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ENDOGENOUS HYDROGEN PEROXIDE AND PEROXIDATIVE METABOLISM IN ADIPOCYTES IN RESPONSE TO INSULIN AND SULFHYDRYL REAGENTS

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Abstract—Evidence is presented that glucose oxidation in adipocytes via the pentose phosphate pathway, known to be regulated essentially by the supply of $NADP^+$ in the presence of oxidized glutathione, is increased in response to insulin due to a peroxidative catabolism of endogenously produced hydrogen peroxide. A similar response was observed with sulfhydryl reagents, e.g. dithiothreitol (DTT) and *p*-chloromercuribenzoate (pCMB), both of which inhibit D-glucose transport and glucose oxidation by the glycolytic pathway, but like insulin enhance the pentose phosphate pathway. A catalase-dependent peroxidative reaction, viz. $^{14}CO_2$ production from sodium [^{14}C]formate, was increased about 2-fold in cells exposed to insulin, or DTT and pCMB. Its partial inhibition in the presence of 50 mM 3-amino-1,2,4-triazole was greater in the insulin-, DTT- or pCMB-treated cells than in the control. This indicated intracellular accumulation of catalase:H_2O_2 complexes. The data on the catalatic and peroxidative activities of catalase in the adipocytes under these experimental conditions, coupled with the preferential stimulation of the pentose phosphate pathway in the presence of inhibitors of catalase, e.g. aminotriazole and azide, indicate a major role of glutathione peroxidase in the disposal of H_2O_2. These findings support our previous report that insulin or —SH reactive agents activate a pyridine nucleotide oxidase in the plasma membrane of these cells [S. P. Mukherjee and W. S. Lynn, *Fedn Proc.* **35**, 1694 (1976); *Archs Biochem. Biophys.* **184**, 69 (1977)]. A close relationship was observed between the cellular content of glutathione (GSH) and glucose utilization. Thus, induction of a peroxidative metabolism within these cells, coupled with glucose oxidation, appears to be a major effect of insulin.

The major biological effect of insulin in adipose cells is enhanced glucose consumption to support the lipogenic and energy-requiring metabolic process [1–3]. It is believed that stimulation of glucose transport by insulin is the rate-limiting step for intracellular glucose utilization in these cells [4, 5]. Mukherjee and Lynn [6, 7] first reported experimental evidence that the effect of insulin on glucose transport parallels that on glucose oxidation via the glycolytic pathway, but that a distinct control for glucose oxidation exists in these cells, apparently independently of the regulation of glucose transport by insulin. This was evident from the finding that, even under conditions designed to inhibit glucose transport, glucose oxidation is preferentially stimulated via the pentose phosphate pathway in response to insulin (this differential effect is seen even at low glucose) or to some agents which are known to mimic the action of insulin on glucose oxidation, such as thiols [8] and other sulfhydryl reagents [9–12]. Earlier, it was shown that insulin-dependent activation of D-glucose transport in skeletal [13] and cardiac muscle [14] requires functional sulfhydryl groups in the plasma membrane. The present report follows our recent discovery that an oxidase activity, utilizing reduced NADP in the plasma membrane of rat fat cells, is stimulated when the intact cells are exposed to insulin or sulfhydryl reagents. A product of this reaction, H_2O_2, may accumulate *in vivo* and should be metabolized within the cells. We provide evidence that these cells can efficiently utilize the endogenous H_2O_2 by peroxidative functions of glutathione peroxidase and catalase, finally to funnel glucose through the pentose phosphate pathway (ppp).

