipose tissue of normal, fed rats was incubated during 3½ hr. The Krebs-Ringer bicarbonate buffer contained 200 mg of albumin per 100 ml and fructose-U-^{14}C in different concentrations. The means of the results of two flasks and their range are given.

Additions to medium	Glycerol release (μmoles/g/hr)	Glycogen of tissue (μmoles/g)	Incorporation of fructose-U-^{14}C (μmoles/g/hr) into				
			CO_2	Fatty acids	Glyceride glycerol	Glycogen	Total
800 mg fructose/100 ml	1.01 ±0.06	2.21 ±0.08	3.54 ±0.25	2.38 ±0.04	0.29 ±0.01	0.45 ±0.03	6.67 ±0.17
800 mg fructose/100 ml + 5-methylpyrazole-3-carboxylic acid 10^{-5} M	0.73 ±0.09	2.59 ±0.09	3.41 ±0.05	2.27 ±0.04	0.31 ±0.01	0.67 ±0.01	6.66 ±0.02
200 mg fructose/100 ml + insulin 1000 μU/ml	0.82 ±0.03	1.22 ±0.06	3.42 ±0.11	2.51 ±0.08	0.29 ±0.08	0.18 ±0.01	6.41 ±0.11
200 mg fructose/100 ml	0.77 ±0.02	1.33 ±0.05	1.74 ±0.02	1.01 ±0.07	0.22 ±0.01	0.28 ±0.01	3.23 ±0.06
200 mg fructose/100 ml + 5-methylpyrazole-3-carboxylic acid 10^{-5} M	0.61 ±0.02	1.55 ±0.07	2.00 ±0.05	1.07 ±0.02	0.19 ±0.02	0.28 ±0.04	3.55 ±0.06
50 mg fructose/100 ml + insulin 1000 μU/ml	0.70 ±0.01	0.64 ±0.02	1.22 ±0.05	0.43 ±0.02	0.17 ±0.01	0.04 ±0.01	1.86 ±0.04

glycogen. The last column in Table 1 shows the sum of the oxidation of glucose-6-^{14}C to $^{14}CO_2$ and of its incorporation into total lipids and glycogen. Maximal stimulation by 5-methylpyrazole-3-carboxylic acid above baseline was 72% compared to 605% by 1 milliunit of insulin per milliliter. The ratio of the stimulatory activity on glucose metabolism to the antilipolytic activity as percentage of the baseline was entirely different for the two drugs, i.e., 5 in the case of 5-methylpyrazole-3-carboxylic acid and 25 in the case of insulin.

The effectiveness of 5-methylpyrazole-3-carboxylic acid on lipolysis of adipose tissue depended on the nutritional state of the animals. Basal glycerol release of fed animals was 2.00 ± 0.19 and it was inhibited to 1.06 ± 0.13 μmoles/g/hr (mean ± SEM, $n = 8$) by 5×10^{-4} M 5-methylpyrazole-3-carboxylic acid. In fasted-refed rats basal glycerol release was 17.3 ± 1.7, and it was decreased to 2.1 ± 0.1 μmoles/g/hr (mean ± SEM, $n = 8$) by 5-methylpyrazole-3-carboxylic acid. When tissue of fed rats was stimulated by 0.1 μg of glucagon per milliliter the glycerol release was 4.86 ± 0.22 and it was inhibited to 2.34 ± 0.40 μmoles/g/hr (mean ± SEM, $n = 4$) by 5-methylpyrazole-3-carboxylic acid. Thus, inhibition of glycerol release was most pronounced in the fasted-refed rats which exhibit a very high rate of spontaneous glycerol release.

The basal glycerol release of adipose tissue of fasted-refed rats and its inhibition by 5-methylpyrazole-3-carboxylic acid were independent of the presence of glucose in the medium. Basal glycerol release of 17.3 ± 1.7 in the absence of glucose was inhibited to 2.1 ± 0.1 μmoles/g/hr ($n = 8$) by 5-methylpyrazole-3-carboxylic acid and from 19.0 ± 0.7 to 2.2 ± 0.1 μmoles/g/hr ($n = 8$) in the presence of 200 mg of glucose per 100 ml.

As shown in Table 2, 5-methylpyrazole-

TABLE 3

Effects of 5-methylpyrazole-3-carboxylic acid, of insulin and of both substances together on the metabolism of fructose-U-^{14}C by adipose tissue of fasted-refed rats

Pooled epididymal adipose tissue of 120 hours fasted–24 hours refed rats was incubated during 3 hours in Krebs-Ringer bicarbonate buffer containing 200 mg of albumin per 100 ml and fructose-U-^{14}C. The results represent the mean values of 4 flasks with the SEM. Student's *t* test was used for statistical evaluation.