Thus, an interesting parallel is found between the action of insulin on a target cell and the metabolic pattern of other cells known to generate H_2O_2. For adipose cells, the pentose phosphate pathway of glucose metabolism and *de novo* fatty acid synthesis are believed to be interdependent for the co-factor requirement. On the other hand, previous studies by Mukherjee [15] indicated that, in ageing and naturally obese rat adipose tissue, a substantial decline in both glucose oxidation via the ppp and in the lipogenic enzyme activities is experimentally distinguishable by adding NADPH-oxidizing agents which increase the ppp without affecting lipogenesis. Thus, the lack of the pentose phosphate pathway activity under the insulin-refractory state appears to be due to a higher cytoplasmic NADPH/$NADP^+$ ratio. The present report demonstrates that insulin may induce a peroxidative metabolism in these cells by modulating the activities of glutathione peroxidase and of catalase. We have also observed that alterations in cellular glutathione (GSH) content are closely linked with glucose utilization. This suggests that a co-ordinate control of the cytoplasmic redox potential by insulin may be a major focus of the biological effects of the hormone.

MATERIALS AND METHODS

Isolated adipocytes obtained by collagenase digestion of parametrial adipose tissue [16] from female rats (CD Strain, Charles River Co.) of 200–225 g body

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weight were employed in these studies. Fat pads were pooled from a number of rats, cut into small pieces, and distributed in 1–4 g into 1 oz polyethylene bottles containing 3–8 ml of 3% bovine serum albumin in Krebs–Ringer bicarbonate buffer and 1 mg of crude collagenase/ml (*Clostridium histolyticum*, Worthington Biochemical Corp, Freehold, NJ), and incubated at 37° for 1 hr on a Dubnoff metabolic shaker with moderate shaking. The composition of the buffer was: 126 mM NaCl, 1.4 mM $MgSO_4$, 5.2 mM KCl, 4 mM $NaHPO_4$ and 12 mM $NaHCO_3$. The buffer was pregassed with a mixture of O_2–CO_2, 95:5. At the end of digestion the cells were filtered through a double layer of cheesecloth and washed twice with approx 4 vol. of 1% albumin buffer and suspended in this medium. Aliquots of 0.2 ml of cell suspension were added to plastic culture tubes (17 × 100 mm) which contained 1 ml of the 3% albumin-buffer plus 0.2 mM ^{14}C-labeled glucose for assay by monitoring the production of $^{14}CO_2$ from [6-^{14}C]- and [1-^{14}C] D-glucose. The tubes were stoppered immediately with rubber serum-stoppers with a center well containing a strip of filter paper. Oxidation of sodium [^{14}C]formate by adipocytes was measured in the presence or absence of insulin or sulfhydryl reagents, e.g. dithiothreitol (DTT) or *p*-chloromercuribenzoate (pCMB), as evidence for H_2O_2 formation. For indirect evidence of endogenous H_2O_2 accumulation, sodium azide or aminotriazole, inhibitors of catalase, were added to the assay of glucose oxidation, in the presence or absense of insulin, DTT or pCMB. After incubating the assay tubes at 37° on a Dubnoff metabolic shaker, the reaction was stopped by injecting 0.2 ml 1 N H_2SO_4 into the medium; the $^{14}CO_2$ liberated was trapped with phenethylamine (New England Nuclear, Boston, MA) which was injected into the filter paper strip.

A blank correction was made by incubating some tubes with the albumin medium containing only [^{14}C]formate. This yielded only negligible counts, indicating that no significant contaminant of carbonate was present in the commercial Na-formate. Uptake of 2-deoxy-D-glucose by adipocytes was measured by the oil-separation techniques of Glieman *et al.* [17]. The possibility of lipid peroxidation was examined by the thiobarbuturic acid procedure [18].

Catalase activity in the soluble supernatant fraction (105,000 *g*) of adipocytes exposed to insulin, DTT or pCMB, with or without Na 3-amino-1,2,4-triazole, was estimated by the procedure adopted from Maehly and Chance [19] as follows: 5 ml of 6×10^{-3} M H_2O_2 in 10^{-2} M Na phosphate buffer, pH 7.0, was incubated with 0.5 ml of experimental samples (approx. 1.0–1.5 mg protein) for 3 min at 0°. The reaction was stopped by the addition of 1 ml of 6 N H_2SO_4. The residual H_2O_2 was titrated with 0.01 N $KMnO_4$. The basal (untreated) activity was assumed to represent 100 per cent activity.

Estimation of cellular glutathione (GSH) was done as follows. Adipose cells, suspended in Krebs–Ringer phosphate buffer, pH 7.4, containing 0.1% bovine serum albumin (cells were intact at this concentration of albumin), were incubated in 17 × 100 mm polypropylene tubes at 37° in a moderately shaking water bath, in the presence of insulin, DTT or pCMB, with or without 0.2 mM D-glucose. At intervals, 1.0 ml of uniform suspensions was withdrawn, diluted four times with warm buffer, and centrifuged for 10 sec; the media were separated by aspiration. The cells were suspended in a small volume of 10 mM phosphate buffer, pH 7.4. The tubes were rapidly chilled in ice-water and homogenized and centrifuged at 0–4° in a Sorvall centrifuge at 27,000 *g* for 5 min. The congealed fat-cakes were removed and 0.98-ml aliquots were added to tubes containing 0.02 ml of a 10 mM solution of 5,5′-dithio-bis-(2-nitrobenzoic acid). After 10 min of incubation at room temperature (25°), 3.0 ml absolute ethanol was added to each tube to deproteinize the samples. The clear supernatant fractions after centrifugation were read at 412 nm on a Beckman spectrophotometer.

The radiochemicals, viz. [1-^{14}C]- and [6-^{14}C]D-glucose, 2-deoxy[H^3]D-glucose and Na[^{14}C]formate, were purchased from New England Nuclear. Dithiothreitol and *p*-chloromercuribenzoic acid were purchased from the Sigma Chemical Co., St. Louis, MO; 5,5′-dithio-bis-(2-nitrobenzoic acid) Ellman's reagent) and 3-amino-1,2,4-triazole were purchased from Aldrich Chemical Company, Milwaukee, WI. The other inorganic chemicals, all of analytical grade, were supplied by Fisher Scientific Co., Fairlawn, N.J.

RESULTS

Glucose oxidation by adipocytes in response to insulin and —SH reagents. The total glucose oxidation by adipocytes was stimulated in the presence of insulin, as expected (Fig. 1). Sulfhydryl reagents, such as dithiothreitol and *p*-chloromercuribenzoate, also produced about a 2.5- to 3-fold increase in $^{14}CO_2$ production from [1-^{14}C]glucose, while they inhibited that from [6-^{14}C]glucose by about 40–50 per cent of the basal rate. The optimal dosage for these differential effects of the sulfhydryl reagents was 10^{-3} M. These data suggest a stimulation of the pentose phosphate pathway activity. Both DTT and pCMB inhibited the uptake of 2-deoxy-D-glucose into fat cells (Table 1) to the same extent as they inhibited the glycolytic pathway, as evident from the decline in the $^{14}CO_2$ production from [6-^{14}C]glucose (Fig. 1). The sulfhydryl oxidants, such as hydrogen peroxide and *O*-iodosobenzoate, on the contrary, stimulated the uptake of the sugar. This analogue of D-glucose is not metabolized after the initial phosphorylation reaction and, therefore, its uptake is a suitable measure of the changes in hexose transport.

This differential effect of the sulfhydryl reagents on glucose utilization was due to their interaction with the plasma membrane components which is also the expected site of action for insulin [1–4]. Neither insulin nor the sulfhydryl reagents affected the rates of glucose oxidation by the cell-free homogenates (not illustrated).