Additions to medium	Release into medium (μmoles/g/hr)		Glycogen content of tissue (μmoles/g)	Incorporation of uniformly labeled fructose-^{14}C (μmoles/g/hr) into				
	Glycerol	Lactic acid		CO_2	Fatty acids	Glyceride-glycerol	Glycogen	Total
800 mg fructose/100 ml	7.20 ±0.11	3.84 ±0.12	30.40 ±0.47	3.90 ±0.08	2.61 ±0.08	1.40 ±0.11	0.15 ±0.01	8.06 ±0.07
800 mg fructose/100 ml + 5-methylpyrazole-3-carboxylic acid, 10^{-5} M	1.75 ±0.13	2.85 ±0.06	40.59 ±1.30	4.48 ±0.16	3.61 ±0.09	0.95 ±0.06	1.16 ±0.08	10.20 ±0.25
P:	<0.001	<0.005	<0.005	<0.05	<0.005	<0.025	<0.001	<0.005
200 mg fructose/100 ml + insulin 1000 μU/ml	1.86 ±0.08	1.45 ±0.09	34.12 ±1.98	3.57 ±0.19	3.35 ±0.31	0.75 ±0.17	0.31 ±0.03	7.98 ±0.42
200 mg fructose/100 ml + 5-methylpyrazole-3-carboxylic acid, 10^{-5} M + insulin, 1000 μU/ml	1.82 ±0.03	1.78 ±0.09	34.00 ±1.16	4.34 ±0.15	3.69 ±0.16	0.73 ±0.10	0.40 ±0.01	9.16 ±0.28
P:	>0.40	<0.05	>0.475	<0.05	<0.25	>0.475	<0.10	<0.10

3-carboxylic acid had only little effect on fructose metabolism of adipose tissue of fed rats *in vitro*. The sum of the incorporation of fructose-U-^{14}C into CO_2, total lipids and glycogen was doubled by increasing the concentration of fructose in the medium from 200 to 800 mg/100 ml. 5-Methylpyrazole-3-carboxylic acid did not stimulate fructose metabolism. Contrary to 5-methylpyrazole-3-carboxylic acid, insulin stimulated total fructose incorporation into these metabolic indices approximately 2-fold. The glycogen content of the tissue at the end of incubation appeared to correlate with the fructose concentration in the medium and did not seem to be influenced by insulin nor by 5-methylpyrazole-3-carboxylic acid. Incorporation of labeled fructose into glycogen was proportional to the fructose concentration in the medium rather than to the sum of the incorporation of fructose-U-^{14}C into CO_2 and total lipids. 5-Methylpyrazole-3-carboxylic acid may have stimulated the incorporation of fructose-U-^{14}C into glycogen at a fructose concentration of 800 mg/100 ml.

The effect of 5-methylpyrazole-3-carboxylic acid on glycerol release, glycogen breakdown and on the incorporation of fructose-U-^{14}C into glycogen became more marked when tissue of fasted-refed rats was used (Fig. 1). Without enhancing the sum of fructose-U-^{14}C incorporation into the various metabolic indices to any great extent, 5-methylpyrazole-3-carboxylic acid stimulated the incorporation of fructose-U-^{14}C into glycogen approximately 5-fold and it partially inhibited the breakdown of tissue glycogen. In contrast to 5-methylpyrazole-3-carboxylic acid, insulin did not stimulate fructose incorporation into glycogen out of proportion to its other metabolic effects. At a fructose concentration of 800 mg/100 ml, 5-methylpyrazole-3-car-

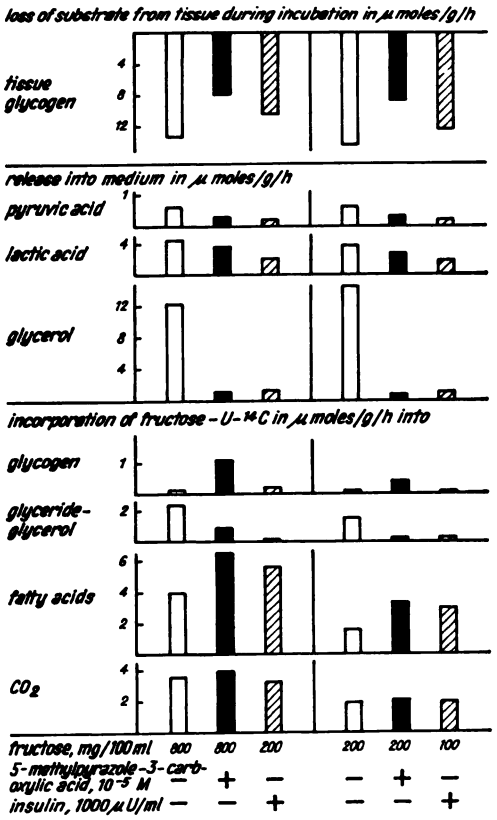

FIG. 1. *Effects of 5-methylpyrazole-3-carboxylic acid and of insulin on fructose-U-^{14}C metabolism of adipose tissue of fasted-refed rats*

Pooled epididymal adipose tissue of 12 rats fasted during 120 hr and refed during 24 hr was incubated in Krebs-Ringer bicarbonate buffer containing 2 g of albumin per 100 ml during 3 hr. The results represent the mean values of two flasks.

boxylic acid stimulated fructose oxidation by 15%, incorporation into fatty acids by 38%, whereas it inhibited its incorporation into glyceride-glycerol to 68% of the baseline. These significant though small effects of 5-methylpyrazole-3-carboxylic acid contrast with the 7½-fold stimulation of fructose-U-^{14}C incorporation into glycogen. Insulin stimulated fructose incorporation into glycogen only 2-fold at comparable rates of total fructose metabolism. The effects of the two drugs do not seem to be additive, as shown in the last two lines of Table 3. Insulin, 5-methylpyrazole-3-carboxylic acid, and both drugs together inhibited the glycerol release to about the same extent, i.e., to approximately 15% of the baseline.

When fructose uptake was stimulated from 8 to 15 μmoles/g/hr by insulin, the inhibition of glycogen breakdown became as marked as under the influence of 5-methylpyrazole-3-carboxylic acid. Still, the latter drug stimulated fructose incorporation into glycogen more markedly than insulin (Table 4). In this particular experiment the basal glycerol release was very high, i.e., 22.85 μmoles/g/hr, and its inhibition by both agents was marked. The antilipolytic effects seemed to correlate with a shift from the incorporation of uniformly labeled fructose into glyceride-glycerol to its incorporation into fatty acids. As also demonstrated by the data shown in Tables 3 and 4, 5-methylpyrazole-3-carboxylic acid, and even more markedly insulin, decreased the release of lactic acid by tissue of fasted-refed rats. In the experiment presented in Table 5, the effects of 5-methylpyrazole-3-carboxylic acid on glucose metabolism of adipose tissue of fasted-refed rats were studied at two concentrations of glucose, i.e., 50 and 800 mg/100 ml. 5-Methylpyrazole-3-carboxylic acid almost doubled the sum of the incorporation of uniformly labeled glucose into CO_2, total lipids, and glycogen at both glucose concentrations. 5-Methylpyrazole-3-carboxylic acid partially blocked the loss of glycogen from the tissue independently of the glucose concentration in the medium. Insulin also reduced the loss of tissue glycogen. However, insulin much more effectively inhibited the loss of tissue glycogen in the presence of 200 mg than of 25 mg of glucose/100 ml. Total glucose metabolism was 24.98 and 12.19 μmoles/g/hr, respectively, under these two conditions. 5-Methylpyrazole-3-carboxylic acid did not significantly stimulate the incorporation of labeled glucose into glycogen, neither did insulin at the low glucose concentration of 25 mg/100 ml. However, when the total glucose incorporation was increased to 25 μmoles/g/hr by 1000 μunits of insulin/ml and 200 mg of glucose/100 ml, incorporation of uniformly labeled glucose into glycogen was markedly stimulated and ac-

TABLE 4

Comparison of the effects of insulin and of 5-methylpyrazole-3-carboxylic acid on fructose-U-^{14}C metabolism of adipose tissue of fasted-refed rats

Pooled epididymal adipose tissue of 120 hr fasted–24 hours refed rats was used. The results represent the mean values of 6 flasks with the SEM. Student's *t* test was applied for statistical evaluation.