We have already shown that exposure of fat cells to insulin or sulfhydryl reagents causes activation of NADPH oxidase in the plasma membrane, yielding H_2O_2 and $NADP^+$ [20, 21]; the intracellular H_2O_2 can be detected by modifying its catabolism. We added two inhibitors of catalase, viz. sodium azide and 3-amino-1,2,4-triazole to the assay medium of glucose oxidation. The basal rate and the insulin stimulation of [6-^{14}C]glucose oxidation were inhibited (Fig. 1) by sodium azide. Our data also suggest a direct

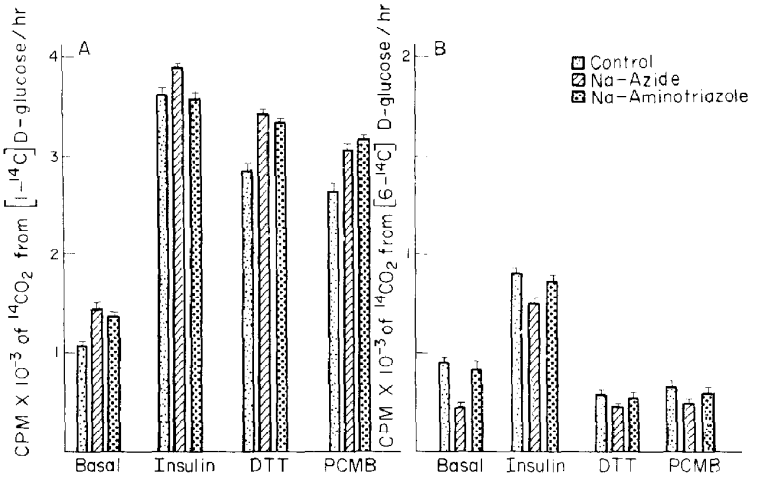

Fig. 1. Effects of inhibitors of catalase activity upon oxidation of glucose by the pentose phosphate pathway and the glycolytic pathway. Sodium azide (10^{-3} M) or sodium aminotriazole (5×10^{-2} M) was added to cells during assay of $^{14}CO_2$ production/hr from glucose (0.2 mM), labeled at the 1-carbon (panel A) and the 6-carbon (panel B). These additions were made to cells which were pre-incubated with or without insulin (0.2 mU/ml) or dithiothreitol or *p*-chloromercuribenzoate (each at 10^{-3} M). There were 2×10^6 cells/assay. Specific activity of [^{14}C] glucose: 10^{-7} Ci/μmole.

Table 1. Modification of 2-deoxy-D-glucose uptake in adipocytes by insulin and sulfhydryl reagents*

Treatment of cells	Glucose uptake (nmoles/4 min/10^6 cells)
Control	2.34 ± 0.62
Insulin	5.76 ± 1.08
H_2O_2	6.54 ± 0.42
O-iodosobenzoate	8.28 ± 1.4
Dithiothreitol	0.82 ± 0.21
pCMB	1.1 ± 0.42

* Cells were incubated with or without insulin (0.24 mU/ml), H_2O_2 (1 mM), *O*-iodosobenzoate (1 mM), dithiothreitol (1 mM) or *p*-chloromercuribenzoate (1 mM) for 30 min, and 2-deoxy-D-glucose (final concentration 0.2 mM, about 1 μCi/assay) was added; aliquots of 50 μl cells were withdrawn after 4 min, and separated by centrifugation in 0.45-ml tubes, on Dow Corning silicon oil (specific gravity: 0.97), followed by a wash with 100 μl of warm buffer. Values are averages ± S. E. of four experiments.

relationship between glucose uptake and the glycolytic pathway. But the stimulation of the pentose phosphate pathway was further amplified in the presence of azide or aminotriazole (Fig. 1). This effect was apparently the result of channelling of the additional H_2O_2 spared by catalase to the glutathione peroxidase system. Aminotriazole did not affect the glycolytic pathway.

Formate peroxidation in adipocytes. Oxidation of formate in cells is recognized as a catalase-dependent peroxidative reaction [22–27]. Our data (Fig. 2) illustrate that this reaction was increased by about 2-fold in the cells exposed to insulin, DTT or pCMB. Inhibition of this reaction by aminotriazole is generally accepted as a measure of hydrogen peroxide accumulation [22, 23, 25–30]. In the presence of 50 mM aminotriazole, this reaction was inhibited to a greater extent (by 25 per cent of the control) in the cells which were exposed to insulin or the —SH reagents than in the untreated cells (by 15–18 per cent of the control). Nitrite inhibits formate peroxidation by substituting for this substrate in the catalase:H_2O_2 complex I [23–28]. This effect is also illustrated in Fig. 2.

The greater inhibition of formate peroxidation by aminotriazole in cells treated with insulin or the sulfhydryl reagents indicates that endogenous H_2O_2 accumulates in these cells as a catalase:H_2O_2 complex

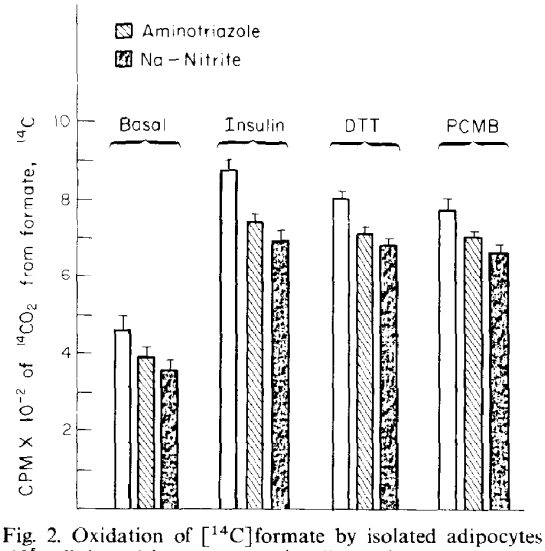

Fig. 2. Oxidation of [^{14}C]formate by isolated adipocytes (10^5 cells/assay) in response to insulin (0.24 mU/ml), dithiothreitol (DDT, 10^{-3} M) or *p*-chloromercuribenzoate (pCMB, 10^{-3} M). Sodium 4-amino-1,2,4-triazole (50 mM) or sodium nitrite (10 mM) was added to the assay medium. The data are averages ± S. E. of six paired experiments. The difference in the inhibition by aminotriazole is significant between basal and insulin ($P < 0.001$) or DDT ($P < 0.005$), or pCMB ($P < 0.02$).

Table 2. Catalase activity in adipocyte supernatant fraction*

Pretreatment of cells	A Untreated	B Aminotriazole	C Nitrate	D Nitrite + aminotriazole
		(Per cent activity)		
(1) Control	100	68	58	86
(2) Insulin	86	45	40	110
(3) DTT	89	46	42	106
(4) pCMB	92	56	46	98

* Values are averages of six paired experiments. The assay procedure is described in Materials and Methods. Values are expressed as per cent of catalase activity in the untreated control. In column D, sodium nitrite (10 mM) was added 30 min before aminotriazole. The total period of exposure to aminotriazole or nitrite was 1 hr. Activity was completely lost in the presence of 0.5 mM sodium azide. The differences are significant between 1A and 1B, 1C; 1A and 1C and 1D; 1A and 2A, 3A; 2A and 2B, 2C; 2C and 2D; 1B and 2B; 3B and 4B; 1C and 2C, 3C ($P < 0.001$); 1C and 2C, 3C, 4C ($P < 0.005$); 1A and 4A ($P < 0.02$).

and participates in the peroxidative metabolism in response to insulin and possibly to some insulin-mimicking agents.

No evidence of lipid peroxidation was noted in these cells after their exposure to insulin, DTT or pCMB, using the thiobarbituric acid procedure.

Aminotriazole does not affect other peroxidase activities in the cell [24–27]. The remaining proportions of formate peroxidation in the presence of aminotriazole reflect, therefore, the share of glutathione peroxidase activity. The latter is also evident from the preferential increase in the pentose phosphate pathway activity (Fig. 1) in response to the sulfhydryl reagents or to insulin, as routinely observed.