Addition to medium	Release into medium (μmoles/g/hr)		Loss of tissue glycogen (μmoles/g/hr)	Incorporation of uniformly labeled fructose-^{14}C (μmoles/g/hr) into				
	Glycerol	Lactic acid		CO_2	Fatty acids	Glyceride-glycerol	Glycogen	Total
I: 200 mg fructose/100 ml	22.85 ± 1.92	7.48 ± 0.69	13.23 ± 2.38	4.16 ± 0.13	1.50 ± 0.06	2.65 ± 0.18	0.02 ± 0.002	8.33 ± 0.35
II: 200 mg fructose/100 ml + 5-methylpyrazole-3-carboxylic acid, 10^{-5} M	2.89 ± 0.32	5.46 ± 0.95	4.21 ± 1.25	4.62 ± 0.16	2.81 ± 0.10	1.20 ± 0.07	0.12 ± 0.004	8.76 ± 0.32
III: 200 mg fructose/100 ml + insulin, 250 μU/ml	5.09 ± 0.82	2.11 ± 0.31	5.03 ± 1.41	7.26 ± 0.13	5.52 ± 0.19	2.03 ± 0.19	0.07 ± 0.003	15.07 ± 0.49
P I ↔ II:	<0.001	>0.10	<0.01	<0.05	<0.001	<0.001	<0.001	>0.20
P I ↔ III:	<0.001	<0.001	<0.025	<0.001	<0.001	<0.05	<0.001	<0.001
P II ↔ III:	<0.025	<0.025	>0.35	<0.001	<0.001	<0.005	<0.001	<0.001

TABLE 5

Comparison of the effects of 5-methylpyrazole-3-carboxylic acid with those of insulin on lipolysis and glucose-U-^{14}C metabolism of adipose tissue of fasted-refed rats at different glucose concentrations

Pooled epididymal adipose tissue of 120 hr fasted–24 hours refed rats was incubated during 3½ hr in Krebs-Ringer bicarbonate buffer containing 2 g of albumin per 100 ml. The results represent the mean values of two flasks and their range.

Additions to medium	Glycerol release (μmoles/g/hr)	Loss of tissue glycogen (μmoles/g/hr)	Incorporation of uniformly labeled glucose-^{14}C (μmoles/g/hr) into			
			CO_2	Total lipids	Glycogen	Total
50 mg glucose/100 ml	23.21 ±0.07	14.96 ±0.39	0.90 ±0.01	0.61 ±0.01	0.07 ±0.001	1.58 ±0.02
50 mg glucose/100 ml + 5-methylpyrazole-3-carboxylic acid, 10^{-5} M	2.19 ±0.15	6.54 ±2.12	1.88 ±0.01	1.96 ±0.04	0.07 ±0.001	3.91 ±0.06
25 mg glucose/100 ml + insulin, 1000 μU/ml	3.79 ±0.19	7.71 ±1.06	5.24 ±0.15	6.78 ±0.30	0.17 ±0.01	12.19 ±0.44
800 mg glucose/100 ml	25.78 ±0.09	11.99 ±1.95	2.53 ±0.16	3.18 ±0.22	0.13 ±0.002	5.84 ±0.39
800 mg glucose/100 ml + 5-methylpyrazole-3-carboxylic acid, 10^{-5} M	2.54 ±0.03	8.82 ±1.76	4.05 ±0.11	5.43 ±0.18	0.28 ±0.003	9.76 ±0.07
200 mg glucose/100 ml + insulin, 1000 μU/ml	3.39 ±0.02	+1.71 ±0.33	9.36 ±0.21	13.19 ±0.42	2.43 ±0.16	24.98 ±0.05

counted for 10% of the total metabolism of glucose. At this high rate of glucose uptake net glycogen synthesis occurred during incubation *in vitro*.

In order to investigate whether 5-methylpyrazole-3-carboxylic acid stimulates fructose incorporation into glycogen also in the presence of glucose in the medium, the experiment represented in Fig. 2 was carried out. 5-Methylpyrazole-3-carboxylic acid increased both glucose-U-^{14}C and fructose-U-^{14}C incorporation into CO_2 and fatty acids while decreasing their incorporation into glyceride-glycerol. Glycogen formation from fructose-U-^{14}C was stimulated 5-fold by 5-methylpyrazole-3-carboxylic acid as in the other experiments in which glucose was absent from the medium. In the presence of fructose the incorporation of glucose-U-^{14}C into glycogen was doubled by 5-methylpyrazole-3-carboxylic acid. Insulin led to a 3-fold increase of the sum of the incorporation of both hexoses into CO_2, total lipids and glycogen. Whereas insulin had not or only slightly stimulated the incorporation of fructose-U-^{14}C into glycogen in the absence of glucose (Tables 3 and 4) it led to a stimulation of this metabolic index in the presence of glucose.

Phlorizin dihydrate was added to the medium in a concentration of 10^{-2} M at which it inhibited glucose metabolism of adipose tissue of fasted-refed rats to 17% of the baseline. Phlorizin decreased the sum of the incorporation of fructose-U-^{14}C into CO_2, total lipids, and glycogen from 7.84 to 4.70 μmoles/g/hr, but it did not affect its incorporation into glycogen (0.16 versus 0.17 μmole/g/hr). 5-Methylpyrazole-3-carboxylic acid stimulated the total fructose metabolism to 9.88 μmoles/g/hr and the incorporation into glycogen to 0.86 μmole/g/hr. In the presence of phlorizin and 5-methylpyrazole-3-carboxylic acid, the total fructose metabolism was reduced to 5.66 μmoles/g/hr but fructose incorporation into glycogen remained at an elevated level of 0.59 μmole/g/hr.

A different experimental approach was used in the last experiment, the results of which are given in Fig. 3 and Table 6. Adipose tissue of 120 hours fasted–12 hours refed rats was preincubated during 4 hours in the presence of 200 mg of glucose and 800 mg of fructose/100 ml, both hexoses

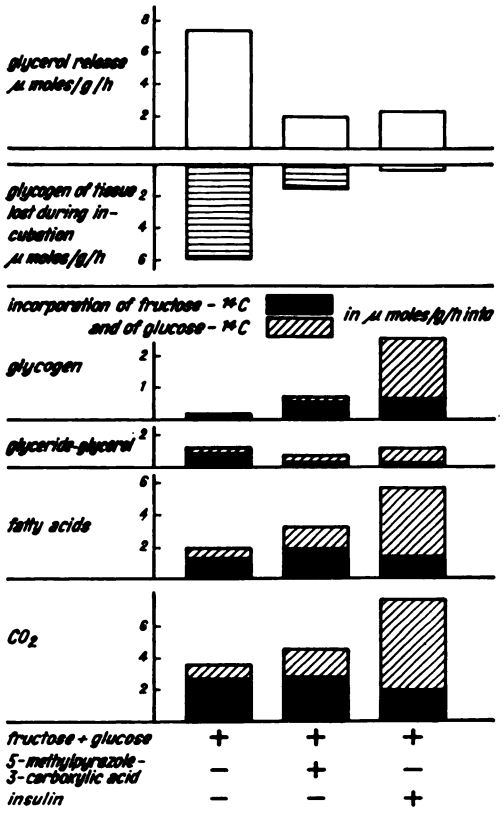

FIG. 2. *Interrelation between the metabolism of fructose-U-¹⁴C and glucose-U-¹⁴C by adipose tissue of fasted-refed rats and effects of 5-methylpyrazole-3-carboxylic acid and of insulin thereupon*

Pooled epididymal adipose tissue of 120 hr fasted–24 hr refed rats was incubated during 2 hr in Krebs-Ringer bicarbonate buffer containing 200 mg of albumin per 100 ml. Insulin was used in a concentration of 1000 μunits/ml and 5-methylpyrazole-3-carboxylic acid in a concentration of 10^{-5} M. The fructose concentration was 800 mg, that of glucose 200 mg, per 100 ml. All flasks contained both hexoses, only one carrying the label in paired flasks. The results of the incorporation of carbon-14 in the paired flasks were added together as shown in the figure. The bars give the mean values of two flasks.

being uniformly labeled. Insulin was added in a concentration of 1 milliunit/ml. At the end of the preincubation a pooled sample of the tissue was used for the measurement of the counts in the total lipids and in glycogen and of the glycogen content. The

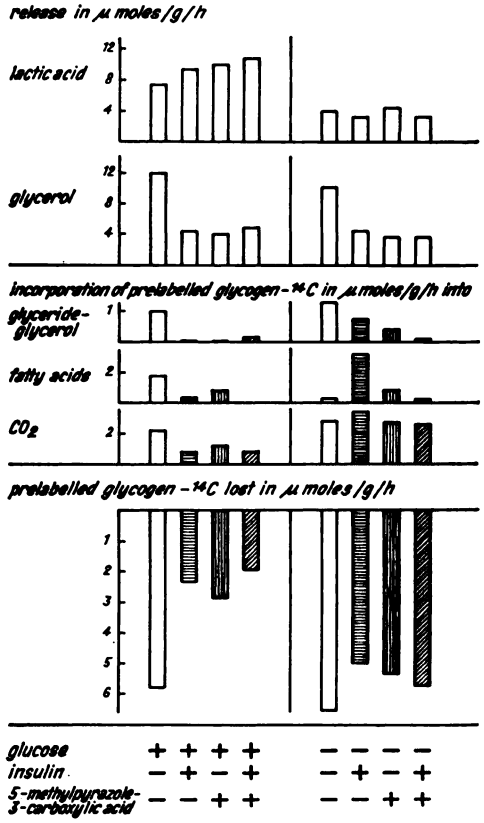

FIG. 3. *Metabolism of prelabeled glycogen of adipose tissue of fasted-refed rats under the influence of 5-methylpyrazole-3-carboxylic acid and insulin in the presence and absence of glucose in the medium*