Catalatic activity. The catalatic activity of catalase in these cells was determined on the basis of the decomposition of H_2O_2 by the soluble supernatant fractions of adipocytes, as documented in Table 2. Interestingly, a partial diminution of the catalatic pathway in response to insulin or the sulfhydryl reagents indicates that a part of the enzyme may be converted to the catalase: H_2O_2 complex II [24–27] which has only the peroxidative function.

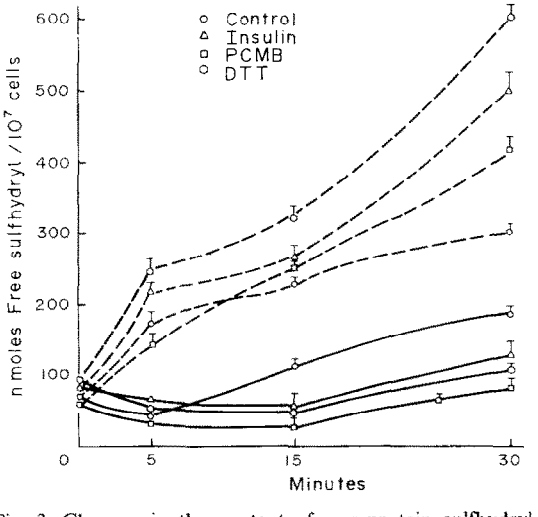

Fig. 3. Changes in the content of non-protein sulfhydryl groups, as a measure of glutathione (GSH), in adipocytes during exposure to insulin, dithiothreitol (DTT) or *p*-chloromercuribenzoate (pCMB), in the presence (broken lines) or absence (solid lines) of 0.2 mM D-glucose. The whole cell contents were estimated after removal of the incubation medium containing these agents, as described in Materials and Methods.

Change in the cellular content of glutathione in response to insulin or the sulfhydryl reagents. The cellular content of glutathione (GSH) was found to change in the course of time, when adipocytes were exposed to insulin or the sulfhydryl reagents. The supply of D-glucose (0.2 mM) to the medium had a profound influence on these changes. Exposure of the cells to insulin or the sulfhydryl reagents, without glucose, led to a gradual decline in the GSH level; in the presence of glucose, this process was reversed, and a rise in GSH was noted (Fig. 3). It is well recognized, however, that rapid oxidation of GSH and its *de novo* synthesis may interfere with its estimation in the cells. The data represent the average non-protein —SH content of the cells in a number of experiments.

DISCUSSION

Regulation of glucose transport and intracellular utilization is recognized as the principal biological action of insulin. Yet, a number of metabolic effects of this hormone in mammalian cells, such as the antilipolytic effect in adipose tissue [1] and activation of the mitochondrial pyruvate dehydrogenase complex [31], are reported to be independent of glucose transport. It is logical to postulate that insulin, by its interaction with specific plasma membrane components, may modify or activate a metabolic process which is distinct from its effect on glucose transport. Pursuit of this question recently led to the finding that insulin or sulfhydryl reagents can activate a reduced pyridine nucleotide oxidase enzyme in the plasma membrane of adipocytes which yields stoichiometric amounts of hydrogen peroxide [20, 21]. The next important questions are: how does this possible action of insulin *in vivo* affect cellular metabolism and how can the resultant H_2O_2 be disposed of. The

present report answers these questions. Sulfhydryl reagents are among the various agents which mimic some actions of insulin in adipose tissue [1, 9, 10, 32]. Earlier reports on the effects of sulfhydryl inhibitors, such as *N*-ethyl maleimide [11], were both interesting and intriguing because the stimulatory effect of sulfhydryl reagents on glucose utilization reported by the authors, while the same agents inhibit D-glucose transport, could not be explained. This led Czech and Fain [33] to hypothesize that sulfhydryl reagents may react with the medium albumin contaminants to produce a stimulatory substance. Speculations about such an artefactual effect are now laid to rest, since we have been able to identify an insulin-sensitive plasma membrane-linked NADPH oxidase activity [20, 21] which appears to be responsible for triggering the pentose phosphate pathway activity by increasing the $NADP^+$/NADPH ratio in these cells. This is a distinct mechanism since the effect of insulin is reproducible with some sulfhydryl reagents, as shown here, and even in the presence of other inhibitors of hexose transport, such as cytochalasin B, although glucose transport and consequently fatty acid synthesis are impaired under these conditions (S. P. Mukherjee, manuscript submitted for publication). Thus, the increase in the ppp activity is apparently one of the primary effects of insulin rather than being secondary to increased provision of $NADP^+$ through *de novo* fatty acid synthesis. A direct relationship between the insulin-responsive glucose transport and the Embden–Meyerhof pathway is evident from these data. Inhibitors of glucose transport and azide depressed this pathway of glucose oxidation (Fig. 1). This finding is consistent with the recent suggestion that activation of hexose transport by insulin in these cells requires a source of high-energy phosphates [34, 35].