Intact epididymal fat pads of 20 rats which were fasted during 120 hr and refed during 12 hr were incubated during 4 hr in Krebs-Ringer bicarbonate buffer containing 500 mg of albumin, 800 mg of fructose, and 200 mg of glucose per 100 ml. Both hexoses were uniformly labeled with carbon-14 and their specific activity was the same. The concentration of insulin was 1000 μunits/ml. At the end of this preincubation 4 pads were used for the determination of the glycogen content and the carbon-14 activity in the glycogen, total lipids, and fatty acids of the tissue. The remaining 16 pads were thoroughly washed during 15 min with an ample amount of buffer and pooled into 16 flasks. These contained 1.5 g of albumin/100 ml, no labeled hexoses, and the additions as mentioned in the text of the figure. The incubation lasted 1½ hr. The difference between the counts recovered in the various metabolic indices after the preincubation and after the final incubation was divided by the specific activity of hexose-

remaining pooled tissue was distributed in flasks containing 3 variables, i.e., glucose, insulin, and 5-methylpyrazole-3-carboxylic acid. The carbon-14 of the glycogen was traced during the final incubation. Insulin and 5-methylpyrazole-3-carboxylic acid inhibited the loss of prelabeled glycogen markedly when glucose was present in the medium of the final incubation. The accumulation of carbon-14 from prelabeled glycogen in glyceride-glycerol was prevented by both antilipolytic agents in the presence as well as in the absence of glucose in the medium, whereas the accumulation of carbon-14 in the fatty acids and in CO_2 was prevented by these drugs only in the presence of unlabeled glucose in the medium during the final incubation. The glycogen balance during the final incubation is given in Table 6. The agreement between the loss of total and of ^{14}C-labeled glycogen during the final incubation in the absence of glucose was fairly good. This indicated that the glycogen at the end of the preincubation with labeled hexoses had the same specific activity as the hexoses in the medium. Therefore, the glycogen turnover in such tissue must be extremely rapid. In this particular experiment approximately 20 μmoles of glycogen were totally replaced by newly synthesized glycogen per gram during the preincubation of 4 hr duration. In the presence of glucose in the medium, the total loss of glycogen was much smaller than in its absence. The breakdown of ^{14}C-labeled glycogen exceeded that of chemically determined glycogen, an indication that synthesis of glycogen from cold glucose was occurring (see column glycogen "formed" in Table 6).

DISCUSSION

Adipose tissue of fasted-refed rats is a useful model for the study of lipolysis and glycogen metabolism *in vitro* (5, 6). This tissue contains large amounts of glycogen which disappear rapidly during incubation *in vitro* (5). Furthermore, the spontaneous glycerol release of adipose tissue of fasted-refed rats is equal or greater than that of tissue under maximal stimulation with epinephrine or other lipolytic hormones. Spontaneous lipolysis *in vitro* of adipose tissue of fasted-refed rats differs from that of hormonally induced lipolysis inasmuch as no free fatty acids are released (5). The fatty acids which are hydrolyzed seem to be instantaneously reesterified by α-glycerol-phosphate originating from the large glycogen stores.

Insulin has been shown to be a potent inhibitor both of lipolysis and of glycogen breakdown in tissue of fasted-refed rats *in vitro* (5). The experiments reported here were undertaken to get a better insight into the mechanism of action of insulin and of another potent antilipolytic substance, 5-methylpyrazole-3-carboxylic acid. The antidiabetic and free fatty acid-lowering properties of this drug were first described by Smith *et al.* (22). An explanation for these effects *in vivo* was offered by Gerritsen and Dulin (23), who found that 5-methylpyrazole-3-carboxylic acid inhibited the release of free fatty acids from adipose tissue *in vitro*.

5-Methylpyrazole-3-carboxylic acid, like insulin, very effectively inhibited the glycerol release and partially blocked the glycogen breakdown of adipose tissue of fasted-refed rats *in vitro* (5). The smallest effective concentration was found to be 1×10^{-8} M, and maximal effects were reached at a concentration between 10^{-7} and 10^{-6} M (Table 1). In contrast to insulin, 5-methylpyrazole-3-carboxylic acid stimulated the glucose uptake of adipose tissue of fed and of fasted-refed rats only to a minor, although significant, degree. The ratios of the stimulatory action on glucose metabolism to the antilipolytic activity were estimated to be 25 for insulin and 5 for 5-methylpyrazole-3-carboxylic acid (glucose uptake as percentage of baseline divided by glycerol release as percentage of baseline).