Our results indicate that endogenously formed hydrogen peroxide is catabolized by the two enzymatic pathways, catalase and glutathione peroxidase, finally to the useful purpose of an efficient utilization of glucose through the oxidative hexose monophosphate shunt. The net reactions involved in this process, as shown for other cells actively metabolizing H_2O_2 [25–30, 36], appear to be valid for these cells also: (1) peroxidation of GSH with H_2O_2 to the glutathione disulfide, GSSG; (2) reduction of GSSG, catalyzed by glutathione reductase, using reduced NADP; and (3) increase in the glucose 6-phosphate dehydrogenase activity due to the increased availability of $NADP^+$ and the protective effect of GSSG [37, 38].

We have some preliminary data suggesting a 2- to 3-fold increase in the soluble glutathione peroxidase and NADPH-specific glutathione reductase enzymes in these cells in response to insulin. However, the relative rates of these two enzymes were found to be variable under different experimental conditions, apparently depending on the availability of glucose and the rate of lipogenesis (S. P. Mukherjee, unpublished observations). Further studies on these possibilities are in progress. The data on the cellular content of glutathione presented here (Fig. 3) indicate its direct relationship with glucose oxidation via the ppp, since, in the presence of glucose, insulin and the sulfhydryl reagents (which inhibit the glycolytic pathway) led to a rapid increase in the cellular content of GSH.

Catalase can exist in the cells either as the free enzyme or as the catalase:H_2O_2 complex I [25–30]. This complex has two possible modes of action: catalatic and peroxidative. In some cells where endogenous H_2O_2 formation and its metabolism have been studied, e.g. red blood cells [22, 23, 25] and alveolar macrophages, the catalase activity has been shown to involve a catalase:H_2O_2 complex II which has only the peroxidative function [23–25]. The formate peroxidation was increased by about 2-fold in cells in response to insulin or the sulfhydryl reagents, and it was partly inhibited in the presence of 50 mM aminotriazole. This inhibition was also greater in the cells exposed to insulin or the sulfhydryl reagents (Fig. 2), reflecting an increased accumulation of H_2O_2 as catalase complex in the treated cells. This experimental approach has been aptly used by other authors to determine H_2O_2 accumulation in various cells [25–28]. Aminotriazole does not, however, affect other peroxidase reactions in the cells [26, 27]. A concomitant rise in the pentose phosphate pathway activity (Fig. 1) in the presence of the catalase inhibitors indicates that glutathione peroxidase activity is the predominant route for H_2O_2 catabolism in these cells and accounts for the higher rate of the pentose phosphate pathway in response to insulin.

A finding of additional interest is that the catalatic function of catalase is partly diminished in cells pretreated with insulin or these —SH reagents (Table 2). This suggests that some of the catalase:H_2O_2 complex I may be converted to complex II, with the peroxidative function alone. The evidence for H_2O_2 formation and the consequent peroxidative reactions in these hormone-sensitive cells have an important bearing on our recent finding that the inhibitory effect of insulin on the adenylate cyclase activity in these cells may be mediated by oxidation (or peroxidation) of the key sulfhydryl groups of the enzyme [21].

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