^{14}C in the preincubation medium and the results are expressed as micromoles of hexose-^{14}C of the prelabeled glycogen. The assumption was made that this was the main or only labeled substrate which was further metabolized during the second incubation. The results give the mean values of two flasks.

TABLE 6

Balance of prelabeled glycogen in adipose tissue of fasted-refed rats under the influence of 5-methylpyrazole-3-carboxylic acid and of insulin in the presence and absence of glucose in the medium

Same experiment as in Fig. 3. For explanation see legend to Fig. 3.

Additions to medium	Glycogen lost (μmoles glucose/g/hr)	Glycogen lost (μmoles glucose-^{14}C/g/hr)	Glycogen "formed" (μmoles/g/hr)	Glycogen lost (μmoles glucose/g/hr)	Glycogen lost (μmoles glucose-^{14}C/g/hr)	Glycogen "formed" (μmoles/g/hr)
	No glucose			200 mg glucose/100 ml		
None	6.83 ± 0.72	6.52 ± 0.07	—	3.35 ± 0.42	5.80 ± 0.12	2.45
Insulin, 1000 μU/ml	2.52 ± 1.02	5.05 ± 0.26	2.53	0.32 ± 0.72	2.36 ± 0.48	2.04
5-Methylpyrazole-3-carboxylic acid, 10^{-6} M	5.88	5.23	—	0.05 ± 0.22	2.83 ± 0.44	2.78
Insulin + 5-methylpyrazole-3-carboxylic acid	5.86 ± 0.72	5.73 ± 0.21	—	+0.09 ± 1.18	1.96 ± 0.12	1.87

The so-called hormone sensitive lipase is generally believed to be the enzyme which limits the rate of lipolysis in adipose tissue [for references see review by Vaughan and Steinberg (24)]. This study demonstrates that lipolysis may be very active in the absence of any activating hormones in the medium. Insulin as well as 5-methylpyrazole-3-carboxylic acid seems to inhibit both the hormone-activated enzyme system and the system responsible for spontaneous lipolysis of tissue of fasted-refed rats. This makes it likely that the enzymes involved in these two types of lipolysis are the same.

Glycogen turnover in tissue of fasted-refed rats is very rapid as demonstrated by the experiments shown in Figs. 2 and 3 and Table 6. After an incubation of 4 hours' duration in the presence of labeled glucose, fructose, and insulin, the tissue glycogen had the same specific activity as the hexoses in the medium. Thus, the total amount of glycogen present in the tissue at the beginning of the experiment, i.e., 20 μmoles of glycogen per gram, was replaced by labeled hexoses from the medium *in vitro*. This would yield a turnover of approximately 5 μmoles of glycogen per gram per hour. This value is somewhat higher than that obtained in the experiment shown in Fig. 2. During this experiment the glycogen content of tissue incubated *in vitro* remained constant at a time when 2.6 μmoles of uniformly labeled hexoses were incorporated into glycogen per gram per hour. These data confirm earlier observations of Frerichs and Ball according to which phosphorylase activity was increased in tissue of fasted-refed rats (25). Nevertheless, net glycogen synthesis occurred in this tissue implying that the enzyme system responsible for glycogen synthesis was even more active than phosphorylase.

Insulin affected fructose metabolism less markedly than glucose metabolism of adipose tissue (5, 20). When fructose served as the substrate similar values of the sum of the incorporation of carbon-14 to CO_2, total lipids and glycogen were obtained with 800 mg and 200 mg of fructose/100 ml, respectively, in the absence and presence of insulin. Incorporation of fructose-U-^{14}C into glycogen was not stimulated, but rather decreased, by insulin (Table 2). If one considers that insulin inhibited the glycogen breakdown, thereby reducing the dilution of labeled hexose esters by preformed unlabeled hexose esters one must conclude that insulin did not activate overall glycogen synthesis.

In contrast to insulin, 5-methylpyrazole-3-carboxylic acid stimulated the incorporation of fructose-U-^{14}C into glycogen out of proportion to its effects on fructose oxidation and incorporation into total lipids. Insulin prevented the stimulatory effect of 5-methylpyrazole-3-carboxylic acid on fructose incorporation into glycogen (Table 3). Phlorizin, which competes with glucose for transport (26) reduced the total metabolism of fructose to a small extent, but did not inhibit the effects of 5-methylpyrazole-3-carboxylic acid on fructose incorporation into glycogen. In other experiments in which glucose uptake and incorporation into glycogen was maximally stimulated by insulin, 5-methylpyrazole-3-carboxylic acid still very effectively increased the simultaneous incorporation of fructose into glycogen.

These data can only be interpreted in the light of earlier experiments which showed that glucose and fructose are transported into the adipose tissue cell in an independent, noncompetitive manner (20). Since insulin does not enhance the incorporation of fructose into glycogen it appears to activate fructose transport into a compartment which does not favor glycogen synthesis. Phlorizin, on the other hand, which reduced fructose uptake only to a small degree, did not diminish the incorporation of fructose into glycogen in the absence as well as in the presence of 5-methylpyrazole-3-carboxylic acid. This finding supports the view that activation of facilitated glucose diffusion may not favor glycogen synthesis because of an unequal compartmental distribution. In contrast to insulin, 5-methylpyrazole-3-carboxylic acid stimulated the incorporation of fructose-U-^{14}C into glycogen several-

fold without enhancing its incorporation into total lipids and CO_2 to a comparable extent.

Insulin and 5-methylpyrazole-3-carboxylic acid inhibit the breakdown of glycogen of tissue of fasted-refed rats, both in the presence as well as in the absence of a substrate in the medium. It is impossible to decide on the basis of our data whether these drugs inhibit phosphorylase or whether they favor recycling of glucose 6-phosphate into glycogen. According to Jungas (27) insulin has a dual effect. It inhibits phosphorylase and increases the activity of glucose 6-phosphate independent glycogen synthesis by decreasing the formation of cyclic 3′,5′-AMP (27). Inhibition of lipolysis by insulin has also been explained by a decrease of the tissue level of cyclic 3′,5′-AMP (28, 29).

We were hoping to obtain a better understanding of the mechanism of action of these drugs by labeling the tissue glycogen during a preincubation and by subsequently following its metabolic fate. The results presented in Table 6 tend to show that the loss of prelabeled glycogen was partially inhibited by 5-methylpyrazole-3-carboxylic acid and by insulin. Both drugs were much more effective in the presence of glucose than in its absence. The loss of total glycogen was smaller in the presence of glucose in the medium, indicating that formation of glycogen during the second incubation occurred. Glycogen synthesis during the second incubation was not further stimulated by insulin nor by 5-methylpyrazole-3-carboxylic acid. As shown in Fig. 3, the carbon-14 of the prelabeled glycogen was traced in CO_2 and total lipids. In the absence of glucose, insulin increased fatty acid synthesis from labeled glycogen, both drugs decreased its incorporation into glyceride-glycerol whereas they did not change oxidation to $^{14}CO_2$. In the presence of glucose in the medium, insulin and 5-methylpyrazole-3-carboxylic acid decreased not only the incorporation of carbon-14 into glyceride-glycerol but also that into fatty acids and CO_2. In the case of insulin this effect might be explained by dilution of the pool of labeled glucose 6-phosphate by the simultaneous stimulation of the uptake of unlabeled glucose. However, 5-methylpyrazole-3-carboxylic acid which does not stimulate glucose uptake of adipose tissue *in vitro* to the same extent also inhibited the conversion of prelabeled glycogen to $^{14}CO_2$, fatty acids and glyceride-glycerol. These results support our concept that insulin and 5-methylpyrazole-3-carboxylic acid influence the glycogen metabolism of adipose tissue of fasted-refed rats mainly by inhibiting glycogen degradation. In addition, accessibility of glucose 6-phosphate for glycogen synthetase may be more important for the glycogen balance than the activity of glycogen synthetase.

Søvik (30) has presented evidence that insulin regulates glycogen synthesis of muscle by increasing the intracellular concentration of glucose 6-phosphate in addition to its effect on glycogen synthetase (31, 32). Two pools of glucose 6-phosphate seem to exist in muscle (33, 34). According to Landau and Sims (34) the insulin responsive pool of glucose 6-phosphate may also exist in adipose tissue. Insulin appears to activate glycogen synthesis primarily by increasing the concentration of glucose 6-phosphate whereas 5-methylpyrazole-3-carboxylic acid appears to activate glycogen synthesis in a compartment of the cell to which only fructose which is not transported into the cell by way of the facilitated glucose carrier has access. The difference between muscle and adipose tissue with respect to glycogen metabolism and the insulin effects thereupon is intriguing and merits further investigation.

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